

*EVALUATION OF ALTERNATIVE FEEDSTOCKS FOR
BIOENERGY PRODUCTION IN PORTUGAL: NON-EDIBLE
OILSEED CROPS, BY-PRODUCTS AND WASTES*

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**EVALUATION OF ALTERNATIVE FEEDSTOCKS FOR
BIOENERGY PRODUCTION IN PORTUGAL: NON-EDIBLE
OILSEED CROPS, BY-PRODUCTS AND WASTES**

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*To my father, mother,
girlfriend, brother and sister
for your love and support*

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ABSTRACT

The increase of energy demand is related with population growth and industrialisation and might be the genesis of international conflicts in the future. Consequently, it is essential to promote a change in the energy paradigm, at a global scale, since the natural resources currently used by the different economic sectors are not infinitely available and the fact that their consumption can lead to significant environmental impacts. Biodiesel is a biofuel with special relevance considering its straightforward replacement of mineral diesel, and hence it becomes essential to promote changes in energy policies. It is estimated that 95% of the world's biodiesel production comes from edible oils and that the cost of these feedstocks represents 70 – 95% of the total cost of biodiesel production. It is therefore paramount to identify non-edible oils, by-products and wastes, with the aim to reduce both food versus fuel competition and economic constraints. In agreement, the present work reports a set of studies related with the development of more sustainable biodiesel production processes, devoted to two fundamental aspects: i) to obtain low-cost oils from alternative sources (such as olive pomace, rice bran and soapstock) and convert them into biodiesel; ii) to evaluate the potential of alternative crops - *Crambe abyssinica* and *Cynara cardunculus* L. - for biodiesel production, including agronomic assessment of their cultivation. Throughout the studies, different processes (including pretreatment processes and biodiesel production) are evaluated and the properties of the oil and biodiesel are monitored.

Agronomic studies indicated that *Crambe abyssinica* can be cultivated in Portugal resulting in seeds with relatively high oil content (> 26 wt.%) and acceptable yields ($742 \text{ kg} \cdot \text{ha}^{-1}$). On the other hand, mild temperatures until flowering and the high-water availability allows to achieve good emergence rates on shorter periods (between 19 – 43 days). On the other hand, the prevalence of cold weather or high temperatures during the germination negatively affects the germination rate of the seeds. The life cycle assessment demonstrated that crambe cultivation under the studied conditions had lower impact for climate change when comparing with other systems in extensive mode. For *Cynara cardunculus* L., the seeds showed a relatively high oil content (from around 22 to 27 wt.%) and seed yields close to $189 \text{ kg} \cdot \text{ha}^{-1}$. The decrease of water availability mostly during the Winter-Spring season might justify the lower yields obtained in 2019 (second harvest). Higher seed yields are expected using a cultivated variety. The nutritional assessment of cardoon and crambe seeds reveals that these materials had a high content of protein (> 16 wt.%) and oil (> 22 wt.%). Meal and presscake wastes generally showed an even higher protein content (> 18 wt.%). Overall, biomass wastes from these two crops are rich in oil, protein and fiber, having the potential to be used as animal feed. Thus, the results demonstrated that the cultivation of these crops can also be exploited beyond the biodiesel purpose, as a source of nutrients for animal production, maximizing their potential.

The biodiesel produced by homogenous alkaline transesterification using crambe oil extracted from the seeds revealed to be of good quality, fulfilling the criteria established in the European standard of quality control, the EN 14214. Enzymatic transesterification proved to be a good alternative process, being possible to obtain 84 ± 5 wt.% of FAME content only after 2 hours, a value that using the predictive model can increase to almost 99 wt.% after 24 h using a methanol:oil molar ratio of 6:1 and 8 wt.% of enzyme (experimental result – 97.5 wt.%). With cardoon oil, the biodiesel produced using alkaline or enzymatic catalysts showed high purity (around 87 wt.%), although lower than that required by the standard EN 14214 (96.5 wt.%).

Typically, oils obtained from wastes/by-products have low quality (high acidity) which is a critical issue when biodiesel production is performed by the conventional process - alkaline transesterification. Consequently, pretreatments are required. Using an oil obtained from soapstock with very high acidity ($128.8 \text{ mg KOH} \cdot \text{g}^{-1}$), homogeneous acid esterification and glycerolysis reaction were performed, allowing to obtain a product with an acidity close to $10 \text{ mg KOH} \cdot \text{g}^{-1}$. After alkaline transesterification, biodiesel with 90 wt.% fatty acid methyl ester content was obtained. When using acid rice bran oil ($47 \text{ mg KOH} \cdot \text{g}^{-1}$), the enzymatic esterification pretreatment (at 35°C , for a 6:1 methanol:oil molar ratio and 5 wt.% of enzyme) reduced the acidity from $47 \text{ mg KOH} \cdot \text{g}^{-1}$ to $8 \text{ mg KOH} \cdot \text{g}^{-1}$ (24 h), being the final FAME content 87 wt.%. Finally, for acid olive pomace oil, an acidity reduction of 85 % was achieved performing an enzymatic esterification with a methanol:oil molar ratio of 6:1 and an enzymatic concentration of 3.5 wt.%, reducing FFA content from $50.6 \text{ mg KOH} \cdot \text{g}^{-1}$ to $7.5 \text{ mg KOH} \cdot \text{g}^{-1}$ (85% reduction), displaying the pretreated oil a FAME content of 84 wt.%.

Considering all the studies developed it was possible to verify that in Portugal there are several types of wastes/by-products (soapstock, rice bran, waste coffee grounds, grape pomace waste or brewery spent grain) with high potential to be used as alternative raw material for biodiesel production, due to their oils and fats content, as well as the enzymatic and acid pretreatment as effective processes to reduce the oil's acidity.

Keywords: Non-edible oilseed crops; agronomic assessment; by-products recovery; wastes recovery; sustainable biofuels.

RESUMO

O aumento do consumo de energia relacionado com o crescimento populacional e desenvolvimento industrial poderá vir a ser o âmbito de futuros conflitos internacionais. Posto isto, torna-se essencial promover uma mudança no paradigma energético, à escala mundial, uma vez que os recursos naturais que actualmente utilizamos, nos diferentes setores económicos, não estão infinitamente disponíveis e o seu consumo levanta diversos problemas ambientais. O biodiesel é um biocombustível com especial importância, uma vez que pode ser usado directamente como substituto do diesel mineral, sendo assim essencial na promoção de mudanças ao nível das políticas energéticas. É estimado que 95 % da produção mundial de biodiesel provem de óleos alimentares e que o custo destas matérias-primas representa 70 – 95 % do custo total da produção deste biocombustível. Deste modo, é fulcral identificar os óleos não-alimentares, subprodutos e resíduos tendo em vista reduzir a competição entre o mercado alimentar vs energético e os constrangimentos de natureza económica. O presente trabalho detalha assim um conjunto de estudos relacionados com o desenvolvimento de processos de produção de biodiesel mais sustentáveis, com dois objectivos principais: i) obter óleos de baixo custo a partir de fontes alternativas (bagaço de azeitona, sêmea de arroz e pastas de neutralização de óleos vegetais) e convertê-los em biodiesel; ii) avaliar o potencial das culturas *Crambe abyssinica* e *Cynara cardunculus* L. - para a produção de biodiesel, incluindo a sua avaliação agronómica. Ao longo dos estudos, os diferentes processos (incluindo pré-tratamento e produção de biodiesel) são avaliados e as propriedades dos óleos e biodiesel são monitorizadas.

Os estudos agronómicos demonstraram que *Crambe abyssinica* pode ser cultivada em Portugal, produzindo sementes com teor de óleo relativamente alto ($> 26 \text{ \% m/m}$) e rendimentos, em termos de sementes, aceitáveis (742 kg.ha^{-1}). As temperaturas amenas até à etapa de florescimento e as elevadas disponibilidades de água proporcionam uma boa taxa de germinação, em curtos períodos (19 – 43 dias). Por outro lado, a prevalência de temperaturas baixas ou demasiado altas afecta negativamente a taxa de germinação das sementes. Da avaliação de ciclo de vida foi possível concluir que o cultivo de crambe nas condições estudadas não contribui significativamente para as alterações climáticas quando comparado com outros sistemas produtivos, em modo extensivo.

Para *Cynara cardunculus* L., as sementes apresentaram um teor de óleo relativamente alto (22 a 27 % m/m) com rendimentos de sementes próximos de 189 kg.ha^{-1} . O decréscimo da disponibilidade de água entre o inverno e a primavera poderá estar na base dos menores rendimentos verificados em 2019 (segunda colheita de sementes). São previstos maiores rendimentos das sementes se usada uma variedade cultivada. A avaliação nutricional das sementes de cardo e crambe revelou que estas apresentam um alto teor de proteína ($> 16 \text{ \% m/m}$) e óleo ($> 22 \text{ \% m/m}$). Os resíduos de torta/farelo apresentaram um teor proteico ainda maior ($> 18 \text{ \% m/m}$). Globalmente, os resíduos de biomassa destas duas culturas são caracterizados por elevados teores em óleo, proteína e fibras, demonstrando potencial para serem utilizados para ração animal. Assim sendo, os resultados demonstraram que o cultivo destas culturas, para além do seu potencial para produção de biodiesel, podem também ser exploradas como fonte de nutrientes para alimentação animal.

O biodiesel produzido por transesterificação alcalina homogénea, utilizando óleo de crambe extraído das sementes colhidas em Portugal, revelou-se de boa qualidade, cumprindo quase todos os limites/requisitos analisados, definidos na norma Europeia de controlo de qualidade EN 14214. A transesterificação enzimática mostrou ser um processo alternativo viável, sendo possível obter um biodiesel com um teor em FAME de $84 \pm 5 \text{ \% m/m}$ após 2 horas, valor este que utilizando o modelo preditivo aumenta quase 99 % m/m após 24 h para uma razão molar óleo:metanol de 6:1, e com uma quantidade de enzima de 8 % m/m (resultado experimental – 97,5 % m/m). Já com óleo de cardo, o biodiesel produzido usando catalisadores alcalinos ou enzimáticos apresentou alta pureza (cerca de 87 % m/m), embora com um teor de FAME inferior ao requerido pela norma EN 14214 (96,5 % m/m).

Habitualmente, os óleos provenientes de resíduos/subprodutos apresentam uma baixa qualidade, característica essencial quando se pretende produzir biodiesel pelo processo convencional - transesterificação alcalina. Por esse motivo, os processos de pré-tratamento são necessários. Para um óleo ácido ($128,8 \text{ mg KOH.g}^{-1}$) extraído a partir de pastas de neutralização, foram realizados os processos de esterificação ácida e de glicerólise, permitindo obter um produto com uma acidez próxima de 10 mg KOH.g^{-1} . Após a transesterificação alcalina, foi obtido um biodiesel com um teor em FAME de 90 % m/m. Quando utilizado o óleo extraído a partir de farelo de arroz (47 mg KOH.g^{-1}) o pré-tratamento por esterificação enzimática (a $35 \text{ }^{\circ}\text{C}$, para uma relação metanol:óleo de 6:1 e uma quantidade de enzima de 5 % m/m) permitiu uma redução de acidez de 47 mg KOH.g^{-1} para 8 mg KOH.g^{-1} (24 h), sendo o teor final de FAME próximo de 87 % m/m.

Finalmente, para o óleo de bagaço de azeitona, foi alcançada uma redução da acidez de 85 % realizando-se uma esterificação enzimática com uma razão molar de metanol:óleo de 6:1 e uma quantidade de enzima de 3,5 % m/m, reduzindo o teor de ácidos gordos de $50,6 \text{ mg KOH.g}^{-1}$ para $7,5 \text{ mg KOH.g}^{-1}$ (85 % de redução), em que o óleo pré-tratado apresenta um teor em FAME de 84 % m/m.

De acordo com os estudos desenvolvidos, foi possível verificar que em Portugal vários tipos de resíduos/subprodutos (sêmea de arroz, pastas de neutralização, borra de café, bagaço de uva e drêche) podem ser utilizados como fonte de óleos para produção de biodiesel, devido ao seu teor em óleos e gorduras além de que a esterificação enzimática ou ácida são processos eficazes com vista à redução da acidez destes.

Palavras-Chaves: Culturas oleaginosas não-alimentares; avaliação agrícola; valorização de sub-produtos; valorização de resíduos; biocombustíveis sustentáveis.

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LIST OF ACRONYMS

Bio-ETBE – Bio ethyl tertiary butyl ether;

CO₂ – Carbon dioxide;

BSG – Brewery Spent Grain;

DG – Diglycerides;

ET_p – Evapotranspiration potential;

EU – European Union;

FAME – Fatty acid methyl esters;

FFA – Free fatty acids;

GC – Gas chromatography;

GDP – Gross domestic product

GHG – Greenhouse gases;

HA – Harvest Aids;

HVO – Hydrotreated vegetable oil;

ILUC – Indirect land-use change;

LCA – Life cycle assessment;

MG – Monoglycerides;

PA – Phosphatidic acid;

PC – Phosphatidylcholine;

PE – Phosphatidyl ethanolamine;

PGR – Plant Growth Regulators;

PI – Phosphatidyl inositol;

SCG – Spent coffee grounds;

SS – Soapstock;

TdB – Biofuel titles;

TG – Triglycerides;

TSW – Thousand seeds weight;

WCO – Waste cooking oil;

WtE – Waste-to-energy technology;

EC – European Commission;

CHAPTER 1. *INTRODUCTION*

1.1 *RELEVANCE OF THE STUDY*

The population growth forecasted for the 21st century, which will correspond to an increase near to 38 % (9.8 billion inhabitants forecasted against the current 7.3 billion) [1, 2], is undoubtedly one of the biggest challenges that humanity will have to deal. This accentuated growth of the world's population raises several sustainability issues due to the formation of big cities, densely populated, increasing the complexity of the transport systems, the access to energy and the waste management. World population growth will be the main prosecutor of ecological diversity losses, climate change effects and decrease of soil quality, accelerated by the erosion processes associated to intensive agriculture practices. These facts can directly influence the production of food products and the access to safe potable water [2, 3].

If the mitigation measures are not implemented, there will be millions of people without the minimum of subsistence conditions or leaving in places with poor environmental conditions. The population growth forecasted will be mainly felt in Africa, where the availability of goods and food will be the key of economic growth among African emerging countries [1, 4].

The increase of energy demand is directly related with the industrialisation and will be the genesis of international conflicts in the future. For this reason, it is essential to promote a change in the energy paradigm, at a global scale, because natural resources currently used, in the different economic sectors, are not infinitely available and their consumption implies, in most situations, environmental problems [3, 5]. Engineering science plays an important role in the achievement of economically and sustainable alternatives pathways for energy production. Renewable energy sources are the main alternative route to fossil fuels, in particular concerning environmental protection, but economic sustainability is not yet ensured [6].

The gradual change of the energy paradigm can be promoted by the consumption of alternative fuels from biomass (biofuels), such as bioethanol, biodiesel, biomethanol, biohydrogen, among others. Biodiesel is a biofuel with special relevance considering its straightforward replacement of mineral diesel, therefore, being essential for promotion of changes in energy policies; however, it is mostly produced using edible oils as feedstock which is a considerable drawback. In fact, it is estimated that 95% of world's biodiesel production comes from edible oils [7]. The use of vegetable

oils allows that carbon dioxide (CO₂) release during the life cycle of the biofuel can be partially captured again by the vegetable crop due to photosynthesis process. In agreement with Directive 2013/18/EU (amending Directive 2009/28/EC of the European Parliament and of the Council on the promotion of the use of energy from renewable sources), the substitution of mineral diesel by biodiesel obtained from refined vegetable oils allows a reduction of greenhouse gases (GHG) emissions between 19% to 56%. The production of biodiesel from edible oils (rapeseed oil, sunflower oil and palm oil) has however some drawbacks due to the potential impacts on food supply [8]. In addition, arable land used for food production is replaced for the production of oilseed crops for energy purpose. Also, in some developing countries, the forest is converted into arable land aiming to plant oilseed crops and causing losses of biodiversity [9]. According to Directive (EU) 2015/1513 (amending Directive 98/70/EC related to the quality of petrol and diesel fuels and amending Directive 2009/28/EC on the promotion of the use of energy from renewable sources), advanced biofuels such as those produced from non-edible oils, wastes (waste cooking oil or fats) or algae contribute to a considerable decrease of GHG with a low risk of indirect land use change. When pasture or agricultural land conventionally used for food and feed markets is diverted for biofuel production, the food demand will still need to be satisfied. Consequently, an intensification of current production will be verified, leading to the conversion of land with high carbon stock or used for food production (indirect land use change), increasing greenhouse gas emissions.

According to the Food and Agriculture Organization, more than 840 million of people in the world do not have enough to eat and over half of child deaths are associated with malnutrition [10]. Considering that fundamental social aspect, the production of biodiesel from edible oils should be avoided. Furthermore, most oilseed crops are produced through intensive agriculture practices, leading to the degradation of soil and depletion of nutrients, affecting the sustainable use of the soils.

The cost of the conventional edible raw materials used for biodiesel production represents 70 – 95% of the total cost of biodiesel production, reason why less expensive alternative raw materials must be identified to reduce the economic constraints associated to the production process [8, 11].

Vegetable oils are mostly constituted by triglycerides (TG); however, low cost raw materials such as crude oils and by-products/wastes with high oil and fat contents, sometimes also have a significant content of free fatty acids (FFA). Such alternative oils and fats might be obtained from several

economic activities, namely from the food sector, dairy industry, municipal wastewater treatment, edible oils refining, olive oil production, fish canning industry and slaughter houses [12].

The recovery of oils and fats from wastes is very relevant, because the lipidic fraction is recovered and used as feedstock for biofuel production, thus avoiding landfill disposal, incineration and all the negative environmental effects that these actions have associated. Therefore, it is possible producing a biofuel and at same time applying more effective waste management practices.

The economic value of these oilseed crops (food and non-food crops) goes beyond their lipidic fraction (oil content), being possible the valorisation/recovery of other parts of the plant, through the production of other added value goods (biorefinery concept). The production of animal feed (seed's hull with high protein content), cosmetic products or natural antioxidants for several purposes, should be highlighted. In addition to those advantages, the increase of feedstock's cost (edible oils market) conventionally used for biofuel production increases the interest in the use of alternative raw materials (non-food crops, wastes, by-products) cheaper.

In most regions of the world, these kind of wastes (with a high content of oils and fats) are not enough to satisfy all the demand of the biodiesel industry [2, 8]. Since the biodiesel production can become more environmentally friendly, several countries have been developing strategies to promote the cultivation of non-food crops, avoiding the competition between food and biofuel market. These oilseed crops present several advantages when compared with the edible crops, because they are not suitable for human consumption, but also because several can be cultivated in marginal lands or contaminated soils [13, 14]. Thus, the main purpose is to cultivate these oilseed crops with low agriculture inputs, allowing soil restoration and avoiding the occupation of arable land or soil impoverishment [15].

In Portugal, the amount of conventional wastes available with high oil content (such as waste cooking oil and fats) is not enough to fulfil all the demand required by the producers for biofuel production (in 2017, the annual biodiesel production installed capacity was $742 \times 10^3 \text{ m}^3$, but only 54% of this capacity was effectively used in 2019 - $402 \times 10^3 \text{ m}^3$) [16, 17]. According to the National Entity for the Energy Sector ("*Entidade Nacional para o Setor Energético*"), in 2017 the amount of waste cooking oil (WCO) and fats used in Portugal for biodiesel production was of $180 \times 10^3 \text{ m}^3$ (corresponding to $164 \times 10^3 \text{ ton}$, considering WCO density as 910 kg.m^{-3}) and $5.4 \times 10^3 \text{ m}^3$, respectively [18]. For this reason, it is very important to study alternative wastes/by-products flows which could complement the amount of raw materials nationally available for biodiesel production. Focusing on

the Portuguese scenario, namely considering the most relevant industrial, agricultural and municipal activities, associated to agriculture, cattle raising and food production, potential relevant alternatives raw materials are: soapstock from edible oils refining, olive pomace, winery waste (grape pomace), rice bran and sludge from waste water treatment plant [19]. Typically, these raw materials have low quality, and the resultant oils have high acidity which is a critical issue when biodiesel production is performed by the classic chemical process of alkaline transesterification (due to production of soaps). For this reason, it is necessary to perform a pre-treatment process aiming to reduce FFA content until an acceptable level (usually lower than 1 wt.%) [12, 20].

The cultivation of non-food crops in Portugal is also justified by the external dependency of our country from the European oilseed market, since the majority of feedstocks used for biodiesel production is imported [16, 18]. The selection of potential crops should also be promoted considering the biorefinery concept, going beyond oil recovery.

The present PhD thesis reports a set of studies related with the development of more sustainable biodiesel production processes, devoted to two fundamental aspects: i) to obtain low cost oils from alternative sources (wastes or by-products) and convert them into biodiesel; ii) to evaluate the potential of two non-edible oils - *Crambe abyssinica* oil and *Cynara cardunculus* oil - as alternative feedstock for biodiesel production, including agronomic assessment.

1.2 OBJECTIVES

The present PhD thesis had as global objective to increase the knowledge about biodiesel production from nonconventional raw materials.

The most relevant specific objectives were:

1. Evaluating different alternative raw materials for bioenergy production (advanced biofuels), namely, non-edible oils and oils obtained from wastes or by-products;
2. Analysing the agriculture viability of selected non-edible oilseed crops (*Crambe abyssinica* and *Cynara cardunculus*) together with their environmental impacts, in particular, those related with the land use caused by crop growth;
3. Identifying, quantifying and characterizing the oils and fats from different sources and developing pre-treatment processes considering those characteristics;

4. Assessing the operational viability and processing constraints of biodiesel production, using as raw materials oils and fats recovered from wastes/by-products and extracted from non-edible oilseed crops;
5. Improving the flow sheet of biodiesel production using alternative oils and wastes/by-products.

Wastes/by-products with high oil content, namely, olive pomace, rice bran, waste coffee grounds, grape pomace, brewery spent grain and acid soapstock oil were assessed to promote the diversification of raw materials used in Portugal for biodiesel production.

The cultivation and use of non-edible oils from *Crambe abyssinica* and *Cynara cardunculus* were studied considering an entire cycle of seeding, harvesting, oil extraction and biodiesel production.

1.3 OUTLINE

The main structure of the work and the topics covered in each chapter, are presented in Table 1.1.

Table 1.1. Outline of the thesis

Chapters of the thesis	Description
Chapter 1 – Introduction	• Presents the relevance of the study, the main objectives of the work and the outline. • Provides an overview related to the energy sector in Portugal and the legal framework regarding biodiesel production. • Reveals the importance of biodiesel production in the world, detailing the advantages and drawbacks of its use, raw materials, their pre-treatment and synthesis reaction. • Provides information about the potential of non-edible crops, wastes and by-products as source of oils/fats for biodiesel production.
Chapter 2 – Non-edible crops as alternative raw materials for biodiesel production – an overview	• An overview about non-edible crops for bioenergy production. • Selection of four non-edible crops with high potential for bioenergy production. • State-of-the art about the selected crops considering their agronomic and edaphoclimatic requirements.
Chapter 3 – Crambe abyssinica crop – Agronomic assessment and bioenergy production	• Cultivation of <i>crambe</i> (annual crop) in Portuguese edapho-climatic conditions. • Assessment of agronomic conditions required by <i>crambe</i> crop. • Comparison between <i>crambe</i> oil from seeds of Brazil and the oil obtained in seeds obtained in Portugal (experimental cultivation). • Characterization of crude oil obtained from harvested seeds. • Study of raw oil refining. • Production of biodiesel from <i>crambe</i> oil by alkaline transesterification and quality assessment according to standard EN 14214. • Study of enzymatic transesterification as an alternative route for biodiesel production from <i>crambe</i> – optimization using response surface methodology.
Chapter 4 – Cynara cardunculus crop – Agronomic assessment and bioenergy production	• Cultivation of <i>Cynara cardunculus</i> (perennial crop) in Portuguese edapho-climatic conditions; • Assessment of agronomic conditions required by <i>Cynara cardunculus</i>; • Characterization of crude oil obtained from the harvested seeds; • Assessment of oil characteristics when obtained from different ecotypes of <i>Cynara cardunculus</i> in Portugal; • Production of biodiesel from cardoon by alkaline and enzymatic transesterification and quality assessment according to standard EN 14214;
Chapter 5 – Crambe abyssinica and Cynara Cardunculus crops – Nutritional analysis of biomass wastes	• Assessment of nutritional value of by-products obtained during <i>crambe</i> cultivation, seed harvesting and oil extraction from seeds; • Assessment of nutritional value of wastes and by-products produced during cardoon cultivation, seed's harvest and seed's oil extraction;

	• Determination of the energetic potential of the solid by-products;
<i>Chapter 6 – Overall assessment of wastes and by-products as alternative raw materials for energy production</i>	• Assessment of wastes and by-products with high potential for valorization as source of oils and fats for bioenergy production: State of the art about new trends and an overview of Portuguese scenario.
<i>Chapter 7 – Selected by-products and Wastes as raw materials for energy production</i>	Chapter 7.1 – Soapstock acid oil by-product for energy production • Characterization of soapstock acid oil; • Study of pre-treatment processes regarding acid oil valorization as raw material for biodiesel production (acid esterification vs glycerolysis); • Characterization of the final product (biodiesel); Chapter 7.2 – Rice bran by-product for energy production • Extraction and characterization of rice bran oil; • Study of pre-treatment processes regarding rice bran oil valorization as raw material for biodiesel production (acid vs enzymatic esterification); • Characterization of the final product (biodiesel); Chapter 7.3 – Olive pomace by-product for energy production • Extraction and characterization of olive pomace oil; • Study of pre-treatment processes regarding acid oil valorization as raw material for biodiesel production (acid esterification, glycerolysis, enzymatic esterification); • Characterization of the final product (pre-treated oil); Chapter 7.4 – Waste Coffee Grounds, Grape Pomace Waste and Brewery Spent Grain – A preliminary Assessment Aiming at Bioenergy Production • Characterization of these wastes/by-products; • Extraction and characterization of the oil recovered from the wastes/by-products;
<i>Chapter 8 – Conclusions and Future Work</i>	• Presents the most important conclusions and suggests future work to complement that developed during the PhD studies.

1.4 BIOFUELS IN PORTUGAL

1.4.1 BIODIESEL CONSUMPTION – LEGAL FRAMEWORK

In European Union, several legal mechanisms regarding biofuels production and its commercialization have been developed. Each member state is responsible for the application of the legal requirements. The first and unique specific European Directive created to promote the use of biofuels in the transport sector was the Directive 2003/30/CE. This Directive dictated that all member states should use 2% of biofuel in relation to the total consumption of fuel (2005), increasing this value for 5.75% (biofuel incorporation calculated on the energy content basis) until 2010. In Portugal, this European Directive was transposed and implemented through the Decree-law nº 62/2006 (revoked by Decree-law nº 152-C/2017), which defined the requirements that should be achieved when biofuel was blended with mineral fuel (for percentage higher than 5%). In this legal rule it was also defined the concept of “small producer of biofuels”. By the Decree-Law nº 69/2016 (amending Decree-Law nº 117/2010, that transposes parts of the Renewable Energy Directive) the sustainability criteria for the biofuels and bioliquids produced in Portugal are defined, together with the incorporation limits and monitoring targets.

Presently, the Directive (EU) 2015/1513 (amending Directive 98/70/EC related to the quality of petrol and diesel fuels and Directive 2009/28/EC on the promotion of the use of energy from renewable sources), established that advanced biofuels such as those produced from algae, non-edible oils and wastes of cooking oil and fats, contribute to a considerable decrease of GHG with a low risk of indirect land use change. In this directive, detailed information concerning the reduction of GHG emissions is depicted. It should be highlighted that the major reduction on GHG (83%) is achieved when biodiesel is produced from waste vegetable oils or animal fats. On the other hand, the biodiesel produced from edible oils is associated to smaller decrease of GHG, being the biodiesel production from sunflower oil the type of biodiesel with the higher reduction (51%). This Directive also defines the share of energy from renewable sources in gross final consumption of energy that Portugal should reach until 2020 (31%). Also for 2020, the percentage of biofuel consumed in the transport should be 10% of the total consumption (calculated on the energy content basis).

Most of the biodiesel is produced using vegetable oil crops, which lead to environmental impacts caused by the use of land. For this reason, an appropriate limit of biofuels produced from oil crops should be established to minimize the overall direct and indirect land-use change (ILUC) impacts. As

referred before, ILUC occurs when the cultivation of crops for biofuels, bioliquids and biomass production substitute traditional production of crops for food and feed purposes.

Nowadays, the main objective is to decrease the use of oil crops (decreasing ILUC impacts) without restricting the overall possibility of use biofuels, promoting the development of alternative feedstocks for biodiesel production. According to the Directive 2018/2001 (EU), “agricultural feedstock for the production of biofuels, bioliquids and biomass fuels should be produced using practices that are consistent with the protection of soil quality and soil organic carbon”. Also, soil quality and soil carbon should be monitored by the operators or national authorities. According the same directive, the level of GHG caused by ILUC cannot be unequivocally determined with a high level of precision. As consequence, the expansion of the production area into land with high-carbon stock should be limited, promoting the use of alternative feedstocks in detriment of food and feed crops.

For the national territory it is currently in force the Decree-Law nº 69/2016 (amending Decree-Law nº 117/2010, which transposes parts of the Renewable Energy Directive) which determines the sustainability criteria for the biofuels and bioliquids produced and establishes the incorporation limits. In order to assess if the percentage of biofuels use in the transport sector is being fulfilled, the legal document determines the creation of Biofuel Titles (TdB). Biofuel producers receive a TdB for each amount of biofuel that generates the same energy as one ton of petroleum equivalent (Toe). The biodiesel produced from waste vegetable oils has a double benefit (double counting). So, each Toe of biodiesel produced from wastes put on the market corresponds to two TdB. For this reason, for two-member states with the same target regarding biofuels utilization, the amount of biodiesel produced could be different, depending on the raw materials used. According to Ordinance no. 301/2011, the biofuels producers that do not produce the foreseen amount of biofuels (stock of TdB) will be economically penalized.

The production of biofuels and bioliquids sometimes is not the better option relatively to the fossil fuels. It depends on the global production chain, since the origin of the raw materials until the biodiesel production at industrial scale and further use. In agreement with the Decree-law nº 117/2010 (amended by Decree-law nº69/2016), the biofuels and bioliquids will be sustainable when their use in substitution of fossil fuels allows a reduction of GHG higher than 60% (since January of 2018). In the same legal rule (article 11th) it is referred the minimum percentage of biofuels incorporated in the transport sector. The percentage of biofuels incorporated in the market should

be 9% and 10% for 2017/2018 and 2019/2020, respectively. However, in the Law nº 71/2018 (article 307º), the target defined for 2019/2020 was abolished (due to national economic constraints) and the incorporation verified for 2019 was adjusted to 7% (share of biofuel in the final market).

In Europe, all the diesel fuel commercialized in the transport sector should fulfill the requirements of EN 590 (Standard for Diesel Fuel). For this reason, when biodiesel is blended with mineral diesel, the mixture must guaranty the quality conditions referred in EN 590. This standard specifies the possibility of blending up to 7% of fatty acid methyl ester. Also considering the climatic conditions of the different countries, for cold weathers, the biodiesel/diesel mixture requires the use of cold flow additives that ensure the fluidity of the mixture in the engine.

1.4.2 ENERGY CONTEXT

The population and economic growth observed since the industrial revolution generated an increase in energy consumption, being the transport sector responsible for most of it. In Portugal, this sector contributes to 74% of the petroleum consumption [21].

Despite the recent find of new oil reserves [22], which increased the world's reserves to the value of 16.96×10^2 billions of barrels (2017) and allowed to maintain the current global energy scenario, a gradual change has been verified in the energy sector due to the development of renewable energy technologies and the use of biofuels [22]. In Figure 1.1 it is possible to verify the increase of world oil reserves over the decades, being 2017 the year with the highest oil reserves ever reported [22].

Despite the high availability of oil, its gradual substitution by other fuels obtained from more environmentally friendly raw materials, such as biofuels, is related with the unanimous knowledge that the climate change is largely caused by GHG emissions, mostly generated during combustion of fossil fuels and that biofuels can significantly contribute to GHG reduction, as previously referred.

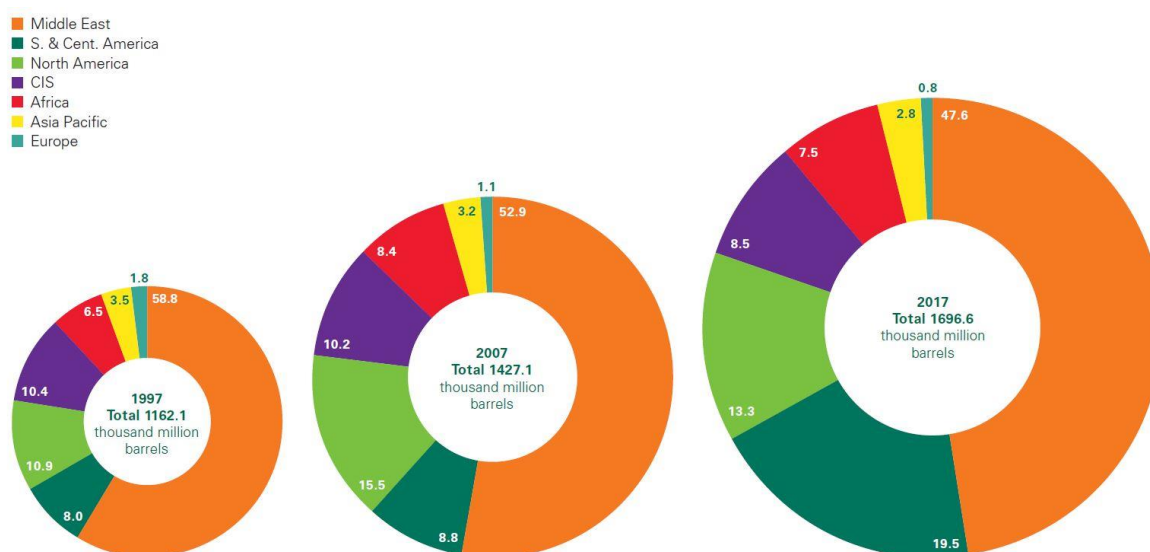


Figure 1.1. Evolution of the world oil reserves, by source, from 1997 till 2017 [22].

In Portugal, biodiesel is the main biofuel used as substitute of fossil fuels, in this case, for diesel engines. According to the Portuguese Association of Biofuels Producers, there is an annual installed biodiesel production capacity of $74.2 \times 10^4 \text{ m}^3$, distributed by eight big producers, namely Iberol ($13.6 \times 10^4 \text{ m}^3$), Biovegetal ($13.6 \times 10^4 \text{ m}^3$), Torrejana ($12.4 \times 10^4 \text{ m}^3$), Prio Biocombustiveis ($11.4 \times 10^4 \text{ m}^3$), Sovena ($10.8 \times 10^4 \text{ m}^3$), Valourodiesel ($57 \times 10^3 \text{ m}^3$), Bioportdiesel ($36 \times 10^3 \text{ m}^3$) and Enerfuel Galp ($31 \times 10^3 \text{ m}^3$). These industries use edible oils as raw materials, like soybean and rapeseed or waste cooking oil. Most of the oils are imported, because the cultivation of oilseed crops in Portugal is very scarce and the available WCO is also limited (soybean - $8\,629 \text{ m}^3$; rapeseed - $6\,640 \text{ m}^3$; WCO – $163\,029 \text{ m}^3$) [18].

Sunflower crop is the main oilseed crop produced in Portugal. In Alentejo region, due to the presence of Alqueva artificial lake (Alqueva Dam), a study from 2013 indicated to be possible to cultivate sunflower, rapeseed and soybean allowing a biodiesel production of around $26.84 \times 10^3 \text{ m}^3$ [23]. Although, at this region, there is a notorious preference for the cultivation of other irrigated crops more economically attractive, like olive trees or maize.

In Portugal, a gradual increase has been verified for seed yields of sunflower crop over the past years, being in 2018 about $1700 \text{ kg} \cdot \text{ha}^{-1}$ ($639 \text{ kg} \cdot \text{ha}^{-1}$ for 2013) [16]. In 2017, each 100 kg of sunflower plant was sold at 38.50 € (2017) [24].

Besides the production of biodiesel, Portugal is also considered to have a high potential for bioethanol production from maize. Bioethanol implementation faces several difficulties, namely,

the economic viability and need to define minimum limits of incorporation in the market or fee exemptions aiming to increase the market share [23].

The European legislation clearly shows that biofuels incorporation in fossil fuels market is essential for the energetic scenario change in the transport sector, although recent developments suggest a change in biofuel's energy policies. Electric vehicles arise as an alternative to the conventional vehicles with combustion engines. The scandals related to the manipulation of GHG emissions by carmakers (the so-called "dieselgate" scandal) increased even more the commitment with the electric vehicles production. Some European countries, such as France, Poland, Holland or Austria, with high biodiesel production capacities, opposed to this abrupt change of energetic policies and conversion of the automobile fleet into electric vehicle. In Europe, 27.45% of electricity produced in 2014 came from renewable sources [25]. Among the 28 countries of European Union, 13 countries produce electrical energy according to the established target [25]. In 2014, Portugal already produced electrical energy above the target, being 51% of electrical energy from renewable sources (wind power – 27%; hydroelectric – 17%; biomass – 5.5% and photovoltaic – 2.1%). For 2020, all 28-member states should reach the target of 31% of renewable energy incorporation into electrical sector. This fact will require high economic investment in a short time and for this reason biofuels should be considered as one more solution to achieve the targets established for 2020. In addition, some environmental problems arise with the utilization of electrical vehicles, namely the exploitation of natural resources (lithium) and the absence of adequate waste management practices for the used batteries [26].

Besides the electrical and transport sectors, GHG emissions are associated to other activities. Therefore, the European Union has developed broad GHG emissions reduction targets. Accordingly, until 2020, 20% of the final energy consumed must be of renewable sources. For 2018, 31% of the final energy consumed in Portugal was from renewable source (UE – 18%) [27]. At the same time, the energy efficiency should be improved to reduce energy demand [28]. In fact, this is only possible with the use of the best available technologies. In this area, Germany has been a leader among the European countries since it has the largest number of patents in the field of mechanical engineering and electrical engineering in Europe [29].

In Portugal, considering the assumptions presented in the legal rule nº 117/2010, several investments in the biofuel industry have been developed. Biodiesel is the main biofuel produced, as previously referred, representing around 95% of the biofuel market in the country. The remaining

fraction of the production corresponded to hydrotreated vegetable oil (HVO) [30]. Bio ethyl tertiary butyl ether (Bio-ETBE) and bioethanol (gasoline substitutes) are not produced in Portugal, being exclusively imported [31].

In Portugal, 43% of the biodiesel produced come from edible oils (2017), most obtained from imported seeds. From the oil extraction, two fractions with economic value are obtained: the vegetable oil and the seed's press cake. The vegetable oil might be used for food or biodiesel production, while the fraction of seed press cake is forwarded to animal feed. This fact implies a high dependence of the animal feed industry from the industry of oilseeds extraction. For the seed press cake, depending on the seed, different protein content can be obtained. For rapeseed, soybean and palm, the protein content can vary between 18% and 40% [32-34]. Proteins are essential for animal feed contributing for the muscular formation. Indirectly, also the human food chain is affected by the relationship between these two industrial sectors (biofuel industry and cattle production) [35].

In Portugal, vegetable protein is mostly produced from cereal grains followed by by-product of oilseeds processing (Figure 1.2). The oilseeds processing corresponds mostly to imported seeds of rapeseed, soybean and sunflower (22% of the animal feed production). In Figure 1.2 the raw materials used in Portugal for animal feed are depicted for 2017 [36].

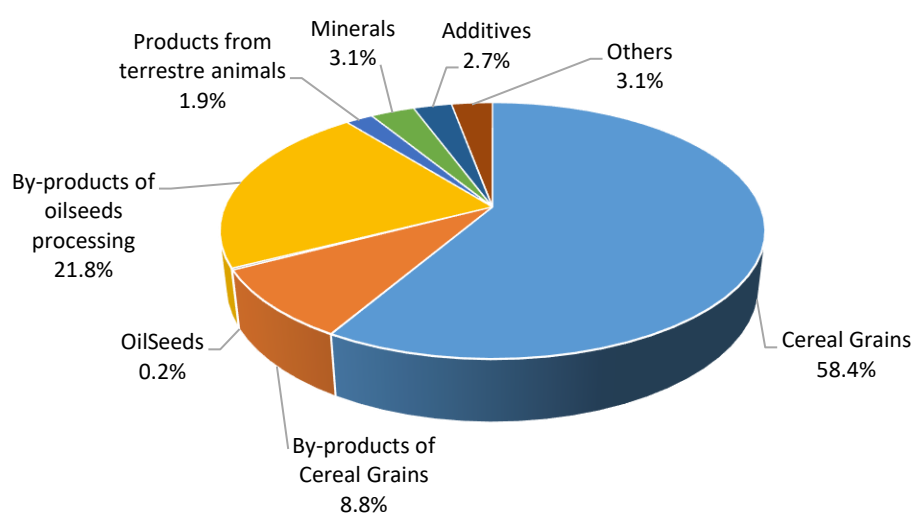


Figure 1.2. Raw material used in 2017 for animal feed production in Portugal [36].

Oilseeds represent only 0.2% of the total of raw materials used for animal feed production. For the year of 2017, the by-products from oil seeds extraction used for animal feed production were mostly

composed by soybean cake (477 876 ton), sunflower cake (96 113 ton), rapeseed cake (62 791 ton) and palm kernel cake (29 298 ton). Considering these values, it is possible to conclude that oil extraction for biodiesel production has a major impact on animal feed production.

According to the Decree-Law nº 117/2010 (amended by Decree-Law nº 69/2016), the credit of two TdB for each Toe of biodiesel produced from waste vegetable oils enables to reach the targeted values of biofuel incorporation through the production of smaller amounts of biodiesel, as previously referred. However, the integral substitution of edible oils feedstocks by waste vegetable oils for biodiesel production is expected to highly impact the animal feed market. In addition, the ability of Portugal to directly buy the seed press cake at international auctions is very small and put in risk the economic sustainability of the livestock sector. In the last case, this could lead to an increase of the animal's feed prices (mostly for white meat).

The political debate about environmental sustainability of biodiesel production in Portugal, reflected in a decrease of related “incentives”. The use of waste vegetable oils or fats for biodiesel production can also directly originate imbalances in the animal feed market, as referred. Fats (category III), due to the small risk of diseases transmission for human beings and animals, can be used to produce animal protein for animal feed or can be used directly as animal feed (Regulation EC nº 1069/2009). Accordingly, the production of biodiesel using fats classified as category III only leads to the emission of one TdB for each Toe of biodiesel produced.

In average, soybean seeds have about 18 – 22% of oil content and 36 – 40% of protein content being suitable for animal feed [33, 34]. According to the information provided by Portuguese Association of Biofuels Producers (“*Associação Portuguesa de Produtores de Biocombustíveis*”), it is estimated that 10 % of oil produced is used for human consumption. Soybean oil together with the protein, will contribute for human consumption and animal feed.

For the rapeseed crop, the cultivation of transgenic cultivars made possible to obtain seeds with higher oil content (above 43%) [37]. The remaining fraction has a protein content around 18 – 25%, used mostly for animal feed. This type of protein has the particularity of being easily digested by the animal organism. [33, 34]. According to the Portuguese Association of Biofuels Producers, 9% of the oil fraction is used for human consumption, mostly in the “Scandinave” countries.

Among the edible oils, the consumption of palm oil for biodiesel production is the most controversial. The production of palm crop is mainly made in Southeast Asia, region where the palm oil production is associated to environmental impacts such as deforestation and land use change,

contributing to the decrease of biodiversity [38]. Also, social aspects such as the poor working conditions are associated to the cultivation of this crop [39].

In 2017, the biodiesel production in Portugal was based mainly in waste cooking oil and edible oils (rapeseed and soybean). Figure 1.3 shows the contribution of each type of raw material for biodiesel production (“others” include acid oils from soapstock) [18]. The most used raw material was WCO (56%), mainly imported. Rapeseed and soybean oils were mostly extracted in Portuguese industrial facilities. Soybean oil is obtained only from imported seeds. Edible oils represent about 39% of the total amount of raw material used for biodiesel production.

In 2016, the amount of edible oils used was higher (55%) and the waste vegetable oils were 45% of the total consumption. Recent investments were made aiming the cultivation in Portugal of rapeseed crop for biofuel production, in order to reduce the dependency of external markets.

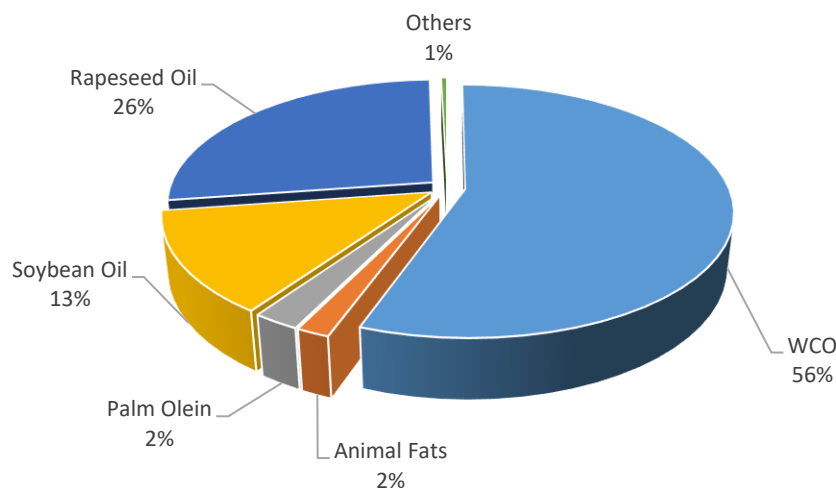


Figure 1.3. Raw materials used in 2017 for biodiesel production in Portugal [18].

1.5 BIODIESEL, A COMPLEMENTARY ENERGY SOURCE TO CONSIDER

Biodiesel is a liquid biofuel, obtained from renewable sources that can be used as a substitute of mineral diesel, being mainly produced from vegetable oils and animal fats. The raw material used for biodiesel production may contain several compounds beyond the triglycerides, namely, free fatty acids (FFA), phospholipids, sterols, water and other impurities [40]. Biodiesel is mostly produced by a chemical reaction called transesterification, discovered by George Chavanne in 1937

(Brussels). This reaction allows to remove the glycerol molecule (from the glycerol ester) present in vegetable oils. From this synthesis, a fatty acid ester (methyl or ethyl) product is obtained, presenting similar physical properties as fossil diesel. The direct use of peanut oil as fuel was also previously tried with success by Rudolf Diesel, showing the potential of this oil as an alternative to fossil fuels [41, 42]. In fact, during the Second World War, due to the scarcity of fuels, the direct use of virgin oils in the engines was a common practice (1939-1945); however, only in 1980, in Brazil, the first patent for biodiesel production was registered (Probiodiesel company) [43].

Biodiesel production by an alkaline catalyzed reaction (route mostly used due to the cost and efficiency), is incompatible with the presence of FFA and water. When FFA are present in the raw materials, the yields of the reaction decrease due to the formation of soaps [44]. Nowadays, biodiesel production from waste vegetable oils is a priority due to their low-cost and environmental sustainability. However, this type of raw material does not have the proper quality to be used directly for biodiesel production due to the high FFA content.

Besides the FFA content, also the presence of high levels of saturated fatty acids can represent an important drawback, because the raw materials, at room temperature, will be in the solid state, creating problems at an industrial scale [45] and during further use of the biofuel. For these reasons, it is very important to study methodologies/processes that allow the reduction of FFA content. The removal of such impurities leads to a pre-treated product with appropriate quality for biodiesel production. In the European Union, biodiesel needs to fulfill the quality requirements imposed by the standard EN 14214:2008. This European standard dictates the fulfillment of limits concerning 25 parameters, being presented in the Annex I.

1.6 BIODIESEL VS MINERAL DIESEL

While fossil fuels, in particular crude oil, can release sulfur compounds into the atmosphere during the combustion, biodiesel produced from vegetable oils is free of sulphur (compound precursor of acid rain), aromatic hydrocarbons (carcinogenic compounds), being for this reason less toxic for living organisms [40]. Biodiesel, according to the Decree-Law nº 152-C/2017, is defined as a “*methyl ester produced from vegetable oils or animal fats, with the proper quality for diesel engines according to EN 14214*”. The most relevant properties of this fuel are comparable with those of fossil diesel. However, in general terms, according to the literature, biodiesel shows in average higher viscosity ($4.03 \text{ mm}^2 \cdot \text{s}^{-1}$) than diesel ($3.43 \text{ mm}^2 \cdot \text{s}^{-1}$), higher flash point (biodiesel: 157°C ; diesel: 58°C)

and higher density (biodiesel: 0.884 g.cm^{-3} ; diesel: 0.842 g.cm^{-3}) [46]. The heating value of biodiesel is also usually 8% lower than the diesel [46].

The use of biodiesel has several advantages compared to fossil diesel; namely, decrease of emissions of carbon monoxide (-11%), particulate matter (-10%), hydrocarbons (-21%) and an overall reduction of GHG since 19% (from palm oil) till 83% (from WCO or animal fats) when compared with the burning of fossil diesel (Directive 2009/28/EC) [47]. The combustion efficiency is directly linked to the availability of oxygen in the internal combustion engine (complete or incomplete reaction). For this reason the overall reduction of GHG emissions, when biodiesel is used, can be slightly different from those previously mentioned [48].

The use of biodiesel allows a better lubrication of the engine components, increasing their lifetime. However, in certain circumstances, due to its solvent properties and composition, it might cause the corrosion of different metallic and polymeric materials (if not prepared for biodiesel) in the automotive engine and admission system [49]. Seals, gaskets, glues or plastics parts are very susceptible to degradation, since biodiesel acts as a solvent of vehicle plastic parts [50]; thus if biodiesel is used, the engine materials must be compatible mainly if high percentages of biofuel are employed.

There are also common problems of clogging due to the formation of catalyst deposits in the tank of fuel or other automotive components, like injectors; for this reason, biodiesel's purification step is fundamental to mitigate this problem.

In cold countries it is advisable to blend commercial biodiesel with additives to prevent the biofuel gelling, improving cold flow properties and consequently allowing a better engine performance [51].

1.7 NON-CONVENTIONAL FEEDSTOCK FOR BIODIESEL PRODUCTION

Nowadays, most of the biodiesel produced in Europe uses as raw material rapeseed oil (edible oil) [52]. The raw material cost is considered to represent approximately 70 – 95% of the total costs of the biodiesel production. Consequently, in addition to the food versus fuel competition already referred, new raw materials (low-cost) should be studied to reduce production costs.

Several efforts have been developed regarding changes in energy policies (fossil fuels vs biodiesel) to increase the contribution of wastes as raw materials.

Non-edible oils are other alternative type of feedstock suitable for biodiesel production that avoids the competition between the energy and the food market. Several non-edible oilseed crops are reported in the literature as having a high potential for oil production. *Jatropha curcas*, *Ricinus communis*, *Crambe abyssinica*, *Simmondsia chinensis* are some of the crops referred in the literature as alternatives to the conventional food crops [53-56]. All of them show a high seed oil content (30 – 60 wt.%) and simultaneously each one has a particular agronomic potential, namely, they could be cultivated on marginal lands (unsuitable for food crops), can provide soil support (perennial), require low fertilizer inputs or can grow in a short period of time [57]. Several non-edible oilseed crops are available all over the world, which can be considered an important benefit, because each region can adopt the most suitable crop according their edaphoclimatic conditions (rainfall, temperature and orography). In Chapter 2 the importance of non-edible oilseed crops as source of oil for biodiesel production will be detailed.

Generally, alternative feedstock for biodiesel production can be divided into three groups: i) non-edible oils (jatropha oil, crambe oil, ricinus oil, karanja oil, etc); ii) waste oils and oils obtained from by-products (WCO, acid oil from soapstocks, fish oil); and, iii) animal fats (beef tallow, pork lard, yellow grease, chicken fats) [57]. According to the Directive (EU) 2015/1513, the production of biofuels using these alternative feedstocks (non-edible oils, fats or waste/recycled oil, algae and microalgae) are called of advanced biofuels. Algae and microalgae are aquatic organisms which show high oil content and a quick growth [57].

As previously referred, in the European Union, through legislation, there is the double counting incentive to produce and use such advanced biofuels, obtained from wastes and by-products.

1.8 BIODIESEL PRODUCTION – TRANSESTERIFICATION REACTION

The transesterification reaction allows to convert triglycerides, diglycerides and monoglycerides (esters of glycerol), in the presence of, more frequently, methanol, into fatty acids methyl esters. If the transesterification reaction is carried out in the presence of ethanol, fatty acids ethyl esters will be produced.

The biodiesel production by transesterification reaction requires that the raw material is constituted by lipids (mostly triglycerides). Lipids are organic molecules with carbon, hydrogen and oxygen

elements (ternary compounds), divided into three major groups, namely, FFA, simple and complex lipids.

The FFA in the triglyceride molecules show hydrocarbon chains with relatively high length, containing in general between 14 and 24 carbons [58, 59]. In Table 1.2 the most common fatty acids found in vegetable oils and fats are presented. FFA are classified as saturated, if their hydrocarbon chains present only single carbon bonds, or unsaturated if it shows double ($-C=C-$) or triple bonds ($-C\equiv C-$). Polyunsaturated FFA present more than one unsaturated bond [58]. When free, at one end of the fatty acids chain the carboxylic group ($-COOH$) is present and in the opposite end a methyl group ($-CH_3$) is present; the triglycerides are esters of glycerol constituted by three fatty acids.

The simple lipids are constituted by fatty acids and a glycerol molecule (alcohol). Glycerol has three sites where hydrocarbon chains (FFA) can be connected (at the level of the three hydroxyl groups). It is possible to establish ester linkages with one, two or three hydrocarbon chains of fatty acids resulting in monoglycerides (MG), diglycerides (DG) or triglycerides (TG), respectively, called sometimes by glycerol esters.

Table 1.2. Molecular formulae of free fatty acids most common in vegetable oils and their correspondent methyl ester

Fatty Acid Methyl ester	Molecular Formulae	Acronym
Palmitic Acid <u>Methyl palmitate</u>	$R - (CH_2)_{14} - CH_3$	C16:0
Stearic Acid <u>Methyl stearate</u>	$R - (CH_2)_{16} - CH_3$	C18:0
Oleic Acid <u>Methyl oleate</u>	$R - (CH_2)_7 - CH = CH - (CH_2)_7 - CH_3$	C18:1
Linoleic Acid <u>Methyl linoleate</u>	$R - (CH_2)_7 - CH = CH - CH_2 - CH$ $= CH - (CH_2)_4 - CH_3$	C18:2
Linolenic acid <u>Methyl linolenate</u>	$R - (CH_2)_7 - (CH = CH - (CH_2))_3 - CH_3$	C18:3

When the ester linkage is established (Figure 1.4), the glycerol molecule loses a hydroxyl group (OH^-) and the fatty acid a H^+ cation leading to water formation (H_2O) [58].

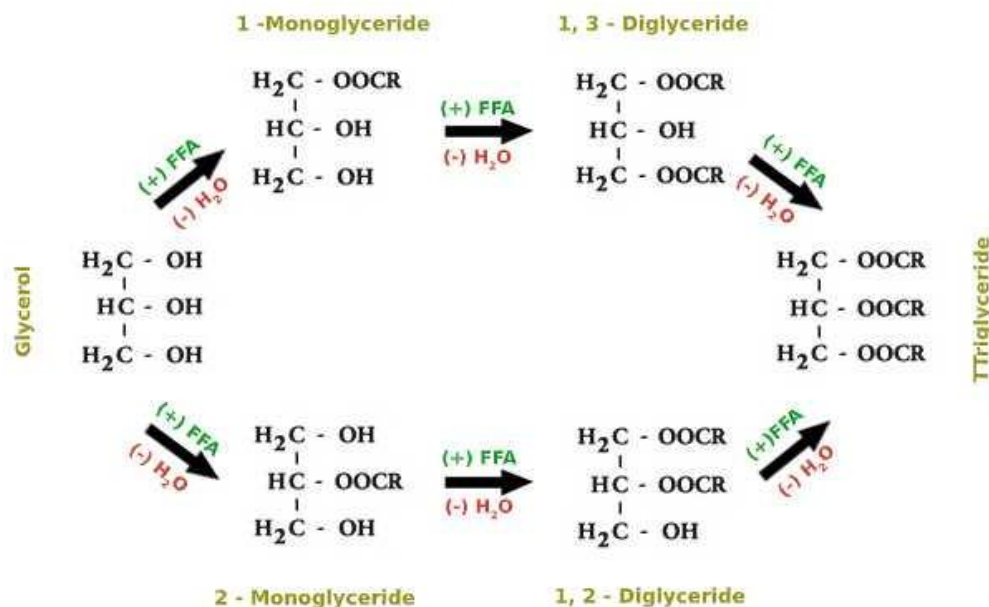
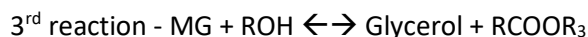


Figure 1.4. Production of mono-, di- and triglycerides from the interaction between glycerol molecule and FFA molecule – adapted from [85].

The complex lipids typically show a fatty acid group, a glycerol group and an additional group (phosphate group, sugar, etc.) [60].

For biodiesel production, TG, DG and MG (glycerol esters) react with the alcohol added to the mixture (methanol or ethanol), producing a new type of ester, depending on the alcohol used. This fact justifies the name given to the reaction, which correspond to a "transformation" of an ester (esters of glycerol) in another type (alkyl ester). The overall reaction of transesterification comprises three consecutive and reversible steps, presented below:



It should be highlighted that the DG and MG that react with alcohol are those that are present in the raw material and those that result from the first and second reaction (DG/MG). For each mol of triglycerides three moles of ester are produced (RCOOR_1 , RCOOR_2 and RCOOR_3). Usually, the reaction is catalyzed by alkaline reagents in order to increase the conversion rate, the most common catalysts being potassium hydroxide, sodium hydroxide and the respective alkoxides, hydrochloric acid and sulphuric acid [40, 61].

1.8.1 BIODIESEL PRODUCTION – ALKALINE TRANSESTERIFICATION

Homogenous alkaline catalysts are the most used due to their low reactivity, when compared with the acid catalysts. Acid catalysts can in fact promote simultaneously esterification (from FFA) and transesterification (from triglycerides) reaction, although these catalysts have associated slow reaction rate, requiring high energy consumption (high temperatures); also leads to problems related with the corrosion of the equipment [62]. The major drawback of using such homogeneous catalysts is associated with the environmental impact caused by the resultant acid wastewater [62]. Alkaline catalysts also require milder reaction conditions such as low operational temperatures [63].

The reversibility of the reaction implies the use of an excess of alcohol to ensure that the chemical equilibrium is directed to the products. Figure 1.5 shows the overall reaction of transesterification where for each mole of triglyceride three moles of alcohol (ROH) are required. Biodiesel production can be also performed using heterogeneous catalysts. This type of catalysts makes the biodiesel purification easier, by simple filtration, also increasing the economic value/quality of the glycerol (by-product), which can be used, for instance, as a reactant in glycerolysis pre-treatment process, as described in the section 1.9.2 [64, 65]. Heterogenous catalyst are still not of transversal use due to the more extreme reaction conditions required, the lower reaction rates and the residual leaching observed [61, 66].

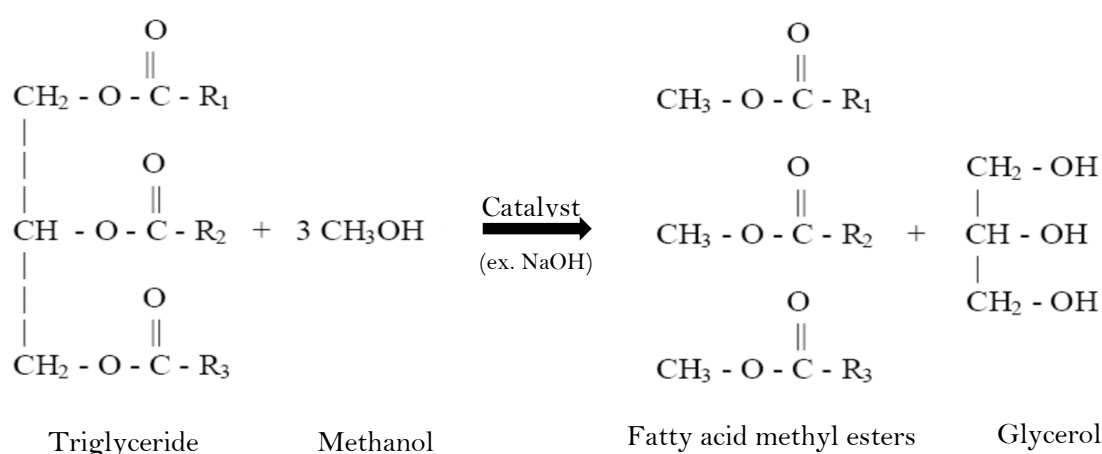


Figure 1.5. Transesterification reaction using methanol as alcohol and sodium hydroxide as catalyst.

The most relevant variables in the transesterification reaction are temperature, stirring speed, reaction time, catalyst type and its concentration, and alcohol type and its concentration. For alkaline transesterification (with NaOH and methanol) the most advisable conditions are the following [67]:

- Temperature: 65 °C (close to the alcohol boiling point);
- Alcohol:methanol molar ratio: 6:1 (the double of the stoichiometric amount);
- Reaction time: 1 hour;
- Amount of catalyst: 1 wt.% ($w_{\text{catalyst}}/w_{\text{feedstock}}$);

At the end of the reaction, the products, namely fatty acid methyl/ethyl esters (biodiesel) and glycerol (by-product) should be separated by density. In case of using homogeneous catalyst, it is also necessary to perform a washing step, to remove it from the product. The biodiesel phase is usually “washed” first with an acidic solution and after with water until close to neutral pH.

Alkaline transesterification, the most widely process used at industrial scale, allows the production of fatty acid methyl esters (FAME) in a short time, however with a major drawback, the consumption of water during biodiesel purification (elimination of the catalyst from biodiesel), producing an alkaline wastewater. Consequently, alternative purification processes have also been developed, such as the use of ceramic membranes or ion exchange resins [80, 81].

When refined oils are used as feedstock for biodiesel production, the transesterification reaction can take place without major drawbacks because the oils are mostly constituted by TG. On the other hand, for waste vegetable oils, the presence of FFA and other impurities decreases the yields of the

reaction and sometimes makes impossible to perform the process without any procedures. For this reason, before the biodiesel production by alkaline transesterification, it is necessary to do a pre-treatment of this raw material aiming, namely, to convert FFA into glycerol esters (feedstock for biodiesel) by the glycerolysis reaction or directly into ethyl or methyl esters (esterification reaction).

The use of ultrasound, microwaves, super-critical conditions, ionic liquids or enzymes have been, until now, poorly studied, due to high associated costs, although these processes could be important in the future regarding the development of more efficient and ecofriendly processes for biodiesel production [68-71].

1.8.2 ENZYMATIC TRANSESTERIFICATION – BIODIESEL PRODUCTION

For the transesterification reaction, enzymatic catalysts arise as a powerful alternative to the conventional chemical catalysts. Using chemical catalysis, the operational temperatures are higher than those verified using enzymatic catalysts and the quality of the oil is a critical factor (low FFA content is required) [72]. Also, biodiesel purification step requires the consumption of high amounts of water producing wastewater that should be treated. Table 1.3 presents the comparison between chemical and enzymatic processes used for biodiesel production.

In fact, transesterification reaction using enzymatic catalyst can solve some of the issues mentioned before, making the reaction more efficient, highly selective, involving less energy and avoiding wastewater generation [73]. Also, the separation between biodiesel and glycerol is easier when enzymatic catalysts were used, since the glycerol will be pure. Lipase also shows an excellent catalytic activity and stability in non-aqueous media [74].

On the other hand, the enzymatic inactivation caused by the alcohol (methanol or ethanol) should be considered. Although, alcohol's stepwise addition can solve this problem [75].

Table 1.3. Properties associated to chemical and enzymatic catalyzed processes used for biodiesel production [73]

Property	Transesterification Process		
	Acid Catalyzed Process	Alkaline Catalyzed Process	Enzymatic Catalyzed Process
Biodiesel yield (%)	Usually > 90	Usually > 96	> 96
Reaction Temperature (°C)	>100	60 – 80	20 – 50
Catalyst cost	Low	Low	High
Purification of methyl esters	Washing with H ₂ O	Washing with H ₂ SO ₄ /H ₂ O	None
FFA content in feedstock	FFA convert into Biodiesel	Soaps formation	FFA convert into Biodiesel
Catalyst recovery	Difficult	Difficult - acid neutralization	Easy

Enzymatic biocatalysts can be divided in two categories, namely extracellular lipases and intracellular lipases. For extracellular lipases, the enzyme is recovered from the microorganism and purified. For intracellular lipase, the enzyme remains inside the cell, although available to promote the reaction [74].

Extracellular lipases are the most common type of enzyme used for biodiesel production, catalyzing also hydrolysis reactions or esterification reactions, depending on the conditions and products present in the mixture [74].

Most of the enzymes produced are from microbial origin (bacteria and fungi). From these, fungal strains are preferable because they produce lipase extracellular, making easier their extraction from the fermentation mixture [76]. Most of lipase used as enzymatic catalyst are produced from microorganisms as *Thermomyces lanuginosus* and *Aspergillus niger* fungi since those microorganisms produce lipases extracellularly and might lead to high resulting yields [76, 77].

Commercial lipases are easy to prepare and use (low-cost), but their reusability is a key aspect aiming economic viability. To enhance those capabilities, immobilization technology has been improved. Different methods can be applied for enzymatic immobilization, namely adsorption, cross-linkage or encapsulation [74]. By supporting the enzyme (immobilization processes), stability is enhanced and reusability is allowed, decreasing industrial costs [78]. Immobilization on solid supports increases the surface area for biocatalytic activity and provide a more uniform distribution [78].

Immobilization processes are essential because allow to recover and reuse the enzymes in the following reactions [79]. The materials/supports used for enzyme immobilization are very expensive and usually the enzymatic activity decreases with the reuse [72].

The lipase activity is related to its three-dimensional structure and the active site (Figure 1.6). Around the active site, it is possible to find a catalytic triad of amino acids, namely amino acid serine, aspartic acid and histidine, which are essential for the catalysis of the reaction [72]. Besides that, the active site is covered by a polypeptide structure (amphipathic structure) called “LID” which protects the active site [80].

In homogenous solutions, in the absence of organic solvents, the active site of lipases is completely isolated of the reaction medium due to the presence of “LID”. Although, in the presence of hydrophobic layer (solvents), the “LID” is partially open (Figure 1.6) [81, 82].

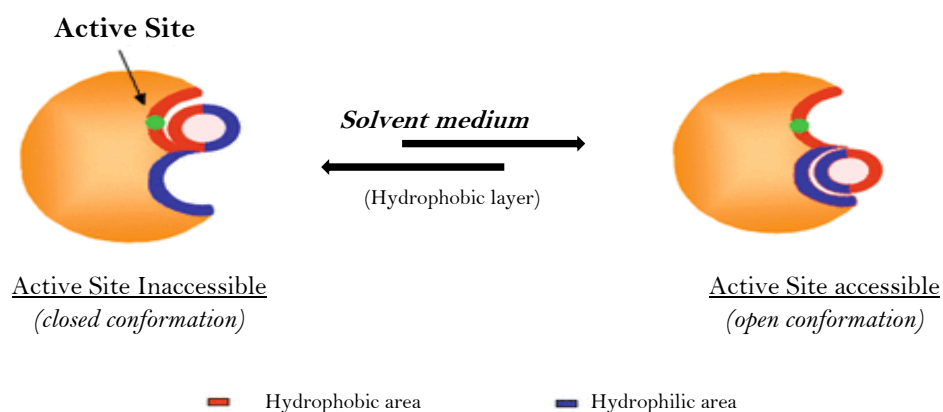


Figure 1.6. Three-dimensional structure of lipase: influence of hydrophobic interface in active site – adapted from [81, 82].

Consequently, in the study developed by Ko *et al.* [83], the transesterification reaction was carried out in the presence of n-hexane because this solvent accelerates acyl migration. For Gog *et al.* [74], the use of organic solvents in enzymatic biodiesel reaction is essential allowing to improve mutual solubility of hydrophobic triglycerides and hydrophilic alcohols. Besides that, organic solvents also protect enzymes from denaturation caused by high alcohol concentration.

The study developed by Lopestro *et al.* [84] evaluated the production of biodiesel using immobilized enzyme. Three mass ratios of biocatalyst/oil were investigated, namely 1%, 3% and 5% (w/w). The

ethanol:oil molar ratio was 3:1, divided in three steps and the reaction temperature was 37 °C. Six cycles of enzyme reuse were assessed, monitoring the FAME content in each cycle. For a biocatalyst/oil ratio of 1 wt.%, the reaction was very slow when compared to the other two higher catalyst concentrations. The concentration of esters was 30% lower than that obtained with 5% catalyst. With 3 wt.% and 5 wt.% of biocatalyst/oil ratio the trends were very similar. The condition using 3% was thus considered the optimal amount of biocatalyst taking in to account the economic and technical aspects.

1.9 PRE-TREATMENT PROCESSES FOR WASTES OR BY-PRODUCTS WITH HIGH ACIDITY

The use of pre-treatment processes has as main objective to decrease the FFA content and valorize waste vegetable oils. The main goal is to convert the raw material in a feedstock with enough quality to be used for biodiesel production. For that, two processes can be performed with the objective to take full use of the materials for biodiesel production, namely esterification and glycerolysis reaction. Saponification reaction might be performed to decrease the FFA content through the removal of FFA from the raw material, however, it results in a loss of part of the raw material as soaps [85].

In Portugal, among the raw materials used for biodiesel production, WCO, palm olein and animal fats are the raw materials which show typically high acidity. WCO usually contains between 2 wt.% and 7 wt.% of FFA and for animal fats the quality of raw material is lower from 5 wt.% to 30 wt.% of FFA [86]. Palm olein is a raw material with an acid value around 4.5 wt.% [87].

The following sections detail the most used pre-treatment processes, previously referred.

1.9.1 PRE-TREATMENT BY ESTERIFICATION REACTION

The esterification reaction allows the production of esters directly from FFA. In the presence of an alcohol, the FFA react with an alcohol, usually methanol, producing methyl esters and water. In Figure 1.7 the esterification reaction is represented. Underlined in red are the species that “change”, producing “new” molecules. It should be referred that the esterification reaction is usually performed in the presence of a catalyst and at mild temperatures.

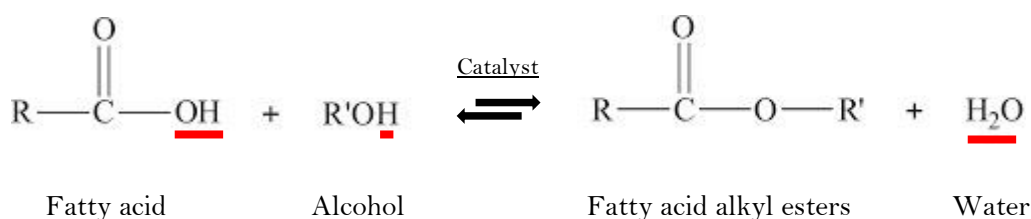


Figure 1.7. Esterification reaction of free fatty acids.

For this reaction, homogeneous (alkaline or acid esterification) or heterogeneous catalysts can be used, namely chemical or enzymatic catalysts [88]. Generally, when the reaction is catalyzed by enzymes (esterification or transesterification reaction), it is reported that ethanol causes less inhibition (reversible inhibition or inactivation) effects than methanol [89, 90]. On the other hand, the addition of methanol in several steps decreases the adverse effects caused by the alcohol on the enzyme, as previously stated for the transesterification.

During the esterification reaction, the main objective is to convert FFA in esters, with the amount of methanol added to the mixture being the stoichiometric required for the reaction of all FFA. At the end of the reaction, it is necessary to perform another reaction – transesterification reaction – converting the remaining mass in esters, i.e. converting TG, DG and MG into alkyl esters.

Typically, the temperatures used during the esterification reaction are lower than 100 °C.

For acid esterification, sulfuric acid is the most used catalyst allowing a quick conversion of FFA into esters [91, 92]. The effectiveness of acid esterification pre-treatment for acid oils will be directly related with the amount of catalyst used, temperature, reaction time and alcohol to FFA molar ratio [73].

According to Marchetti *et al.* [93], the esterification reaction with sulphuric acid allows to obtain a reduction of FFA content near to 95% with a final level of acidity of 0.50 wt.%, using a mixture of oleic acid and pure sunflower oil, at 55 °C, with an ethanol:oil molar ratio of 6:1 and a catalyst amount equal to 2.3 wt.%, relative to oil. In the study developed by Lin *et al.* [94], the production of biodiesel in two steps was started with a pre-treatment by acid esterification using a 6:1 methanol molar ratio during 60 min at 50 °C, being achieved a reduction of acid value from 40 to 4.8 mg KOH.g⁻¹, for rice bran oil (with an initial acidity of 40 mg KOH.g⁻¹). In the second stage (alkaline transesterification reaction), using the raw material with lower levels of FFA, it was possible to produce biodiesel with high purity (around 98.7% of fatty acid methyl ester content).

More recently, in the study published by Chai *et al.* [64], that had as main goal to verify and optimize the acid esterification procedure suggested by the National Renewable Energy Laboratory, the best conditions suggested are methanol:FFA molar ratio of 40:1, catalyzed by 10 wt.% of sulfuric acid in relation to the content of FFA, at temperatures of 55 – 65 °C. The methanol:FFA molar ratio of 40:1 is very different from that indicated by the National Renewable Energy Laboratory (methanol:FFA molar ratio of 20:1). It was also concluded that the methanol:FFA molar ratio of 20:1 is only suitable for oils/fats with FFA levels of 15 – 25 wt.%.

For raw materials with low FFA content (although higher than the threshold value of 1 wt.%, equivalent to around 2 mg KOH.g⁻¹) it is often recommended to blend the acid oil with vegetable oils that typically have low levels of FFA [95]. As a result, the mixture could be used for biodiesel production, avoiding the pre-treatment processes.

The study developed by Chowdhury *et al.* [96], aimed to evaluate the effectiveness of the WCO bioanalyzed esterification process with octanol as well as the possibility of reusing the enzyme (biocatalyst used – Novozyme 435). The operating conditions were: temperature – 60 °C; percentage of enzyme – 5 wt.% ($w_{\text{enzym}}/w_{\text{FFA}}$); molar ratio octanol:FFA of 2.5:1 and stirring at 400 rpm. At the end of the reaction 95% of conversion in octanol esters was obtained as well as the effective reuse of the enzyme during seven consecutive cycles (each cycle of esterification with a duration of 3 hours). In this study, the temperature was significantly higher than that usually used for biocatalysts. Although, high conversion was achieved.

According to López *et al.* [97], the esterification of vegetable oils (initial FFA content around 12%) can be performed using the following optimal conditions: (i) methanol:FFA molar ratio of 1.5:1; (ii) temperature: 25 °C; and (iii) enzyme's concentration: 2.5% ($w_{\text{enzym}}/w_{\text{FFA}}$). At the end of the reaction, the amount of FFA converted was higher than 90% using as catalyst enzyme "Novozyme 435".

Mulalee *et al.* [89], using an ethanol:FFA molar ratio of 2:1, at 45 °C and with enzyme's concentration of 5 wt.% obtained a conversion of free fatty acids (initial FFA content of 93 wt.%) in esters higher than 90%. The enzyme ("Novozyme 435") was reused during 13 cycles (each cycle with duration of 8 hours). Also, the presence of enzyme catalysts simultaneously promote the esterification reaction (converting FFA) and transesterification (converting MG, DG and TG) until the total alcohol consumption [62, 98].

When compared with the alkaline transesterification reaction (60 min), the enzymatic transesterification requires a much higher reaction time (24 h) [61, 62]. The reuse of the enzyme

allows the reduction of operational costs; however, it is necessary to optimize the number of reusing cycles according to the raw material used.

1.9.2 PRE-TREATMENT BY SAPONIFICATION REACTION

It is possible to decrease the acidity of oils and fats through saponification. However, if the main objective is to produce biodiesel, the saponification reaction should only be performed for raw materials slightly acid because this reaction implies the elimination of FFA from the mixture, decreasing the amount of raw material available for biodiesel production. This methodology cannot be considered exactly as the best pre-treatment of the raw material to maximize alkyl esters, because it does not convert FFA into alkyl esters but soaps. Thus, the elimination of FFA is considered inefficient in the biodiesel production chain because there are processes which can convert the FFA into TG, DG and MG (raw materials of biodiesel production). During transesterification, the FFA in the presence of an alkaline catalyst produce soaps (saponification reaction), becoming more difficult biodiesel purification (catalyst removal) with consequent yield's decrease. Figure 1.8 schematizes the saponification reaction. If soaps are formed during transesterification by reaction between FFA and the homogeneous catalysts, their presence in the final product also difficult the separation between glycerol and biodiesel [95, 99]. As previously referred, the production of soaps during the transesterification can be avoided using an acid catalyst, however this route presents low yields when compared with alkaline route, requiring much more reaction time or temperature [99, 100].

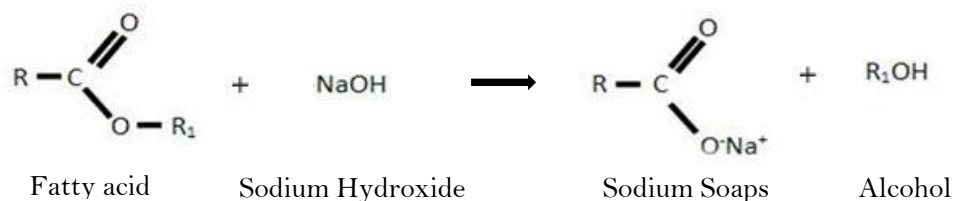


Figure 1.8. Saponification reaction of FFA in the presence of sodium hydroxide.

1.9.3 PRE-TREATMENT BY GLYCEROLYSIS REACTION

The glycerolysis reaction can be used for the pre-treatment of oils and fats with high FFA content. This process is also an alcoholysis; however, the synthesis of glycerides is achieved in the presence of glycerol [101]. The pre-treatment promotes the conversion of FFA into MG, DG and TG and water. Taking into consideration the reversibility of the reaction, it is important removing water during the reaction, to ensure that all FFA are converted into glycerides. The resulting mixture of the reaction depends on the amount of FFA present; also, a high amount of glycerol promotes mainly the formation of monoglycerides instead of diglycerides or triglycerides. Lower amounts of glycerol promote the free fatty acids linkage to the same glycerol molecule (leading to the formation of DG or TG) [101]. Figure 1.9 shows as the glycerolysis reaction is.

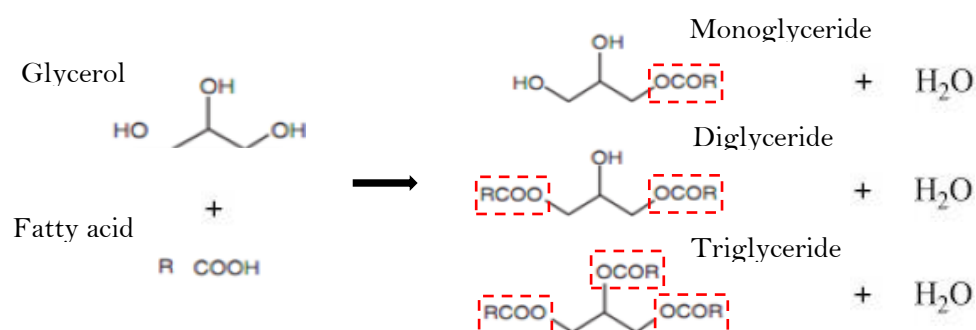


Figure 1.9. Glycerolysis reaction: production of MG, DG and TG - adapted from [101].

As the pre-treatment processes referred previously, also in the glycerolysis reaction it is possible to use catalysts (0.1 to 0.4 wt.%), decreasing the operational temperature (< 150 °C) and reaction time (90 min) [102-104]. Metal oxides (metallic zinc, zinc chloride) with different alkalinity can be used as catalyst; however, it is quite difficult the separation of the catalyst from the mixture.

Glycerolysis reaction might be conducted without catalyst; however, high temperatures are required (200 °C). Since high temperatures can promote undesired decomposition of the raw material, it is advised that reaction temperatures should be lower than 250 °C [102]. According to Costa *et al.* [67], it is possible to reduce effectively the FFA content of fats from sludges produced in waste water treatment plants and dairy industry, using a glycerol:fat ratio of 1:2, at a temperature of 200 °C during 120 min, obtaining a FFA reduction between 85 wt.% to 96 wt.% (corresponding to final acid values of 6.13 – 1.37 mgKOH.g⁻¹). In that study, after this treatment, it was possible to

produce biodiesel by transesterification reaction with yields between 48% and 80% ($w_{\text{biodiesel}}/w_{\text{feedstock}}$). In the study from Islam *et al.* [101], the pre-treatment was performed using an excess of glycerol near to 4% taking into consideration the stoichiometric molar ratio glycerol:oil, at 200 °C during 90 min. Under such conditions, an acidity decrease from 85 mgKOH.g⁻¹ to 1.5 mgKOH.g⁻¹ was achieved. This study was developed in a reaction system with nitrogen atmosphere (reactor with purging).

Aiming at more sustainable and integrated production of biodiesel, the pre-treatment by glycerolysis can be very useful in the valorization of glycerol, the by-product from transesterification. The technical viability of glycerol valorization should however be carefully analyzed because the glycerol by-product generally presents several impurities, namely, phosphorus, soaps or other impurities originally present in the raw material.

1.9.4 PRE-TREATMENT BY HYDROESTERIFICATION REACTION

The benefits obtained by the producers of biodiesel, namely, the biofuels titles, when producing biodiesel with raw materials recovered from wastes (low-cost feedstock) is a very relevant aspect. However, most of these raw materials impose several problems during the production of biodiesel from the conventional route (alkaline transesterification) for the reasons previously stated.

Hydroesterification emerges as an alternative way of producing biodiesel directly from acid oils/fats. In a first phase, this process aims to transform the oil mostly constituted by triglycerides in FFA, through the hydrolysis of the glycerides (see Figure 1.10). The hydrolysis reaction might occur in three different steps, depending upon the type of hydrolyzed glyceride. For each mol of triglyceride hydrolyzed, 3 mol of FFA and 1 mol of glycerol are produced [105]. After that, an esterification (2nd phase) of all FFA present in the mixture is promoted. The procedure described has special relevance for oils and fats very acid, because most of the oil is constituted by FFA and, for this reason, only the remaining glycerides fraction is hydrolyzed. From the 1st phase of the process FFA and glycerol are produced. At the end of esterification reaction (2nd phase) it is only necessary to recover the alcohol in excess.

Hydrolysis (1st phase) of the oils can be performed in the presence of an enzymatic catalyst or using an alkaline reactant. For alkaline reaction, a saponification reaction occurs, being the glycerides

converted into salts (soaps) and glycerol. Aiming to obtain FFA, the soaps produced can be converted into FFA in the presence of an acid. The presence of the acid allows the breaking down of the soaps into free fatty acids and salt. This methodology has associated high energy consumptions and requires the referred acidification step [106, 107]. After that, the available FFA are converted into esters by esterification reaction (2nd phase).

Esterification reaction (2nd phase) can be performed by enzymatic or acid esterification reaction, as previously described (section 1.9.1).

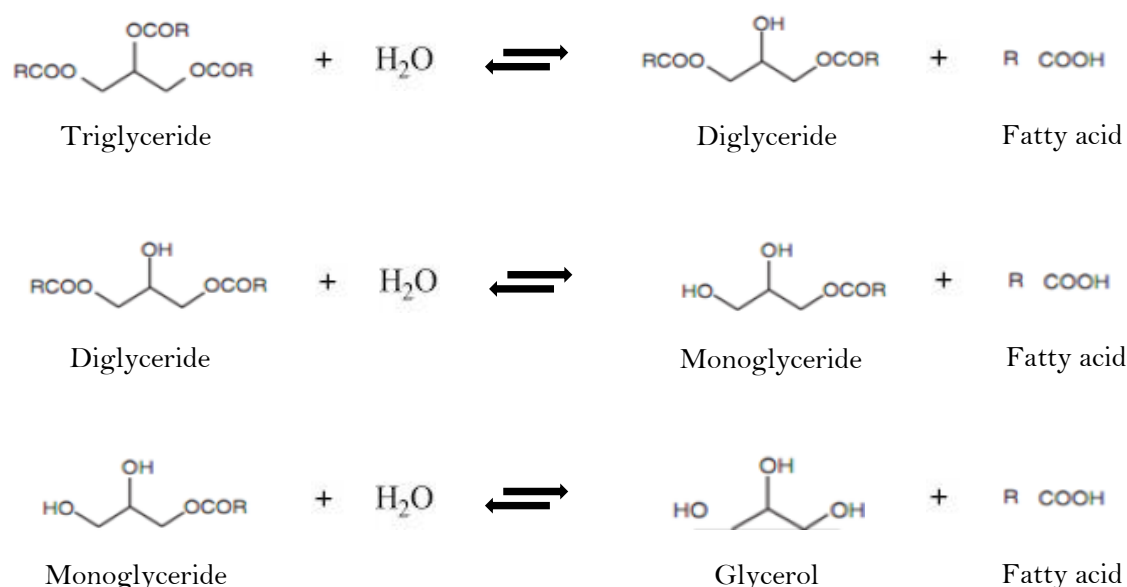


Figure 1.10. Hydrolysis reaction for mono-, di- and triglycerides — adapted from [67, 105].

The use of enzymatic catalysts allows decreasing the energy consumption and obtaining a food-grade glycerol [108]. At the end of hydroesterification process, pre-treated oil, water and glycerol fraction should be separated by centrifugation (immiscible fraction).

The hydrolysis of the glycerides is achieved using hydrolytic enzymes that operate at mild temperatures (35°C) which might contribute to make the process economically viable [109]. Enzymatic hydrolysis can also be performed at room temperature in the presence of water. The use of biological catalysts becomes possible to reuse the enzyme in subsequent cycles [82]. To increase the efficiency of enzymatic hydrolysis, it is advisable to have a high interfacial area between the oil and the aqueous phase that contain the enzyme, promoting higher conversion rates (higher enzyme activity) [82]. Figure 1.11 depicts the hydroesterification schematic procedure [108].

In the study developed by Cruz *et al.* [19], enzymatic hydrolysis (1st step) was performed during 24 h (35 °C at 200 rpm) with 3 wt.% of enzyme and 1:0.5 (w:w) of water relatively to the oil. Following, by enzymatic esterification (2nd step), at 35 °C with 2 wt.% of enzyme concentration and 2:1 methanol:acid molar ratio, it was possible to obtain 83.6 wt.% of fatty acid methyl ester in the final product. Another study, developed by Ting *et al.* [110] used an hybrid system of hydroesterification, which considered enzymatic hydrolysis followed by acid esterification. In this case, hydrolysis was catalyzed by binary immobilized lipase, resulting in the hydrolysis of 88% of the soybean oil after 5 h. The hydrolysate was further converted in methyl esters through acid esterification during 12 h at 50 °C. Using 15:1 methanol:oil molar ratio and sulphuric acid concentration of 2.5% biodiesel with purity of 99% was produced [108, 110]. In the literature other studies producing biodiesel with high purity by hydroesterification (> 90 wt.%) are found, becoming clear the relevance of this process [111-113].

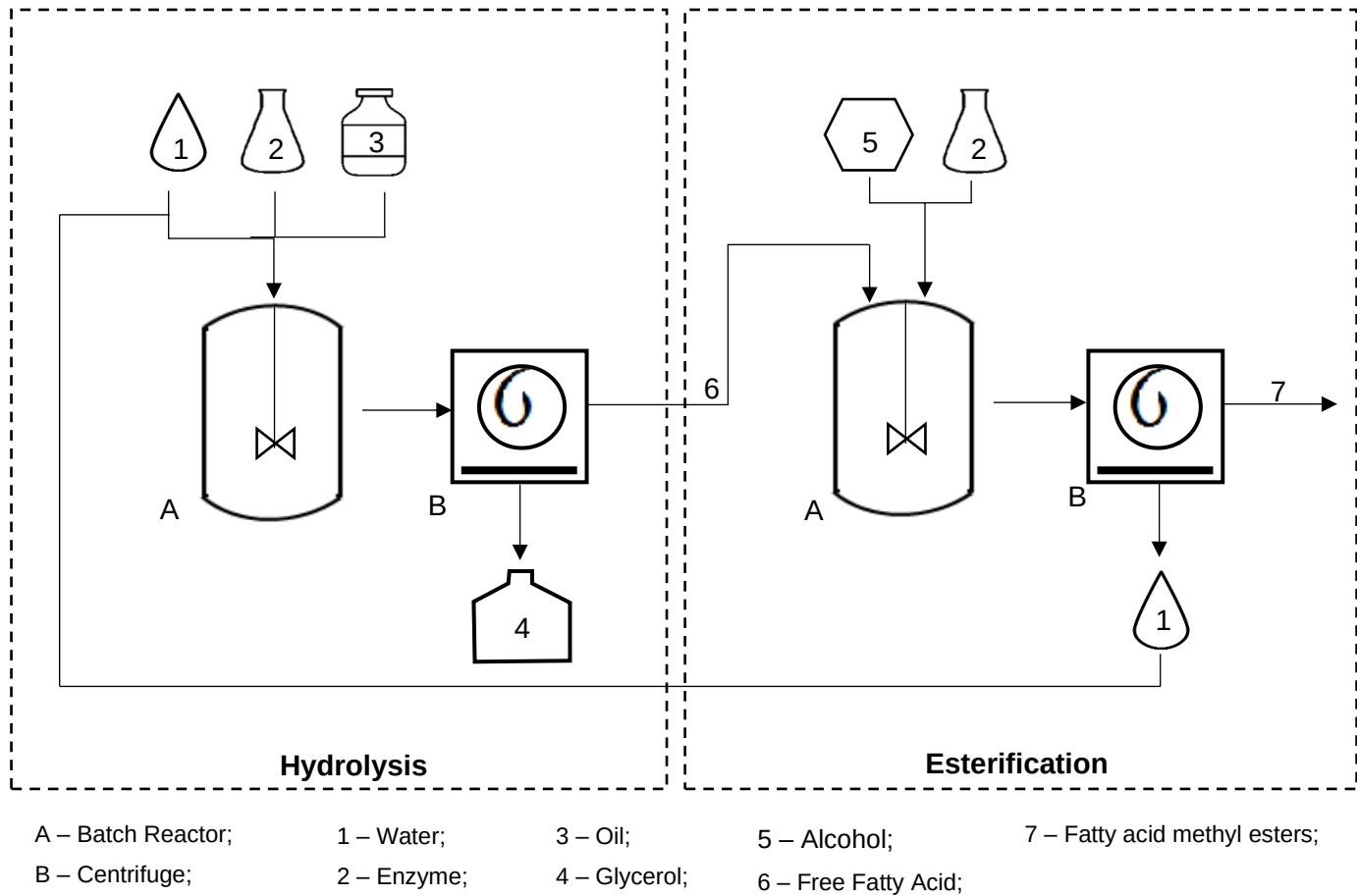


Figure 1.11. Hydroesterification schematic procedure – adapted from [108].

1.10 PRE-TREATMENT PROCESS FOR OIL WITH HIGH PHOSPHORUS CONTENT

The direct use of vegetable oils, after extraction, for biodiesel production is not common because the raw material (oils) typically has a high diversity of constituents, such as tocopherols, metal compounds, FFA or phospholipids. The existence of these compounds is linked to the seed's composition, being also influenced by the type of extraction process, such as mechanical extraction (high pressures) or chemical solvent (at high temperature). Both extraction processes, besides the oil, separate other compounds present in the seeds, which might represent 1 – 3 wt.% of the total oil mass extracted, being generically designated by gums (or phosphatides) [114, 115]. Gums are a variety of compounds which are removed during the refining process that include hydratable and non-hydratable phospholipids, lecithin and other impurities [116]. Some gums present in vegetable

oils have economic value, like lecithin or phosphatidic acid that can be used as a dietary supplements [114].

These compounds are not advisable during transesterification reaction, since they affect the yield of biodiesel reaction. Besides that, the presence of some of these compounds in the final biodiesel produced leads to problems during its use in combustion engines, causing clogging and engines deposits, and decreasing the vehicle's performance [105]. Because of that, oil refining should be carried out with the objective of removing such impurities from the vegetable oils. The refining process is also very important to prevent the deterioration (hydrolysis) of the oil during the storage. Also, the presence of water in the oil might promote hydrolysis with the consequent production of FFA [105]. This fact is explained by the presence of *in-situ* lipases in most oilseeds (the lipase is present in the seed's tissues of different plant species), leading to the hydrolysis of triglycerides in the presence of water. [106]. The refining process also decreases the concentration of metals (sodium, potassium, calcium, magnesium, zinc) in the final biodiesel (usually present in the crude vegetable oils) [117]. Most of these impurities are hydrophilic compounds which can easily be hydrated by water and removed (water degumming) [118, 119].

The oil refining by degumming improves the quality and yields of the biodiesel. The separation between the biodiesel and glycerol can also be affected due to the formation of emulsions during the transesterification reaction [120, 121]. The efficiency of degumming process can be directly measured by the phosphorus content present in the biodiesel. Thus, the presence of phosphorus substances is reported as affecting the transesterification yields [122-124]. According to EN 14214, phosphorous concentration in commercial biodiesel cannot exceed 4 ppm.

Among the impurities before mentioned, phospholipids have special relevance, because in some cases their elimination is difficult, requiring specific procedures. Phospholipids present in the oils can be divided in two distinct forms: hydratable fraction and non-hydratable fraction. The hydratable fraction can be removed with addition of water (polar molecule). The non-hydratable fraction represents the phospholipids that form complexes with certain metal cations such as Ca^{2+} or Mg^{2+} (alkali earth metals) which are poorly hydratable. These phospholipids can only be removed by the addition of an acid which converts them into polar molecules and consequently more hydratable (with affinity to the water molecule). When the phospholipids are hydratable, it is possible to remove them from the oil adding water and performing a filtration or centrifugation step [121, 125].

For water degumming (physical process), the removal of phospholipids is only possible for the phospholipids with polarity, which show affinity with the water molecule (polar molecule). This water-phospholipids complex is heavier than the triglyceride (oil fraction) causing phase separation. In polar molecules the dipole moment is different from zero, for this reason ionic species can link to some phospholipids. The polarity evidenced by the phospholipids is due to the presence of polyatomic ions (ClO^- , OH^- , NH_4^+ , etc.) in the terminal part of the hydrocarbon chain. If the terminal part of the phospholipid is constituted by a cation (polyatomic), the oxygen specie (O^{2-}) from the water molecule will establish a bond with that cation, whereas in the presence of an anion (polyatomic), H^+ will establish a bond with that anion, converting the phospholipid into hydratable form.

The hydration process of phospholipids consists in the interaction/affinity between molecules. Consequently, the hydration process of the phospholipid does not change the lipid structure; thus, at the end of the degumming process it is possible to obtain phospholipids with some economic interest. The hydratable phospholipids are mainly of two types: phosphatidylcholine (PC) and phosphatidyl inositol (PI). PC is easily hydrated because it contains a quaternary amino group with a positive charge for any pH value. For PI, the same characteristics is verified, but in this case it is the presence of inositol group with five free hydroxyl groups that ensure the hydration of the phospholipids independent of pH value [126]. Figure 1.12 shows the molecular structure of the main types of hydratable phospholipids.

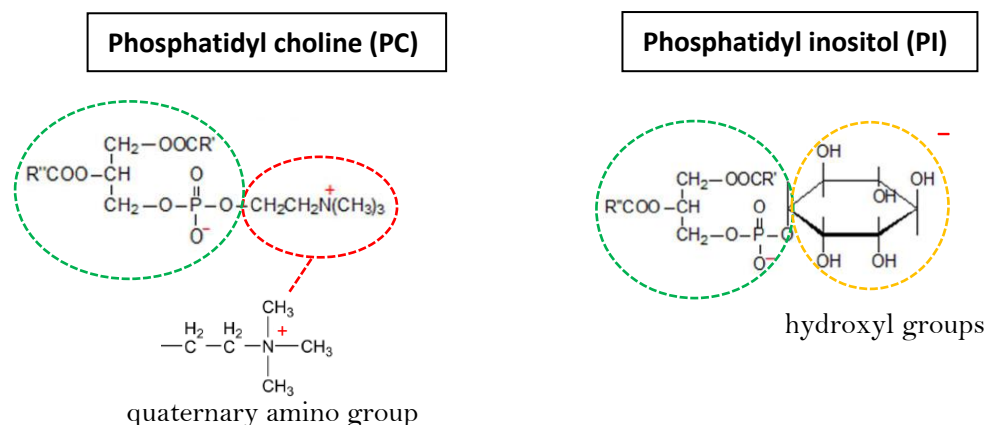


Figure 1.12. Molecular structure of hydratable phospholipids: phosphatidylcholine and inositol — adapted from [126].

Two of the major parameters that affect the water degumming process are the temperature of the mixture and the amount of water added [127]. The optimum temperature of the process will also depend on oil's viscosity. Usually the mixture is heated at 55 – 70 °C, although high temperatures will affect gum's miscibility in the oil (less recovery of gums) [115, 122, 128]. The amount of water added to the oil can range 3 – 4 wt.% ($W_{\text{water}}/W_{\text{oil}}$) [115, 128]. Water degumming process is typically very fast, with the phospholipid's hydration occurring in a short time (5 min.) [129]. The contact time between the water and the hydratable phospholipids is not a critical issue because are enough some minutes for the hydration to be complete; however, it is usual to find in the literature large periods of contact (30 min.) [114, 122, 128].

Besides the hydratable fraction, in the crude oils are commonly found non-hydratable phospholipids. In this case, a chemical degumming process is necessary, to convert the non-hydratable phospholipids into hydratable ones, as already mentioned. The principle of the process consists in rearranging the molecular structure (three-dimensional arrangements) of the phospholipid or in promoting a change of their charges, converting them into the hydratable form. The promotion of these changes is achieved with the addition of chemicals (pH variation) which leads to a molecular structural change, allowing the formation of a water-phospholipid complex, easily separated from the oils, as explained before. Two non-hydratable phospholipids are usually found in crude oils, namely phosphatidyl ethanolamine (PE) and phosphatidic acid (PA) (Figure 1.13). In practice, phosphoric acid, citric acid, oxalic or tartaric acid can be used to reduce the pH, although the most used in chemical degumming is phosphoric acid due to legal requirements imposed by the food industry and due to its lower cost compared to the others reactants [114].

For PE, the terminal part of the hydrocarbon chain shows a chemical structure with two opposite charges (dipolar ion, internal salt or amphoteric ion) in non-adjacent atoms, leading to a charges balance null, for an oil mixture with neutral pH (Figure 1.12) [130]. When the pH is below 3 or above 9, the phospholipid molecule becomes hydratable because the charge balance of the molecule is no longer null [126, 127]. For an intermediate pH range, the dissociation of the phosphate group (PO_4^{3-}) and the formation of internal salt (few hydratable) is observed [129, 131]. Internal salt is defined as an electrically neutral chemical structure with opposite charges at both ends of the molecule.

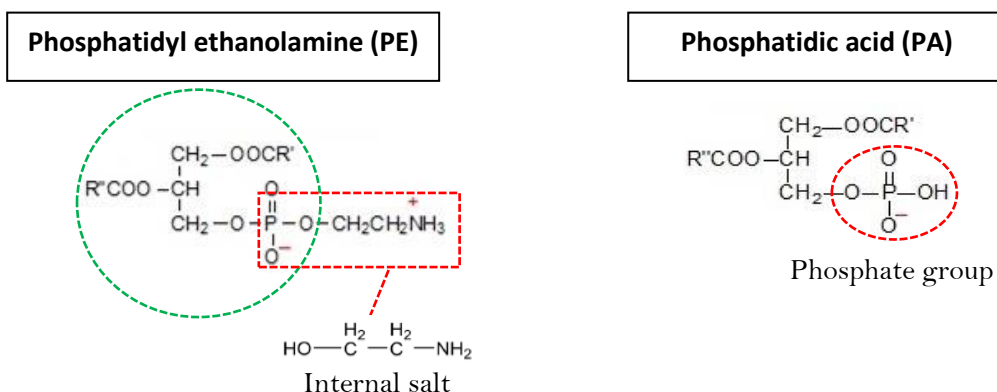


Figure 1.13. Molecular structure of non-hydratable phospholipids: phosphatidyl ethanolamine and phosphatidic acid — adapted from [126].

For the phosphatidic acid, present in an oil, the use of low pH does not convert it into a hydratable form, despite the presence of two OH^- group in its structure [132]. This is the major disadvantage of the chemical degumming using an acid [126]. For alkaline oil mixtures ($\text{pH} > 7$), it is possible to dissociate PA showing two negative charges (hydratable). PA is usually present in the oils as a calcium salt (without charges); however, when alkali refining is performed, these salts can be removed [129].

For chemical degumming several methodologies are proposed by different authors [115, 122, 128, 133]. The proposed dose of commercial acid (phosphoric acid) varies between 0.1 wt.% and 0.5 wt.% ($W_{\text{acid}}/W_{\text{oil}}$) [122, 128, 133]. The addition of acid will allow pH variation and consequently the conversion of the non-hydratable fraction of phospholipids into the hydratable one. Usually, after the addition of acid to oil, a subsequent addition of an alkali reactant (base) is referred in several studies. The main purpose is to neutralize the mineral acidity caused by the acid present [114, 128]. The amount of alkaline reactant added depends on the amount of soaps (resulting from the saponification reaction) which is acceptable to have in the gums [114, 127]. If the objective is the recovery of the gums, for further use, then it is advisable to use small amounts of base. At an industrial level, neutralization is usually performed using sodium hydroxide solution with a concentration of 5 — 8% and a dosage of 2% ($V_{\text{NaOH}}/V_{\text{oil}}$) [114, 128]. The final stage of the procedure requires the addition of water with the objective of removing the hydratable fraction produced at chemical step. Water dosage suggested ranges 2 — 15% (wt.%) [122, 127, 128]. The sequence of reagents added can vary, although the acid must be added initially and only after the water or the

alkaline reactant, due to the processes previously described. Finally, the temperature of chemical degumming referred in several studies is between 50 °C and 90 °C [114, 122, 127].

The influence of the different conditions referred before in the degumming process can be directly assessed by the phosphorus content present in the final oil, parameter that might be determined according standard NP 1994:2000. The procedure involves the calcination of the sample to eliminate organic matter followed by phosphorus determination using UV spectroscopy.

According to what was referred before, several wastes and by-products might be studied aiming at raw material diversification in the bioenergy sector, or more specifically, the biodiesel sector. Also, several non-edible oilseed crops with potential to be used as feedstock for biodiesel production are reported. Due to the high importance of biofuels in the actual world energy scenario, in the present work the viability to use alternative non-edible oilseed crops (*Crambe abyssinica* and *Cynara cardunculus*) in Portugal and to recover oils from wastes or by-products for bioenergy production was studied.

Agronomic studies were conducted, raw materials were characterized and according to the characteristics of each type of oil, raw oil refining and different pre-treatment processes were performed. Following, biodiesel was produced, and the product characterized aiming to produce biodiesel with higher quality from alternative and possibly complementary feedstocks.

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CHAPTER 2. *NON-EDIBLE CROPS AS ALTERNATIVE RAW MATERIALS FOR BIODIESEL PRODUCTION – AN OVERVIEW*

ABSTRACT

Biodiesel is a mixture of fatty acid alkyl esters which can be used as diesel substitute, being produced mostly from edible oils. In agreement, many research efforts have been made to find alternative feedstocks for biodiesel production which: (i) do not compete with the food market; (ii) are cheaper, reducing production costs; and (iii) can be cultivated on non-agricultural or marginal land. According to the revision performed, among more than 300 oilseed plant species, only 40 have the potential for biofuel production, based on the oil content of the seeds. A relevant example of non-edible crops with high potential for biodiesel producing are *Ricinus communis*, *Crambe abyssinica*, *Jatropha curcas* and *Simmondsia chinensis*. This review provides a comprehensive assessment of these non-edible crops suitable for biodiesel production due to their high seed oil content and details their typical characteristics as well as the advantages and constraints of their use for biodiesel production. *Ricinus communis* and *Crambe abyssinica* can be cultivated as annual crops and their plant growth cycle vary between 90 and 180 days. This species should be cultivated in regions with moderate rainfall and temperature. *Jatropha curcas* and *Simmondsia chinensis* are perennial crops which survive during several years. These species are adopted to semi-arid regions, although to maximize seed's yields, rainfall or irrigation should compensate crop evapotranspiration potential. Among the non-edible crops analyzed, *Crambe abyssinica* and *Jatropha curcas* showed special interest to be cultivated in Portugal, due to their edaphoclimatic requirements; also, crambe has a short growing cycle and *Jatropha curcas* requires low agronomic inputs during its growing cycle.

2.1 SCIENTIFIC RELEVANCE

The population growth forecast for the 21st century, estimates an increase around 38% until 2050 [1]. Such increase will lead to the emergence of several problems, such as the increase of population density in cities (impacting transport, energy and waste management), the decrease of ecological diversity (climate change) and the higher pressure exerted on fertile soils (affecting food production) and water resources (affecting drinking water). If appropriate measures are not taken, there will be millions of people without the minimum resources to subsist and living in degraded environmental conditions. Such growth will be especially felt in Africa and Asia and it will be vital for emerging countries the access to energy [1-4].

The access to energy and industrialization can be considered the exit to poverty, boosting international conflicts. Thus, it becomes essential to change the world energy paradigm, once the resources currently exploited are not infinitely available and their consumption causes high environmental deterioration [5]. It is therefore appropriate to consider new forms of energy that go beyond the fossil resources, where engineering is expected to have a major role in the development of new alternatives, which should be economically viable. In fact, renewable energy sources are presented as technologies with small environmental impact; however, only few of them are considered economically sustainable [6].

The production of biofuels from vegetable oils (renewable energy sources) started to have a high impact by the end of 20th century since they are, in several cases, environmentally and economically viable. In what concerns biodiesel, which replaces fossil diesel, it can be obtained from several food plant species, mostly from *Brassica napus* (colza/rapeseed), *Glycine max.* (soybean), *Helianthus annuus* (sunflower) and *Elaeis guineensis* J. or *Elaeis oleifera* (African and American palm). In fact, it is estimated that around 95% of biodiesel production is made with these oils [7]. Once these species are also used for human consumption, a competition between the energy market and the food market occurs, causing the increase of prices due to high demand; on the other hand, it is not reasonable to use food oils for biofuels production, considering that globally there are problems of food shortages. Furthermore, fertile soil is used for the production of oil and usually intensive farming practices are adopted leading to the soil impoverishment and erosion problems [8, 9].

In some developing countries, the cut of forests is observed aiming to sow energy/food crops, thus causing the loss of biodiversity [10]. Therefore, non-edible crops emerge as an important alternative for the sustainable production of biofuel, especially when planted or sown in marginal lands/wastelands [11, 12]. Some examples of non-edible crops, starting from the most used and

studied, are: *Jatropha curcas* L. [13-16], *Pongamia pinnata* [15, 17, 18], *Calophyllum inophyllum* [13, 15, 19], *Crambe abyssinica* [20, 21], *Ricinus communis* [22, 23], *Azadirachta indica* [15, 24, 25], *Gossypium hirsutum* (cotton seed) [26, 27], *Croton megalocarpus* [16, 28], *Madhuca indica* [29], *Hevea brasiliensis* (rubber seed) [30, 31], *Simmondsia chinensis* (jojoba) [4, 32], *Eruca Sativa* [33, 34], *Sapindus mukorossi* (Soapnut) [32, 35], *Moringa oleifera* [32, 35], *Cerbera adollam* [4, 13], *Thevetia peruviana* [4, 36] and *Nicotiana tabacum* [37, 38]. These crops are from different origins, adapted to different ecological niches. The knowledge about the environmental requirements of each crop allows to maximize biomass yields and the profits of their cultivation.

The use of non-edible vegetable oil for biodiesel production is extremely attractive because they are expected to present a lower cost when compared with the traditional feedstocks. In addition, the economic value of the non-edible crops goes beyond its content in oils, being possible to produce simultaneously others products with high economic value like alcohol extracts with antibacterial action or natural antioxidants [39, 40].

In the literature is possible find several studies (paper reviews) on the use of non-edible oils for biodiesel production [13, 17, 19, 21-23, 30, 33, 37, 41], although some take into consideration the agronomic conditions, no studies were found where such information is compiled.

In agreement, this study presents a review of agronomic studies on using non-edible crops, namely those with higher potential for biodiesel production, previously refereed. An overview and assessment of the agronomic conditions required by each crop was performed, considering the adequate establishment of the selected crops at different parts of the world.

2.2 GENERAL CONSIDERATIONS ON THE CULTIVATION OF NON-EDIBLE CROPS

Several non-edible crops are currently studied by the scientific community for the production of biofuels, but among them, few are considered a real alternative taking into account the quality of biofuel produced and the economic aspects (costs with the crop establishment and oil extraction/refining). The study presented in this chapter provides a detailed analysis on the agronomic studies of the most promising plants for biodiesel production: *Ricinus communis* L., *Crambe abyssinica*, *Jatropha curcas* L. and *Simmondsia chinensis*. The crops *Ricinus communis* and *Jatropha curcas* are the crops with the highest yields and oilseed contents which might be cultivated in marginal lands. *Crambe abyssinica* has a special interest because it can be harvested in a short period of time (90 days) which allows crop rotation with other species, being also possible, in certain

edaphoclimatic conditions, to harvest seeds two times per year [42]. Crambe's seeds show a high oil content (26 – 34 wt.%) [42]. *Simmondsia chinensis* is a crop which requires low agronomic inputs also presenting high seed and oil yields (seed oil content higher than 40%) [43]. In this last crop, the ability to grow in dry regions, associated to an easy harvest and oil extraction (mechanical processes) shows it as an interesting source of oil for biodiesel production. These four non-edible crops show therefore a high seed oil content and simultaneously each one has a particular agronomic characteristic which might be of relevance for the purpose of biodiesel production (cultivation on marginal lands, low nutrition requirements or a short time for development).

The length of the growing cycle is extremely important to assess if the crop rotation strategy can be adopted (annual crops) aiming a more ecofriendly agricultural practice. Crop rotation is in fact a common practice to establish more sustainable agricultural practices (soil preservation and use of less amounts of chemical fertilizers). This strategy allows to balance plant's nutritional needs, simultaneously ensuring soil preservation. However, the integration with other agricultural practices should be assessed aiming to guarantee the recovery of non-lipidic products with high economic value. Crop rotation is especially important when crops with a short growing cycle are cultivated.

Table 2.1 presents the general conditions employed for the cultivation of non-edible crops in the revised studies.

Table 2.1. General conditions for the production of selected non-edible crops

	Parameters	<i>Ricinus communis</i> L.	Ref.	<i>Crambe abyssinica</i>	Ref.	<i>Jatropha curcas</i> L.	Ref.	<i>Simmondsia chinensis</i>	Ref.
Agronomic	Sowing	After middle of Apr. ¹	[44]	Cool season/Spring sowing	[45]	Nov. – Mar. (Rainy season)	[46]	Late spring	[47]
	Harvest	End of Sept. and middle of Oct. ¹	[44]	Dependent on the date of the sow	–	Mar. – July (South Africa)	[48]	At full maturity	[49]
	Crop Cycle (days)	Perennial	[50]	Annual: 120	[45]	Perennial: 120 – 150	[44]	Perennial	[47]
	Nutrient (kg/ha)	N: 45 – 134; P: 40 – 80	[51]	N:100; P:70	[52] [53]	Requires low amounts of fertilizer	[54]	N: 75	[55]
Production	Oil content (% dry seed)	40 – 60	[56]	36 – 43	[57]	30 – 48	[46]	50 – 56	[58]
	Yield(kg/ha)	1500 – 5000 in irrigated Conditions	[59]	1125 – 5000 in irrigation/fertile conditions	[60]	2000 – 5000 well managed plantation	[61]	400 – 5260 well managed plantation	[58]
Edaphoclimatic	T (°C)	7.0 – 27.8	[51]	5.7 – 16.2	[60]	10 – 35	[62]	21-31	[47]
	Rainfall (mm)	Tolerate: 200 – 4290 Ideal: 750 – 1500	[51]	350 – 1200	[60]	Tolerate: 944 – 3121 Ideal: 1207 – 2001	[62]	Tolerate: 254–380 Ideal: > 600	[63]
	Frost	Tolerant	[56]	Sensitive	[64]	Very Sensitive	[62]	Very sensitive	[65]
	Drought	Tolerant	[66]	Tolerant	[60]	Tolerant	[67]	Tolerant	[47]
	Susceptibility to Pests/Diseases	Fungal diseases	[68]	Insects/Fungal diseases	[69]	Insects/Fungal diseases	[48]	Insects/Fungal diseases	[47]
	pH soil	4.5 – 8.3	[70]	5 – 7.8	[60]	6 – 8/8.5	[71]	5–8	[63]

¹ For the Mediterranean region;

2.2.1 *Ricinus communis* L.

Ricinus communis L. can be considered, at an industrial level, the most important non-edible crop, growing in different environmental niches (tropical, sub-tropical and warm temperatures) [72]. The oil has more than 700 industrial uses beyond biodiesel production (e.g. house roofing, building huts, ingredient for cosmetics and drugs, lubricant, use in insecticidal formulations, use as rubber substitute, use as food additive) [73, 74]. The reported productivities are between 1500 kg.ha⁻¹ (dry conditions) and 5000 kg.ha⁻¹ (irrigated conditions). Seed oil content varies between 40 and 60 wt.% [56].

This crop is known by several names, according the country: *castor*, *mamona*, *ricino*, *mamoneira*, *tártago*, *higuereta*, *castor bean* or *palma-christi* [56]. It belongs to the family of *Euphorbiaceae* (originated from tropical countries) and can grow until 4 – 5 m of height. The fruit is a three-lobed (green or red capsule) and the photosynthesis is the type of C3 (uses the C3 pathway), suggesting that under high temperature and relative humidity it has high photosynthetic rates. Under low relative humidity photosynthetic rate suffers a marked reduction, due to the stomatal closure [75]. The global production of castor seed between 2013 and 2015 was around 1.5 Mt, being India, China and Brazil the main producers [76]. The European Community uses nearly 40% of the castor oil produced in the world castor oil for different purposes (excluding biodiesel production), being dependent on the imported oil and seeds [40, 77].

Ricinus communis L. has a high intraspecific variability which allows its development in several ecological scenarios. It is considered a perennial crop but usually grows as annual crop for a better economic profit. Seed yield depends directly of the number of racemes per plant. Frequently, the plant has many racemes depending on the number of existing branches. This fact represents a disadvantage for commercial production, since a high number of racemes become very difficult mechanical harvest. It is thus advisable to use genotypes (varieties) to obtain plants with fewer branches (up to three racemes), making the plant also more resistant to shattering [44]. At the Mediterranean area, the cultivation of *Ricinus communis* L. starts in April with a vegetative cycle duration of 140 – 180 days. This crop can easily grow in regions with high rainfall intensity (750 – 1500 mm) being however drought tolerant. In India (the largest producer) the crop is cultivated between October and November (after the monsoon season) in arid and semi-arid regions (500-600 mm of precipitation) [55]. For a correct establishment of the *Ricinus communis* is advisable to

fertilize the soil with nitrogen ($45 - 134 \text{ kg.ha}^{-1}$) and phosphorous ($40 - 80 \text{ kg.ha}^{-1}$), ensuring a soil pH between 4.5 and 8.3.

For mechanical harvesting, the plant height should be low. For perennial ecotypes, the plant height varies between 3 and 10 m, being higher than the height of annual plants [78].

The seed oil content (oil yield) depends on the environmental conditions, agricultural practices and the time at which the harvesting is performed. It is indicated that the number of days until the flowering affects negatively seed oil content [44].

This crop should not be cultivated for more than two years in the same location, in order to avoid the proliferation of diseases. After the harvest, the land is used for pasture or for crop rotation using the following crops: maize, peanut, soybean, herbaceous cotton (*Gossypium hirsutum*), fava or sorghum [79].

2.2.1.1 Agronomic Studies

Due to its high intraspecific variability, it is possible to find *Ricinus communis* L. in different parts of world, from semi-arid areas (Argentina) to wetlands (India) [56, 72]. The optimal agricultural conditions will therefore vary in accordance with the location, depending on several features, such as climatic conditions, quality of the soil, date of sowing, amount of nutrients provided, among others. Table 2.2 and Table 2.3 summarize the work carried out by several authors about the influence of different agronomic conditions in the cultivation of *Ricinus communis* L. concerning agricultural and edaphoclimatic conditions and agricultural management and productivity, respectively. The references of the respective studies are presented in Table 2.3.

Table 2.2. A review of the agronomic conditions applied to *Ricinus communis* L. – Agricultural and edaphoclimatic conditions

<u>Country (City)</u>	<u>Agricultural Conditions</u>						<u>Edaphoclimatic Conditions</u>			
	Date of Sowing	Harvest (Days)	Type of Harvest	Flowering (Days)	Density of Sow (plant/ha) x 10 ³	Geometry of the plot	Soil pH	Temperature	Rainfall (Mean, mm)	Water supply
Italy Osimo	April-Early May (1993/94)	90	–	43	–	0.7 m between rows/0.28 m between plants	–	–	320	Dry conditions
Italy Sassari	April-Early May (1993/94)	105	–	48	–	0.7 m between rows/0.28 m between plants	–	–	320	Full Irrigation
Greece Thess.	10,18 and 26 Apr. (1995-97)	122-137	Mech. ^(a)	52-73	–	0.33 m between rows/10 m long/ 6 rows	7.85	1421– 1792 d°C	–	280-300 mm (70-75% ET _c)
Greece Loudias	11, 23 and 25 April (1995-97)	121-151	Mech. ^(a)	42-68	–	0.25 m between rows/ 10 m long/6 rows	7.65	1377 – 1895 d°C	–	140-250 mm
India Palem	(A) 1Oct.(B) 15 Oct. (C) 1Nov. (D) 15Nov. (08-09/09-10)	120	–	61.3-72.1	–	Spacing between seeds 0.90x0.60 m	6.5	15.6 – 37.2 °C	92.7 and 216.8	3 scenarios: (E) 50 (F) 75 (G) 100 mm
USA Texas	(A) 28/05/09 (B) 24/05/10	175	Manual	–	12	0.9 m between rows/0.90 m between plants	8.6	(A) 14.0 – 27.1 °C (B) 17.1 – 27.1 °C	(A) 188 (B) 259	7 scenarios: (35 after planting) 0.1.2.3.4.5.6 mm/day
USA PSREU	(A) 5/05/11 (B) 1/05/12	(A) 138 (B) 57	Manual	–	(A) 66 (B) 33	7.6 m long/0.9 m between rows/6 or 3 plants.m ⁻¹ (row)	–	–	–	–
USA WFREC	18/05/11	155	Manual	–	66	7.6 m long/0.9 m between rows/6 plants.m ⁻¹ (row)	–	–	–	–
Italy Osimo	(A) 28/04/92 6/05/93	Before the end of Sept.	–	–	51	4.2 m wide/7.56 m long/ 0.7 m between rows	–	–	(A) 317 (B) 224	(A) 879 (B) 1278 (RDI)
Italy Sassari	(A) 12/05/92 (B) 15/05/93	Before the end of Sept.	–	–	51	4.2 m wide/7.56 m long /0.7 m between rows	–	–	(A) 111 (B) 126	(A) 441 (B) 592 (RDI)
Usa Texas	May 2007, 2008 and 2009	Last week of Oct.	Mech. ^(a)	–	–	4 rows/10 m long/1 m between rows/6 seeds.m ⁻¹	–	–	314 (2007) 552 (2008) 236 (2009)	123 (2007) 82 (2008) 144 (2009)
USA Texas	11/05/2009 7/05/2010	Last week of October	Mech. ^(a)	–	–	4 rows/6 m long/1 m between rows	–	–	236 (2009) 259 (2010)	144 (2009) 218 (2010)

^(a) Mech. - Mechanical harvesting; ET_c – Crop evapotranspiration; RDI - Regulation Deficit Irrigation.

Table 2.3. A review of the agronomic conditions applied to *Ricinus communis* L. – *Agricultural Management and productivity*

	<u>Agricultural Management</u>					<u>Productivity</u>			
<u>Country (City)</u>	<u>Fertilization (kg/ha)</u>	<u>Type of fertilization</u>	<u>Herb./Fungicides (kg/ha)</u>	<u>Pruning/others processes</u>	<u>Method of Irrigation</u>	<u>Seed yield (t/ha)</u>	<u>Oil yield (t/ha)</u>	<u>Seed oil content (%)</u>	<u>Ref.</u>
Italy Osimo	P ₂ O ₅ :100 N:80	Chemical	Trifluralin: 1.2 Linuron: 0.70	–	–	1.06	0.49	47	[80]
Italy Sassari	P ₂ O ₅ :100 N:80	Chemical	Trifluralin: 1.2 Linuron: 0.70	–	–	1.77	0.86	48	[80]
Greece Thess.	N: 80 P: 48 K: 91	Chemical	Trifluralin: 1.2 Linuron: 0.68	–	Sprinkler Irrig. (BPE ⁽⁷⁾) Drip Irrigation (APE ⁽⁸⁾)	2.46 – 4.35	–	46.3 – 54.2	[44]
Greece Loudias	N: 100 P: 24	Chemical	Trifluralin:1.2 Linuron: 0.68	–	Sprinkler Irrigation (BPE ⁽⁷⁾ and APE ⁽⁸⁾)	2.99 – 5.14	–	44.5 – 53	[44]
India Palem	N: 40	Chemical	–	–	–	(A) 2.34; (B) 1.96 (C) 1.95; (D) 1.61 (E) 2.13; (F) 1.98 (G) 1.78	(A) 1.17; (B) 0.98 (C) 0.92; (D) 0.81 (E) 1.07; (F) 0.99 (G) 0.89	(A) 49.8; (B) 50.1 (C) 50.0; (D) 50.2 (E) 50.0; (F) 50.0 (G) 49.8	[72]
USA Texas	N: 67	Chemical	No herb./fungicide applied	–	Subsurface drip irrigation	Estimated ⁽¹⁾	Estimated ⁽¹⁾	Estimated ⁽¹⁾	[81]
USA PSREU	N: 146	Chemical	Trifluralin: 0.56	PGR ⁽²⁾ : (GA ⁽⁴⁾ + MC ⁽⁵⁾) 140.9 g/ha; HA ⁽³⁾ : (DEF ⁽⁶⁾) 1509.7 g/ha; (Paraquat) 671.0 g/ha;	–	0.686 – 1.357	–	42.9 – 47.6	[82]
USA WFREC	N: 112	Chemical	Trifluralin: 0.56	(A) PGR ⁽²⁾ : (GA ⁽³⁾ + MC ⁽⁵⁾) 135.8 g/ha; HA ⁽³⁾ : (DEF ⁽⁶⁾) 1509.7 g/ha; (Paraquat) 671.0 g/ha; (B) No treatments;	–	(A) 0.746 – 0.990 (B) 0.960 – 1.236	–	(A) 44.7 – 45.8 (B) 45.8 – 46.5	[82]
Italy Osimo	–	–	–	–	Drip Irrigation	1.7 (1992/1993)	–	50 (1992/1993)	[83]
Italy Sassari	–	–	–	–	Drip Irrigation	1.4 (1992) 2.5 (1993)	–	48 (1992/1993)	[83]
USA Texas	N: 32	Chemical	–	PGR ⁽²⁾ applied in 3 stages: Pix [®] and Stance [®] - 0.38; 0.51; 0.73 L/ha;	Drip Irrigation	Estimated ⁽¹⁾ 0.2 – 5.5 t/ha/y	–	–	[84]
USA Texas	N: 32	Chemical	–	HA ⁽³⁾ : ET [®] - 0.081 L/ha; Ginstar [®] (0.21 L/ha) + Finish [®] (0.71 L/ha); Paraquat - 0.62 l/ha;	Drip Irrigation	ET [®] : 2.289; Ginstar [®] + Finish [®] : 2.390; Paraquat: 2.736;	–	–	[84]

⁽¹⁾ Estimated by predictive models (linear correlations); ⁽²⁾ PGR- Plant growth regulators; ⁽³⁾ HA- Harvest aids; ⁽⁴⁾ GA- giberellic acid; ⁽⁵⁾ MC- mepiquat chloride; ⁽⁶⁾ DEF- tribufos; ⁽⁷⁾ BPE - Before plant emergence; ⁽⁸⁾ APE – After plant emergence.

Most of the agronomic studies (Europe and USA) performed the sowing between the months of April and May. The exception happens in the studies carried out in the region of India, mostly due to the different weather conditions. The right time for harvesting is based on the color of crop. The ideal moment occurs when the crop shows a predominantly a chestnut color. Depending on the geometry and area of sowing adopted, some studies adopted mechanical harvesting. For small areas, manual harvesting was performed. To avoid competition between lines, a distance between rows in the range of 0.25 – 0.90 m is advised.

Usually, atmospheric temperature and soil pH are two fundamental parameters for an adequate plant growth [56], however in most of the studies these values are not referred. The analyzed studies showed that the crop can grow in a wide range of temperatures, between 14°C and 37°C. High temperatures (above 35°C) and high water stress can reduce the seed oil content [85]. When reported, soil pH varied between the 6.5 and 8.6, although the studies show that it is possible to cultivate *Ricinus communis* L. in soils with a pH range between 4.5 to 8.3 (thus presenting a high tolerance to pH variation). In this case, pH parameter is not a critical issue for the efficient growth of this crop.

The amount of water supplied to the crop will be decisive to maximize yields. Some authors chose only to provide a fraction of the water required by the plant (Regulation Deficit Irrigation). This methodology aims to promote the growth of the seed (oil content) at the expense of the growth of other parts of the plant (e.g. canopy).

According to revised literature, the amount of water supplied varies between 50 mm and 592 mm, being the highest productivity achieved in Loudias (5.14 t.ha⁻¹) - 250 mm, followed by Thessaloniki (4.35 t.ha⁻¹) - 300 mm [44].

The soil composition is essential to evaluate the amount of fertilizer which should be added, considering that a poor soil will naturally need a higher supply of nutrients. The addition of nutrients can be made by adding chemical or natural fertilizers (in the form of green manure). In all revised studies, chemical fertilization was the selected method of fertilization. The doses applied varied according to the type of macro-nutrient required: nitrogen (40 – 146 kg.ha⁻¹) and phosphorous (40 – 100 kg.ha⁻¹). A potassium fertilizer (91 kg.ha⁻¹) was only applied in one study (Thessaloniki, Italy).

Most studies used herbicides (Trifluralin) to avoid the competition phenomena caused by the presence of weeds.

The pruning technic, for some species, allows an harmonious development of the plant [84]; however, this technique was not applied among the studies analyzed. An alternative process to pruning is the use of chemicals to control the growth of plant (Plant Growth Regulators - PGR), aiming mechanical harvesting. It is also possible to apply Harvest Aids (HA) to guarantee a homogenous level of maturity and a small amount of vegetative material. For the studies which applied these substances the results obtained were not always according to those expected. PGR were inefficient to control the height and the results using HA in many studies were inconsistent, not allowing to obtain the homogenous level of maturity expected.

2.2.1.2 Valorization of non-lipidic components

The cultivation of *Ricinus communis* goes beyond the oil value which is used in several industrial applications such as feedstock for biofuel industry, chemical industry, cosmetics industry, among others [86], as already referred. In addition to the lipidic component it is possible to recover other products (non-lipidic) or use other parts of the plant with economic interest.

According to the study by Rana *et al.* [40], the following alternatives seem relevant (i) use of the leaves for medicinal purposes as well as the production of natural fungicidal and antibacterial, animal feed and insect repellent products (ii) use of the cake as manure/fertilizer (rich in nitrogen) to obtain natural organic amendment/fertilizer products, with examples of cultures benefiting being tobacco, sugarcane and paddy (rice); (iii) use of roots in medicinal purposes; and (iv) use of dried stems and branches for construction activities [40].

2.2.2 *Crambe abyssinica*

This specie, usually known as “crambe”, belongs to the Cruciferous family. It is originally from the Mediterranean region (native of Ethiopia and Tanzania); however, it is able to adapt to the most diverse climatic conditions (cold and dry regions) [52, 87]. Crambe has high potential to be used as feedstock for biodiesel production due to its short annual cycle (90 – 120 days).

It has a moderately branched straight stalk and can reach heights between 70 – 90 cm [45]. The oil extracted from crambe is competitive in comparison with other oilseed crops (peanut, sunflower, among others), since typically, crambe seeds shows an oil content higher than 30 wt.%. Also, it has

several applications such as lubricant, corrosion inhibitor or for the manufacturing of synthetic rubber, adhesives and nylon (high erucic acid content, between 50 – 60%).

The seeds are surrounded by small capsules (hulls – typically light brown) and each capsule only contains one greenish brown spherical seed with 0.8 – 2.6 mm diameter. The capsules represent 25 to 30% of the total volume [60]. Due to its origin (Cruciferous), the seeds of crambe are very small (1000 seeds weight about 6 – 10 g) [60].

Crambe abyssinica is a crop characterized by having low intravariability. The genotypes available are mainly from USA, country where the crop was very cultivated in decade of 60s. In Europe, crambe seeds are not commercially available.

Crambe grows in places with rainfall and temperature in the range between 350 to 1200 mm and 5.7°C to 16.2°C, respectively, growing in soils with a pH between 5.0 and 7.8 [60]. This crop also shows tolerance to draught (roots can reach more than 15 cm depth) and frost (-4°C to -6°C) [51, 60]. The cultivation of crambe is usually made in rainfall conditions. In agreement, this species can grow as a spring crop in Europe.

Typically, crambe shows the first flowers after 52 days, having the flowering stage the length between 12 and 15 days [57]. The seed yield varies according to the region where crambe is sown: Russia (1125 – 1622 kg.ha⁻¹); USA (450 – 2522 kg.ha⁻¹); Austria (970 – 3330 kg.ha⁻¹). Irrigation and use of nitrogen fertilizer lead to an increase of the yields [60]. Table 2.4 summarizes the conditions for *Crambe abyssinica* growth. The productivity of this crop can be increased under a spring favorable weather (rainfall) with an ideal average temperature between 10°C to 14°C.

Table 2.4. A review of the agronomic conditions applied to *Crambe abyssinica* – *Agricultural and Edaphoclimatic conditions*

<u>Country (City)</u>	<u>Agricultural Conditions</u>						<u>Edaphoclimatic Conditions</u>			
	Date of Sowing	Harvest (days)	Type of Harvest	Flowering (days)	Density of Sow (plant/ha) x 10 ³	Geometry of the plot	Soil pH	Temperature (°C)	Rainfall (Mean, mm)	Water supply
Brazil Parana	04/2010; 05/2011;	Around 90	Manual	–	800	6 rows/5 m long/0.25 m between rows	4.32	(Average) 22.1	–	–
USA Arizona	3/11/1995; 24/11/1995; 15/12/1995.	173 (24/04/96) 157 (29/04/96) 137 (30/04/96)	–	84 (26/01/96) 88 (20/02/96) 79 (3/03/96)	–	8 rows/10 m long/0.25 m between rows	–	-5 – 40	33; 33; 33;	636 mm 578 mm 504 mm
	21/10/1997; 5/11/1997; 21/11/1997; 7/12/1997.	175 (14/04/98) 173 (20/04/98) 160 (30/04/98) 140 (6/05/98)	–	100 (29/01/98) 100 (13/02/98) 104 (5/03/98) 92 (19/03/98)	–	8 rows/10 m long/0.25 m between rows	–	0 – 30	143; 143; 141; 117;	295 mm 274 mm 218 mm 305 mm
USA N. Dakota	4/05/1989 and 1990; 15/05/1989 and 1990; 31/05/1989 and 1990.	90; 85; 80;	–	54; 48; 43;	–	6 rows/4.90 or 7.60 m long/0.30 m between rows	–	–	–	–
Italy Bologna	Spring 1994/95/96 (before 15 Mar.)	120; 119; 118; (1994/95/96)	–	71; 78; 75; (1994/95/96)	1000	Plots of 14 m ² /rows with 0.35 m of wide	–	2 – 18	160	Unnecessary
Netherland Wagenin.	19/03; 23/04; 23/05; (1990)	112; 108; 100	–	74; 59; 58;	800 Mar./Apr.; 1200 (May);	Plots of 14 m ² /0.125 m between rows	–	1495 °Cd; 1641 °Cd; 1612 °Cd;	–	Irrigated occasionally
	29/03/1991; 2/04/1991.	130; 113	–	92; 67	800 Mar.; 1200 May	Plots of 14 m ² /0.125 m between rows	–	1708 °Cd; 1724 °Cd;	–	Irrigated occasionally
	29/03/1991; 11/04/1991; 13/04/1991.	–	–	–	800 Mar./Apr.; 1200 May	Plots of 14 m ² /0.125 m between rows	–	–	–	Irrigated occasionally

°Cd – Thermal time units (from emergence to seed ripeness).

Table 2.4. A review of the agronomic conditions applied to *Crambe abyssinica* – *Agricultural and Edaphoclimatic conditions* (continued)

	<u>Agricultural Conditions</u>						<u>Edaphoclimatic Conditions</u>			
<u>Country (City)</u>	Date of Sowing	Harvest (days)	Type of Harvest	Flowering (days)	Density of Sow (plant/ha) x 10 ³	Geometry of the plot	Soil pH	Temperature (°C)	Rainfall Mean (mm)	Water supply
Italy Bologna	First two weeks of Mar. (from 1999 to 2001)	110 – 115 (1999 to 2001)	–	20 – 22	–	–	–	–	250 – 410	Not employed
China Sichuan	1/10.; 11/10;21/10; 1/11; 11/11	–	–	–	150	Plots of 13.33 m ²	–	Early Oct. To Mid Oct. (15 – 18)	–	Precipitation was enough
	–	–	–	–	120; 150; 180;	Plots of 13.33 m ²	–	Early Oct. To Mid Oct. (15 – 18)	–	Precipitation was enough
Brazil Parana	22 /04/2010; 13/05/2011;	90	Manual	–	1000	4 rows/4 m long/0.25 m between rows	–	22.1	–	–
Austria Enzersd.	1 st week April 1988/89/90/91	2 nd half of July each year	–	–	2000	6 rows/Plots of 10 m ²	7.5	9.8	572	–
Austria Gleisdorf	1 st week Apr.1990/91	2 nd half of July each year	–	–	2000	6 rows/Plots of 10 m ²	6.0	8.6	831	–
Brazil São Paulo	29/04/2010; 3/05/2011	118; 113		56; 56		10 rows/4 m long		10 – 33	–	–
Brazil Dourados	26/3; 9/4; 23/4; 8/5; (2012)	–	–	–	625	16 rows/4 m long/0.40 m between rows	–	–	–	–
Brazil Midwest	Mar./April 2011	–	–	–	1200	11 rows/10 m long/0.20 m between rows 5 rows/10 m long/0.45 m between rows	–	–	–	–
	29/04/2011	92	–	–	–	4 rows/10 m long/0.40 m between rows	5.85	–	843	–

The rotation of crambe with other crops allows to prevent the proliferation of pests, diseases and weeds. It is very important, after the crambe growing cycle, to use crops from a family or specie different of crambe. For this reason, crops such as canola or mustard should be avoided [88]. Due to its fast growth, crambe can be sown during the “unseeded period” (fallow system), without affecting the yields of future growing seasons of others crops. Typically, crambe is sown after the cultivation of small grains, such as maize, rapeseed, oatmeal or grain legumes. It is also possible to cultivate simultaneous crambe with other crops such as alfalfa or other forage-type. This crop controls also the soil erosion caused by the snow [69].

2.2.2.1 Agronomic Studies

Crambe is a cool-season crop, that is sown among late autumn and early spring at the Northern hemisphere. For the Southern Hemisphere (Brazil), studies were carried out in autumn when rainfall is higher (March to May). Table 2.4 and Table 2.5 present a revised information concerning the agricultural conditions found for the crambe crop.

Theoretically, it is possible harvest crambe’s seeds after 90 days (North Dakota, Parana), in optimal agronomic conditions, however this is uncommon. For most of the works analyzed, crambe was harvested after 120 – 180 days

Crambe crop allows an adoption of high plant densities (between 1000×10^3 and 2000×10^3 plants.ha⁻¹); however, benefits concerning seed yields were not evident.

Table 2.5. A review of the agronomic conditions applied to *Crambe abyssinica* – Agricultural management and productivity

	<u>Agricultural Management</u>					<u>Productivity</u>			
<u>Country (City)</u>	<u>Fertilization (kg/ha)</u>	<u>Type of fertilization</u>	<u>Herb./Fungicides (kg/ha)</u>	<u>Pruning/others processes</u>	<u>Method of Irrigation</u>	<u>Seed yield (t/ha)</u>	<u>Oil yield (t/ha)</u>	<u>Seed oil content (%)</u>	<u>Ref.</u>
Brazil Parana	P ₂ O ₅ : (A):0; (B):15; (C):30; (D):60; (E):90; N: 9; K ₂ O: 30	Chemical	No herb./fungicide applied	–	–	0.30 – 1.40	0.1 – 0.6	27 – 43	[52]
USA Arizona	N:280 (95/96)	Chemical	No herb./fungicide applied	–	Sprinkler Irrigation	3.20; 2.42; 3.13	–	36.4; 33.8; 39.5	[64]
	N:168 (97/98)	Chemical	No herb./fungicide applied	–	Sprinkler Irrigation	1.44; 1.77; 1.90; 1.79	–	44.0; 48.4; 37.7	[64]
USA N. Dakota	–	Chemical	Trifluralin/hand weeding	–	–	–	–	29.0; 28.3; 26.7	[89]
Italy Bologna	N: 80 (94/95/96)	Chemical	Hand weeding	–	–	3.25 (1994) 2.34 (1995) 2.56 (1996)	1.20 (1994) 0.78 (1995) 0.88 (1996)	36.8 (1994) 33.9 (1995) 34.7 (1996)	[45]
Netherland Wagenin.	N: 160 – 180 (1990)	Chemical	Metazachlor: 1.3	Monitoring developmental stages	–	–	–	–	[90]
	N: 160 – 180 (1991)	Chemical	Metazachlor: 1.3	Monitoring developmental stages	–	–	–	–	[90]
	N: 160 – 180 (1991)	Chemical	Metazachlor: 1.3	Monitoring radiation use efficiency	–	2.49; 2.91; 2.97	–	36.1; 36.1; 35.2	[90]
Italy Bologna	N: 80	Chemical	–	–	–	3.60 – 4.10	–	–	[53]
China Sichuan	–	–	–	–	–	1.56; 1.08; 0.85; 0.83; 0.61	–	–	[91]
	–	–	–	–	–	1.197; 1.965; 1.494	–	–	[91]
Brazil Parana	KCl: 0; 15; 30; 60; 90; N: 9; P ₂ O ₅ : 30;	Chemical	Hand weeding	–	–	0.70 – 1.40	–	32 – 45	[92]
Austria Enzersd	N: 70; P ₂ O ₅ : 70; K ₂ O: 140;	Chemical	–	–	–	2.321 (88); 1.331 (89); 2.330 (90); 2.644 (91)	–	45.1	[93]
Austria Gleisdorf	N: 70; P ₂ O ₅ : 70; K ₂ O: 140;	Chemical	–	–	–	1.398 (90); 2.530 (91)	–	45.1	[93]

Table 2.5. A review of the agronomic conditions applied to *Crambe abyssinica* – *Agricultural management and productivity* (continued)

	<u>Agricultural Management</u>					<u>Productivity</u>			
<u>Country</u> <u>(City)</u>	Fertilization (kg/ha)	Type of fertilization	Herb./Fungicides (kg/ha)	Pruning/others processes	Method of Irrigation	Seed yield (t/ha)	Oil yield (t/ha)	Seed oil content (%)	Ref.
Brazil São Paulo	N – P ₂ O ₅ – K ₂ O (8:28:16): 0; 150; 300	Chemical	Hand weeding	–	–	1.31; 1.58; 1.85 (2010) 0.54; 1.99; 2.21 (2011)	–	35.2; 35.4; 36.2 (2010) 34.4; 36.2; 36.4 (2011)	[94]
Brazil Dourados	N – P ₂ O ₅ – K ₂ O (8:20:20): 300	Chemical	–	Seeds treated Vitavax [®] /Thiram [®] (400 ml/100 kg seed)	–	0.654; 0.952; 0.846; 0.672	0.196; 0.303; 0.289; 0.204	32.7; 32.4; 34.0; 29.5	[95]
Brazil Midwest	N – P ₂ O ₅ – K ₂ O (10:43:00): 110; KCl: 60; NO ₃ NH ₄ : 25	Chemical	–	Seeds treated Protreat [®] (4 ml/kg seed)	–	1.195 (spacing 0.20 m) 1.077 (spacing 0.45 m)	–	–	[96]
	Not applied	–	NIHIL [®]	Seeds treated Derosal [®] (4 g/kg seed)	–	1.731	–	–	[96]

Regarding edaphoclimatic conditions, there is a lack of information in most of the studies found. This fact can be explained by the fact that crambe crop can tolerate low temperatures and drought and for this reason the majority of the studies does not present information. As most studies showed, no irrigation scheduling was pre-established. Irrigation was thus occasional and even sometimes unnecessary (rainfall was enough).

Taking into consideration the crop nutritional needs (Table 2.5), crambe is particularly sensitive to the lack of the macronutrient nitrogen. In most of the studies, nitrogen was added to the soil during sowing. The quantities applied depend on soil composition, being the typical dose between 80 and 280 kg.ha⁻¹. Also, the phosphorous macronutrient can be added to the soil (in different forms, e.g. K₂O, KCl, P₂O₅). In the studies developed in Paraná (Brazil), the productivity was found to be directly dependent upon the amount of fertilizer (P and K) applied; seed oil content did not, however, seemed to be affected by the nutrient inputs.

To decrease the competition with invasive species (weeds), it is possible to use herbicides. Some studies chose to perform weeds control by manual removal. In addition, the treatment with chemical products which allow increase the resistance to pests and diseases was also observed. The study developed by Meijer *et al.* [90] (Netherlands) analyzed in detail the different stages of crambe growth as well the radiation use efficiency (the average value of dry matter production per unit of intercepted radiation) at different sowing dates. According to this study, the major constraint to achieve a high productivity for crambe appeared to be the inefficient use of radiation during seed formation.

The seed yields varied very much between the revised studies (from 0.54 t.ha⁻¹ [São Paulo, Brazil] to 4.10 t.ha⁻¹ [Bologna, Italy]) [53, 94]. Seed yields variation is attributed mostly due to the differences of weather conditions, dates of sowing and agronomic procedures adopted (Table 2.4 and Table 2.5). Based on the results, the highest seed's yields were obtained when chemical fertilization was performed. The availability of water was also very important, although only sometimes irrigation was adopted, depending on weather conditions.

2.2.2.2 Valorization of non-lipidic components

Since crambe production has small expression, few studies were found related with the potential of the non-lipidic fraction of the crop.

Most of the research has been focused on the use of crambe seed cake (resulting from the oil's mechanical extraction) as protein source for animal feed. When the mechanical extraction is made with the seed hull, seed cake shows a protein content from 25 to 31%. Using dehulled seed, protein content increases to a range between 46% and 58% [97, 98].

The presence of glucosinolates compounds (sulfur-containing substances) in the cake makes it unsuitable for swine or poultry, due to its toxicity. Thus, such type of food is more suitable for ruminants which exhibit a higher tolerance to such compounds [69]. In addition, it is possible to perform a detoxication by different extraction procedures to remove glucosinolates [98]. Crambe cake can also be used as an organic fertilizer for agriculture, although in doses lower than 2 t.ha⁻¹, due to allelopathic effects. The use of crambe cake as fertilizer should be avoided after the sowing of maize since it was also observed that when the cake doses are higher than 2 t.ha⁻¹, maize productivity decreases [99].

In crambe oil it is possible to find high concentrations of tocopherols - Vitamin E [39], presenting high added value; for the Human being, this substance acts as a natural antioxidant ensuring the maintenance of the cell walls.

2.2.3 *Jatropha Curcas* L.

Jatropha curcas L. is a small tree belonging to the family of *Euphorbiaceae*. Nowadays this crop is suffering a massive investment by governments and private investors; however, several times without the support of the scientific community [100]. *Jatropha* is native of Mexico and Central America, supporting the livelihood in many rural areas (small-scale farmers) [48]. The seed of *Jatropha curcas* is rich in oil (35 – 48%) which can be used, for instances, for biodiesel production. This species is reported as able to grow in infertile soils and consequently reclaiming degraded soils, preventing soil erosion, promoting biodiversity and improving soils quality [101]. *Jatropha curcas* can be found in several regions of the world, from tropics to sub-tropics, in different Continents. It may be cultivated in many environmental conditions; however, it is mostly adapted to regions with arid and semi-arid climates (native regions) [67].

The high seed oil content associated to *Jatropha curcas* increase the interest around this specie leading to the planting of large areas in Asia, Africa and America. Despite the benefits associated to this crop, there are still many issues which need to be clarified, such as the excessive use of water, the seeds yields and the economic potential [48]. This perennial plant can live during several years, being consequently more resistant to pests and diseases. It can grow in regions with precipitation from 200 to over 1500 mm (per year), being perfectly adapted to dry climates [101]. For humid climates, a better crop performance is however achieved [102].

In an ideal scenario, due to its characteristics, *Jatropha curcas* can grow in soils with low quality (marginal land), such as soils with a pH higher than 9 [102].

Jatropha curcas is a large plant which can reach 5 m of height. This tree/bush lives over 50 years, showing high growth rate in youth, decreasing gradually with the age.

This specie tolerates a wide range of agronomic conditions, growing almost wild, and requiring low agronomic inputs. It is considered an undomesticated plant, being variable the productivity between plants [103]. Despite that, seed yields between 0.4 t/ha/year (low rainfall) and 12 t/ha/year (after five years of growth) are estimated [104].

Usually, the sowing/planting is performed in the warm season before or at the onset of the rainy season [101]. When the sowing is before the rainy season, it is essential to irrigate the plant. The emergence rate of *Jatropha* seeds increases in hot and humid climates [67]. It is possible to cultivated *J. curcas* in a large range of rainfall conditions, from dry to humid areas [105]. *Jatropha* tolerates temperatures between 18 – 40°C; however, it is for temperatures between 26 – 28°C where the productivity is maximized [67]. This plant is very sensitive to the frost [106].

This perennial crop is sometimes infected by virus being susceptible to the attacks by sapsuckers and bark eaters [107]. It is advisable to perform pruning annually [101]; being perennial crop, is naturally not used for crop rotation. The ability to grow in poor and contaminated soils is due to the fact that it develops a deep root system that can reach 100 cm, collecting water and nutrients at this soil layer [108]. This ability is maximized by symbiosis relationships between the root (crop) and fungus (mycorrhiza) which allow the plant to absorb unavailable nutrients. *Jatropha* plant produces the first seeds only after 2 to 3 years.

2.2.3.1 Agronomic Studies

Scientific information about *Jatropha* cultivation is scarce, being this specie still considered a semi-wild undomesticated plant, as referred. In Table 2.6 and Table 2.7 are described the agronomic conditions and the results gathered from the revised studied.

In India, most of the studies performed the sowing/planting after the monsoon season (June-September), reducing irrigation. The propagation of this specie is made sowing directly in the soil or by propagation using plant cuttings. In Mozambique the sowing was performed in December (dry weather) while in Egypt the sowing was in March (associated to mild temperatures). Most of the studies did not referred a specific time for fruit harvest. The harvest stage is prolonged because the maturity of the fruit is not homogenous among the plants. It was described in one study that the harvest is performed approximately 90 days after flowering [109].

The density of sowing varies in the studies between 625 and 2500 plant.ha⁻¹. In the study conducted in India, it was verified that the increase of density is followed by an increase of productivity [67].

According to the studies analyzed, soil pH varied between 5.9 to 8.9, which demonstrates that this crop can grow in a high range of soil conditions, including degraded soils. Temperature varied greatly, between 7°C and 45°C, being *Jatropha* also tolerable to low rainfall conditions (455 mm). Due to its semi-wild characteristics, *Jatropha* is currently cultivated with low agronomic inputs. For this reason, the yields obtained were quite variable among the studies revised. The highest seed's yields were obtained in rainy regions and under mild temperatures.

Table 2.6. A review of the agronomic conditions applied to *Jatropha curcas* L. – *Agricultural and Edaphoclimatic conditions*

		<u>Agricultural conditions</u>					<u>Edaphoclimatic Conditions</u>			
<u>Country (City)</u>	<u>Date of Sowing/Planting</u>	<u>Harvest (days)</u>	<u>Type of Harvest</u>	<u>Flowering (days)</u>	<u>Density of Sow (plant/ha) x 10³</u>	<u>Geometry of the plot</u>	<u>Soil pH</u>	<u>Temperature (°C)</u>	<u>Rainfall (Mean, mm)</u>	<u>Water supply</u>
India	Bhavnagar	August to October 2007	–	–	2.5; 1.6; 1.1; 0.625 (at each city)	2x2 m; 2.5x2.5 m; 3x3 m; 4x4 m;	6.9	27	700	–
	Hyderabad						6.1	25.5	800	
	Dehradun						6.3	21	2000	
	Jorhat						6.0	23.5	1600	
	Naharlagun						5.9	23.5	3100	
India	Bhavnagar	August to October 2007	–	–	1.6	2.5x2.5 m;	6.9	27	700	–
	Hyderabad						6.1	25.5	800	
	Dehradun						6.3	21	2000	
	Jorhat						6.0	23.5	1600	
	Jodhpur						8.2	27.6	400	
India	Bhavnagar	August to October 2007	–	–	–	–	6.9	27	700	–
	Hyderabad						6.1	25.5	800	
	Dehradun						6.3	21	2000	
Ukulinga South Africa	Feb. to April 2005	Mar. to Jul. 2007; Apr. to Jun. 2008; 2009; May 2010	Manual	–	1.1	50x25 m;	–	18.4	680	–
India Patancheru	Nov. 2004	Started producing seed at the 3 th year Sept. – Dec.	–	–	–	3x2 m;	8.9 ¹	5.0 – 43.0	875 (2006); 712 (2007); 1102 (2008); 998 (2009);	–

Table 2.6. A review of the agronomic conditions applied to *Jatropha curcas* L. – Agricultural and edaphoclimatic conditions (continued)

	<u>Agricultural conditions</u>						<u>Edaphoclimatic Conditions</u>			
<u>Country (City)</u>	<u>Date of Sowing/Planting</u>	<u>Harvest (days)</u>	<u>Type of Harvest</u>	<u>Flowering (days)</u>	<u>Density of Sow (plant/ha) x 10³</u>	<u>Geometry of the plot</u>	<u>Soil pH</u>	<u>Temperature (°C)</u>	<u>Rainfall (Mean, mm)</u>	<u>Water supply</u>
Egypt Sharkiya	12 Mar. 2003	Sept. 2005	–	–	–	2 m between rows/2 m between trees in the row;	7.5 ¹	–	–	In the growing season of 2005: 125% ET _p ; 100% ET _p ; 75% ET _p ; 50% ET _p ;
India Bawal	October 2009	–	–	–	1.1	3x3 m;	8.14	21 – 46 (May/June); 0 – 15 (Dec./Jan);	350 – 550 (July/Sept.)	I ₀ – No irrigation; I ₁ – One irrigation (May); I ₂ – Two irrigations (Oct.; (dependent on rainfall)
India Gurgaon	July – Aug. 2005	–	–	–	2.5	2x2 m;	7.3 – 7.86	7 – 45	455	Irrigation was done in 15 day intervals
Mozambique Búzi	December 2010	31 Jan./3 Mar./3 Apr./12 Jun./ 27 Jun./ 3 Aug. 2012	–	–	0.83	4x3 m	6.3	24.7	628	–

ET_c – Crop evapotranspiration;

Table 2.7. A review of the agronomic conditions applied to *Jatropha curcas* L. – *Agricultural management and productivity*

		<u>Agricultural Management</u>					<u>Productivity</u>			
<u>Country (City)</u>		<u>Fertilization (kg/ha)</u>	<u>Type of fertilization</u>	<u>Herb./Fungicides (kg/ha)</u>	<u>Pruning/others processes</u>	<u>Method of Irrigation</u>	<u>Seed yield (t/ha)</u>	<u>Oil yield (t/ha)</u>	<u>Seed oil content (%)</u>	<u>Ref.</u>
India	Bhavnagar	–	Compost manure	No herb./fungicide applied	Not applied	–	1440; 1089; 1004; 421;	–	–	[67]
	Hyderabad						326; 216; 173; 68;			
	Dehradun						208; 142; 114; 52;			
	Jorhat						447; 232; 68; 90			
	Naharlagun						88; 0; 0; 0; 0;			
India	Bhavnagar	–	Compost manure	No herb./fungicide applied	Winter season - Pruning height (ref: ground): Control; 30cm; 45 cm; 60cm;	–	357; 178; 204; 283;	–	–	[67]
	Hyderabad						18.32; 34.18; 41.19; 36.04;			
	Dehradun						140.39; 107.89; 120.39; 120.79;			
	Jorhat						521.63; 0; 0; 0;			
	Jodhpur						0; 0; 118.72; 64.24; n.a.;			
India	Bhavnagar	–	Compost manure	No herb./fungicide applied	–	Interval days: no irrigation; 15 days, 30 days, 45 days, 60 days	858.3; 921.1; 1130; 960.9; n.a.;	–	–	[67]
	Hyderabad						69.41; 92.39; 92.40; 92.42; n. a;			
	Dehradun						189.46; not available; 165.06; 230.69; 238.46;			
South Africa Ukulinga		Not applied	–	No herb./fungicide applied	Pruning to a height of 1.0 m	–	89.9; 104.4; 348.8; 10.0;	–	–	[48]
India Patancheru		N: 76 (2005/06/07); P: 10 (2006/07); N: 92 (2008/09); P: 50 (2008/09);	Chemical	–	One year old pruning to a height of 0.5 m (winter)	No irrigation applied	Started producing seed from 3 th year 600 (2007); 1560 (2008); 1000 (2009)	–	–	[100]

Table 2.7. A review of the agronomic conditions applied to *Jatropha curcas* L. – Agricultural management and productivity (continued)

	<u>Agricultural Management</u>					<u>Productivity</u>			
<u>Country (City)</u>	<u>Fertilization (kg/ha)</u>	<u>Type of fertilization</u>	<u>Herb./Fungicides (kg/ha)</u>	<u>Pruning/others processes</u>	<u>Method of Irrigation</u>	<u>Seed yield (t/ha)</u>	<u>Oil yield (t/ha)</u>	<u>Seed oil content (%)</u>	<u>Ref.</u>
Egypt Sharkiya	–	Farmyard manure; Chemical (NPK);	Hand weeding; Pesticides applied in 2 times;	–	Microsprinkler irrigation – 1 for each tree	114.75; 195.08; 103.00; 90.42;	28.69; 58.39; 30.17; 22.15;	–	[110]
India Bawal	N: 0; 30; 60; 90; K: 0; 30; 45; 60	Farmyard manure; Chemical;	Chlorpyrifos 10 mL/plant	Pruning;	–	48 results (combinations with irrigation, N and P fertilization treatments);	48 results (combinations with treatments)	48 results (combinations with treatments)	[111]
India Gurgaon	–	Farmyard manure;	–	–	–	–	–	17.11– 31.63	[112]
Mozambique Búzi	Not applied in the 1 st year	–	Hand weeding Pest control against flea beetle (acetamiprid/cypermethrin)	–	Not applied	–	–	Mean of 6 harvest's moments: 40.81	[113]

According to the studies, the cultivation of this specie, for commercial purposes, should be performed using fertilizers and providing water (maximization of the yields of the crop). Under such conditions, a relevant increase of yields is verified (Egypt – Sharkiya; India – Bawal) [110, 111]. Soil fertilization can be done by adding chemical or organic fertilization (e.g. farmyard manure). Most of the studies chose organic fertilizers as source of macronutrients, promoting the recycle of green wastes resulting from the pruning process.

The high growth rate verified, mainly in the first years after the planting, leads to the development of a large canopy and for this reason it is advised to perform pruning after the fall of the leaf. This process turns easier the manual harvesting and also mitigates the proliferation of pests and diseases [100, 114]. Pruning was conducted in most of the revised studies. To control the proliferation of pests and diseases it is possible to use herbicides and fungicides. In the studies revised, the control of weeds was made by manual removal. In some studies, the control of pests and diseases was made using chemical products.

To maximize the yields, some studies performed irrigation. Among the different systems of irrigation (sprinkler, drip irrigation, subsurface drip irrigation), drip irrigation is not advisable to use because induces a big vegetative growth [110]. In the study developed in India (Jodhpur, Bhavnagar, Hyderabad, Dehradun), it is recommended to carry out irrigation in intervals from 30 to 45 days [67]. In Egypt the results showed that an excess of water did not bring any productivity increase. The treatment that was irrigated with 100% of ET_p (evapotranspiration potential) corresponded to the highest value of seed oil content (29.93 wt.%) [110].

Seed yields were quite variable, ranging from 10.0 kg.ha⁻¹ (without fertilization or any pesticide) to 1440.0 kg.ha⁻¹ (organic fertilization and high density of sow). These results were also strongly influenced by the edaphoclimatic conditions. The highest seed's yields (> 1000 t.ha⁻¹) were obtained for the studies where the annual rainfall was higher than 700 mm and the temperature was between 21 °C and 27 °C [67, 100]. Under favorable soil moisture content, *Jatropha curcas* uses large amounts of water for higher growth, with consequently higher seed's yields [100].

2.2.3.2 Valorization of non-lipidic components

The large-scale cultivation of *Jatropha* in many countries (Brazil, India, China) for bioenergy purposes resulted in an increase of *Jatropha* seed cake production and thus the interest of its use. This by-product is characterized by having high protein and nutrient contents and high calorific value [115]. *Jatropha* seed cake can be used for biogas production (namely for electric power generation) [116]. It is also very relevant the use of this by-product as organic fertilizer due to its macronutrients content (6 wt.% N, 3 wt.% P and 1 wt.% K) [101]. As an example, one study reports that the incorporation of 0.75 – 10 t ha⁻¹ of seed cake leads to an increase of *Jatropha* seed production between 13 – 120 % [117]. The presence of curcumin, a toxic protein, makes inviable its use for animal feed. However, some studies have been developed in order also to promote the detoxification of seed cake [118].

Jatropha is known for its application in traditional medicine [110, 119]. Plant parts like leaves, bark, roots or seeds when properly prepared have a purgative and laxative effect [120]. The leaves are reported in applications to mitigate the effects of diseases such as malaria, rheumatism and muscular pains [105, 121]. From the leaves its possible to obtain alcohol extracts with antibiotic activity, against microorganisms such as *Staphylococcus aureus* and *Escherichia coli* [115]. The latex extracted from this plant (from the stem) can be applied in wounds due to its coagulation properties [115]. From the leaves, the latex extracted is also known to have anti-cancerous properties and can be used as a disinfectant for mouth infections in children as well as to treat skin conditions [109, 121]. The extracts from the roots can be applied in oral health being also known to contain an antidote against snake venom [119]. *Jatropha* wood can also be used for domestic heating or to support vines [101].

2.2.4 *Simmondsia Chinesis*

Simmondsia chinensis, also known as Jojoba, is a perennial and dioecious shrub, endemic from the Sonoran Desert in Mexico and from the Southwestern of the USA (semi-arid regions) [58]. This specie presents economic interest because their seeds can contain up to 50% of wax esters (which comprise jojoba oil), used in several industrial applications: cosmetics, pharmaceuticals and biodiesel production [58, 122]. Currently, Jojoba is present in several countries (USA, Mexico, Israel and Argentina), however the world production of this oil is low. Due to cross-pollination, has a high intraspecific variability and the domestication process is difficult. Only a small fraction of the

population is developed from seeds of native plants (less than 1%) and the yields are known to be very variable [123]. In order to maximize the potential of this crop, in the short time, it is advisable to select plants with the best characteristics and propagate them asexually [124]. The first trial studies developed with this crop used seeds from wild plants which resulted in low yields. This problem can be mitigated using only cuttings from plants with high yield potential (as previously mentioned), upgrading older plantations and establishing new ones [125]. After sowing, the first flower buds appear after from 9 to 24 months [126]. After 3 growing cycles, the first fruits can be collected, being the maximum maturity reached after 10 – 12 years. Some clones can reach 100 years of life [127]. Jojoba can, in natural environment, reach a height of 5 m. However, for an easy harvesting, the height should not exceed 2 – 3 m [47].

This specie is well adapted to arid and semi-arid climates. Plants older than 3 years can survive at low temperatures, but the flower buds can die (between -3°C and -5°C) [65]. In cold seasons, it is not advisable to irrigate the crop, because flower buds became more sensitive to frost [128].

In order to maximize the potential of Jojoba cultivation, it is necessary to consider its characteristics. Usually, seed/planting should be done in the spring season due to associated temperatures. For temperatures between 21°C and 31°C , germination can occur in a one week and the plants are visible on the surface after 20 days. In environments with low temperatures, germination can take several months [47]. In the early months it is important to perform irrigation steadily. After, irrigation should be performed in a monthly basis [47]. This specie can tolerate high temperatures ($35 - 48^{\circ}\text{C}$). Despite being very sensitive to frost, Jojoba can survive and grow in this kind of environmental conditions if the field has some slope and sun exposition [47, 63]. Studies developed in Mexico and USA showed that the plant can grow in soils with a pH between 5 and 8. In fact, usually, the acidity or alkalinity of the soil is not considered a problem for this crop [47, 63].

Jojoba is found naturally in arid areas, although this crop should be irrigated. The biggest plants of Jojoba (5 m) can be found in areas with an annual rainfall between 254 and 380 mm [63]. For areas with an yearly rainfall lower than 640 mm, it is advisable to provide water to the plant [47]. Usually, drip irrigation is considered the best irrigation system for arid climates, avoiding water losses by evaporation. For jojoba crop, there is a lack of information about the nutritional requirements of the plant. In this regard, it is pointed out that Jojoba growth mostly depends on the nitrogen macronutrient and zinc micronutrient [47]. The addition of nitrogen to the crop might result in an seed productivity increase of 65 % compared with unfertilized crop [129].

Even after the crop is fully established, the seed yields can vary in consecutive years in the range between 0.40 and 5.26 t.ha⁻¹ [58, 63]. In wild conditions, seed yields range from a few seeds to more than 0.014 t.plant⁻¹ [58, 63]. Plants with higher height, usually have higher productivity than the small bushes (1.8 kg.plant⁻¹) [63].

This perennial crop needs several years to be completely established; also, due to the characteristics of the plant, crop rotation is not adopted. However, in some cases, the disposition of the crop in the field (rows separated 5 – 7 m) might allow to sow other small crops. The establishment of other crops, between lines, is beneficial for nitrogen fixation, control of weeds and protection of the crop from the desert winds. Crops with economic interest such as alfalfa, asparagus, sesame, safflower or sorghum have been used in such conditions [47].

2.2.4.1 Agronomic Studies

According to the studies analyzed, the environmental conditions and the methodology of crop propagation influenced the beginning of the agronomic studies. Table 2.8 and Table 2.9 compile the gathered information about the agronomic inputs and the climacteric conditions verified during the cultivation of Jojoba crop.

Table 2.8. A review of the agronomic conditions applied to *Simmondsia chinensis* – Agricultural and edaphoclimatic conditions

		<u>Agricultural conditions</u>					<u>Edaphoclimatic Conditions</u>				
<u>Country (City)</u>		<u>Date of Sowing/Planting</u>	<u>Harvest (Days)</u>	<u>Type of Harvest</u>	<u>Flowering (Days)</u>	<u>Density of Sow (plant/ha)x10³</u>	<u>Geometry of the plot</u>	<u>Soil pH</u>	<u>Temperature (°C)</u>	<u>Rainfall (Mean, mm)</u>	<u>Water supply</u>
Argentina Aimogasta		–	–	Manual	18.75		5 m between rows/1.5 between plant/ 15 plants per row	–	11.3 (winter) to 30.4 (summer)	60	32 l/plant (summer) 8 l/plant (winter)
Turkey Kumluca		March 1990	Middle of Sept.	Manual	–		–	8.3	Max.: 28.8-29.0; Min.: 7.2-7.5	1168 (1999); 685 (2000); 1500 (2001);	No irrigation
Saudi Arabia	Hada	20/03/2001	At full maturity (don't have specific date)	Manual	–		4 m between rows/2 m between plant/ 5 plants per row	–	30.3	–	Irrigation done when necessary
	Alyoutamah	28/03/2001							24.7		
	Hail	28/08/2003							24.3		
Chile	(A) Saline conditions	–	4 th year after planting	–	–		4 m between rows/4 m between plant	–	–	0.6	Irrigated every 10 days
	(B) Non saline site	–								140	6 l.plant ⁻¹ .week (20 mm.year ⁻¹)

Table 2.8. A review of the agronomic conditions applied to *Simmondsia chinensis* – *Agricultural and edaphoclimatic conditions* (continued)

<u>Country (City)</u>	<u>Agricultural conditions</u>						<u>Edaphoclimatic Conditions</u>			
	Date of Sowing/Planting	Harvest (Days)	Type of Harvest	Flowering (Days)	Density of Sow (plant/ha)x10 ³	Geometry of the plot	Soil pH	Temperature (°C)	Rainfall (Mean, mm)	Water supply
Saudi Arabi Qassim	Between Oct. 27 to 30 of 2008	2 nd week of May 2010; 4 th week of May 2011; 1 st week of June 2012;	Manual	–	–	3 m between rows/2 m between plant/ 26 plants per row	9.54	14.2 – 37.4	58 – 150	Once every 10 days
Israel Hazerim	May – Jun. 1992	–	Manual	Between Feb. – Mar.	1110	4.5 m between rows/2 m between plant/ 7 – 8 plants per row	–	3 – 34	–	92: 580 m ³ /ha; 93: 624 m ³ /ha; 94: 1660 m ³ /ha; 95: 3380 m ³ /ha; 96: 3970 m ³ /ha; 97: 4000 m ³ /ha;;
USA Arizona	Planting established in 1984	July 1990 and Aug. 1991;	Manual	–	–	4.1 m between rows/0.5-1 m between plant/ 7 – 8 plants per row	–	–	161 (1988); 99 (1989); 232 (1990);	For 4 years [1988/89/90/91] - WT ¹ (20 no./yr.); MT ² (4 no./year); DT ³ (2 no./year);
Argentina Cordoba	Oct. 1981	–	Manual	–	–	4 m between rows/1 m between plant/	–	12 – 30.0	462 – 654	Four irrigation treatments: T1:0 mm; T2:300 mm; T3:600 mm; T4:900 mm; Twelve equal applications every 30 days.

¹WT – wet treatment; ²MT – Medium treatment; ³DT – Dry treatment;

Table 2.9. A review of the agronomic conditions applied to *Simmondsia chinensis* – Agricultural management and productivity

		<u>Agricultural Management</u>				<u>Productivity</u>			
<u>Country (City)</u>		<u>Fertilization</u>	<u>Type of fertilization</u>	<u>Herb./Fungicides</u>	<u>Pruning/others processes</u>	<u>Method of Irrigation</u>	<u>Seed yield (kg/plant)</u>	<u>Seed oil cont. (%)</u>	<u>Ref.</u>
Argentina Aimogasta		–	–	–	–	Drip Irrigation	For 14 Jojoba clones: [0.80 – 1.41];	–	[124]
Turkey Kumluca		Not applied	–	–	No pruning; Dried shoots removed;	–	For 19 Jojoba clones (mean for 3 years - 1999/2000/01): [0.28 – 1.17];	43-58	[130]
Saudi Arabi	Hada						For 9 Jojoba clones: 1.22 (2003/04); 0.92 (2004/05); n.a. (2005/06);	03/04:44.5 04/05:47.4	[49]
	Alyoutamah	Not applied	–	Weed control	–	Drip Irrigation	0.29 (2003/04); 0.32 (2004/05); 0.61 (2005/06);	04/05:42.6 06/07:42.8	
	Hail						0.18 (2003/04); 0.59 (2004/05); 0.55 (2005/06);	04/05:47.4 06/07:48.9	
Chile	(A) Saline cond.					Furrow irrigation	For 21 jojoba clones: between 0.081 – 0.517;		[131]
	(B) Non saline site	–	–	–	–	Drip Irrigation	For 21 jojoba clones: between 0.202 – 1.070;	–	
Saudi Arabia Qassim		DAP ¹ : 100g/plant;	Chemical	–	Hand weeding	Drip Irrigation	For 7 jojoba clones: [0.00233 – 0.00508] (2010); [0.0787 – 0.2624] (2011); [0.3353 – 0.8216] (2012)	–	[132]
Israel Hazerim		–	–	Weed control	Pruning	Drip Irrigation	[0.07 – 0.55] (1994); [0.36 – 1.34] (1995); [0.65 – 2.85] (1996); [1.67 – 3.35] (1997);	–	[133]
USA Arizona		N: 75 kg/ha (May 1987);	Chemical	–	–	WT: Drip Irrigation; MT/DT: Flood irrigation	For 6 clones (mean): Wet treatment: 0.120 (1990); 0.138 (1991); Medium treatment: 0.418 (1990); 0.450 (1991); Dry treatment: 0.430 (1990); 0.132 (1991);	–	[55]
Argentina Cordoba		Don't apply	–	–	–	–	T1:0.107; T2:0.139; T3:0.194; T4:0.199; (1986) T1:0.136; T2:0.161; T3:0.232; T4:0.241; (1987)	–	[134]

¹DAP - Diammonium Phosphate;

The establishment of the crop can be done through three processes: direct seedling, seedling transplants and cuttings. The first process, applied in the study developed by Ayerza *et al.* [134] in Cordoba (Argentina), is cheap and easy to perform, although the number of plants that survive was low. The seedling transplants consists in performing a sowing in the nursery bed, under optimal growth conditions. The plants are ready for transplant after 2 – 4 months. This method was applied in Saudi Arabi, Israel and Turkey studies [130, 132, 133]. The last process of propagation, using plant cuttings, is performed using a cut from a superior bearing from one plant completely established [47]. The cuts are then inserted in the soil. This technic was used in the study developed in Aimogasta (Argentina) and Chile [124, 131]. Usually, the sowing is done during the late-spring (October for Argentina). The transplanting and cuttings must be done before the warm season. Table 2.8 presents the dates adopted by different studies for the establishment of the plants. In some studies, the harvest was not performed in the first years. Usually, harvesting is performed during several weeks (one time for year), by hand, collecting seeds after they had fallen to the ground.

Only the studies developed in Turkey and Saudi Arabia (Qassim) assessed soil pH [130, 132]. For both studies, the soil presents an alkaline pH.

Most studies did not share any information about control of diseases and weeds. Only in the study performed in Qassim (Saudi Arabia) it is specified the type of weed control [132]. Pruning is advised and becomes easier the control of weeds, between lines. Only the study developed in Israel performed pruning [133].

The most common irrigation system adopted for Jojoba crop is drip irrigation. This method of located irrigation minimizes water loss through evaporation and allows a more efficient watering of the plant. The use of flood/furrow irrigation is unusual since it implies a high waste of water.

Jojoba is originally native from arid and semi-arid areas. For this reason, the crop can grow in a large range of temperatures. In the study developed in Cordoba (Argentina), Jojoba survived to temperatures that varied between -3°C and 47°C. For the other studies, the thermal amplitude observed was smaller. Depending on the plant phenological stage, Jojoba can be very sensitive to the frost. Considering the annual rainfall recorded in the different studies, it was possible to verify that the values are quite low, except for the study carried out in Turkey [130]. Due to the general dry conditions, most of the studies chose to perform irrigation, adopting a customized irrigation

schedule. In the study developed in Cordoba (Argentina), Arizona (USA) and Hazerim (Israel) the research focused in understanding how the irrigation affected the productivity of the crop.

As referred before, few information was found about the nutritional needs of Jojoba crop. Only the studies developed in Arizona (USA) and Qassim (Saudi Arabia) referred the type and amount of fertilizer used [55, 132].

The productivity achieved with Jojoba crop depends strongly on the type of clone (ecotype) used. There are few clones commercially available (domesticated clones). Consequently, seed production varies in a wide range because the quality of the clones cultivated was low. Among the studies revised, seed yields varied between 0.00233 kg.plant⁻¹ (residual value) and 1.41 kg.plant⁻¹ (commercial viability). Some of analyzed studies (Saudi Arabia, USA, Argentina-Cordoba) showed that seeds productivity increases with the age of the cop (higher maturity) [55, 124, 132]. In the study developed in Cordoba (Argentina), 600 mm was determined as the amount of water that allowed to obtain the highest productivity [124]. Higher values did not show a significant increase of productivity. For this study, the seed yields varied in the first year between 0.107 – 0.194 kg.plant⁻¹ and in the second year between 0.136 – 0.241 kg.plant⁻¹. The study developed in Chile showed that the soils salinity (water stress) affects the final productivity, being smaller than the obtained in soils without salinity problems [131]. Based on the results, a selection of Jojoba clones was performed regarding future planting under saline conditions.

2.2.4.2 Valorization of non-lipidic components

Beyond the lipidic fraction, others fractions of the crop have economic interest. The cake resulting from oil extraction has few viable applications reported. Jojoba cake has a protein content between 20 – 25% [58]. The recovery for animal feed is rarely used due to the presence of 4 anti-nutritional substances (collectively known as simmondsins) which cause starvation, inhibition of the appetite and may lead to animal death [58, 135]. The use of this by-product is of specially interest in arid areas, where feed availability is low, but only after being properly detoxified [136]. Some research showed the potential of using the cake as a source of protein for fish or broiler feeding [135, 137, 138].

It is also possible to recover secondary metabolites present in Jojoba crop, such as flavonoids. Typically, these substances are present in fruit (pericarp) and show anti-oxidant and hepato-protective activity, used as human weight control, food additive or as a therapeutic agent [139, 140].

The latex which can also be obtained from this plant, shows anti-bacterial and anti-fungal activity against microorganisms like *Staphylococcus aureus*, *Escherichia coli* or *Candida albicans*. From the roots of Jojoba, it is possible to recover aqueous extracts with similar characteristics. These substances (extracts and latex) can be also used as natural preservatives for food [141].

2.3 CONCLUSIONS

In this review, agronomic and edaphoclimatic conditions concerning four non-edible oilseed crops with high potential: *Ricinus communis* L., *Crambe abyssinica*, *Jatropha curcas* L. and *Simmondsia Chinesis*, were comprehensively presented and discussed. The information gathered will assist the different stakeholders, namely, researchers, investors and biodiesel producers.

This study aimed to revise the most recent information about such non-edible crops, specifically, the agronomic inputs required and the edaphoclimatic conditions tolerated. The following findings/conclusions can be achieved after performing this review:

- The proper selection of a non-edible crop according to its characteristics is essential to maximize yields, namely if the final purpose is biodiesel production;
- *Ricinus communis* is known by its high seed oil content (40 and 60 wt.%), high height (4 – 5 m of height), being cultivated in the Mediterranean region in the beginning of April. The cultivation only should be done during two consecutive years, with the plant growth cycle having the duration of 140 – 180 days. This crop can easily grow in regions with a rainfall between 750 – 1500 mm and a temperature between around 16 °C to 37 °C. Soil fertilization is usually performed before sowing.
- *Crambe abyssinica* is an annual non-edible crop with high interest due to its short annual cycle (90 – 120 days) with high seed oil content (30 wt.%). It is considered a small plant (70 – 90 cm) which can grow in regions with a rainfall and temperature in the range between 350 to 1200 mm and from around 6 °C to 16 °C (Spring), respectively, being very sensitive to frost. For a quick germination, this crop should be sown in the beginning of April with some agronomic inputs (irrigation and fertilization).

- *Jatropha curcas* L. is a large plant (up to 5 m) able to grow in infertile soils having the potential to reclaim degraded soils, improving soil's quality. This crop produces a seed rich in oil (35 – 48%). It is well adapted to arid and semi-arid climates with a precipitation between 200 mm and 1500 mm (perennial crop). Usually, the crop is sown/planted during the warm season before the rainy season. The maximum productivity is achieved for temperatures between 26 – 28°C. This specie can grow with low agronomic inputs (semi-wild characteristics).
- *Simmondsia chinensis* is a perennial crop (1 – 2 m) which can survive during decades. Usually the propagation of this specie is made by cuttings from plants with high yield productivity. The first fruits can only be collected after 3 years and the maximum productivity is achieved after 10 – 12 years, being possible to obtain a seed oil content higher than 50 wt.%. This specie is well adapted to arid and semi-arid climates, although for areas with a rainfall lower than 640 mm, it is advisable to perform irrigation. It can grow in regions with temperature between 5°C and 35°C. The use of nitrogen fertilizer increased 65% the productivity of the crop making this aspect relevant to consider in future studies.

The use of non-edible crops as source of oils for biodiesel production reduces the impact of its production in the food market. This study identified four non-edible crops with high potential for bioenergy production and summarizes the most important agronomic characteristics for a successful cultivation.

Although from different species, these four crops have the same common characteristic, i.e. high seed oil content (source of oils). Additionally, some of them can be used in crop rotation regimes, be cultivated in marginal or poor lands (not suitable for food production) or do not require high agronomic inputs.

Among the non-edible crops analyzed, *Crambe abyssinica* and *Jatropha curcas* show special interest to be cultivated in Portugal. These crops show edaphoclimatic requirements, similar to the weather conditions verified in Portugal. *Crambe*, due to its fast growth (short growing cycle), guarantees a quick economic return, allowing also crop rotation with other crops. Among the perennial crops, *Jatropha curcas* corresponds to the crop which grows with the lower amount of rainfall (200 – 1500 mm), ensuring their growth also in dry weathers. Portuguese weather conditions typically are of a

rainy winter and a dry and not too hot summer. However, dry weather conditions are nowadays more frequent (climatic change) and for this reason crops with low water requirements should be chosen.

Final remark

Apart from the mentioned crops, in the literature other crops can be identified as having high potential to be cultivated in Mediterranean region, like *Cynara cardunculus* L., a crop already cultivated in Portugal for the production of the flower used in the traditional production of milk cheese. The seeds of this crop are not usually collected, although they have relevant oil content; for this reason, further studies with *Cynara cardunculus* L. are considered relevant.

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CHAPTER 3. *CRAMBE ABYSSINICA* CROP – AGRONOMIC ASSESSMENT AND BIOENERGY PRODUCTION

3.1 CULTIVATION OF *CRAMBE ABYSSINICA* IN PORTUGAL

ABSTRACT

Crambe abyssinica is a non-food crop referenced in the literature as having high potential for renewable energy production. A comprehensive study regarding cultivation of crambe was developed considering agronomic aspects (plant density, irrigation and fertilization), climatic aspects (weather conditions) and environmental impacts (life cycle assessment). This is probably the first study carried out with this specie in Portugal aiming to evaluate its adaptability and the potential impacts caused by its cultivation. Crambe was cultivated during four growing seasons using seeds of FMS Brilhante cultivar with a germination rate of 84%. The seeds yields were 742 kg.ha⁻¹, 119 kg.ha⁻¹ and 95 kg.ha⁻¹ for the first, second and third growing seasons, respectively, lower than that reported in Brazilian studies (989 – 1347 kg.ha⁻¹). The fourth growing season did not produce seeds due to prevalence of cold weather. The seeds oil content varied between around 26% and 34%. The first growing season showed the best growing cycle, evidencing that weather conditions, namely temperature and soil's moisture, possibly influence not only seeds yield but also oil yield. Mild temperatures until flowering and high water availability allowed achieving good emergence rates on shorter periods (between 19 – 43 days). Life cycle assessment performed according ISO 14040:2006 showed that the impacts related to crambe cultivation in extensive farming mode (with organic fertilization and small inputs of labor) are mainly related to the consumption of diesel and electricity used in the preparation of field for sowing and its irrigation. Overall, crambe cultivation showed lower impact for climate change than that verified for others systems in extensive mode.

This chapter was based on:

Emanuel C., Manuel F. Almeida, Maria C.M. Alvim-Ferraz, Joana M. Dias, “*Cultivation of Crambe abyssinica non-food crop in Portugal for bioenergy purposes: agronomic and environmental assessment*”, Industrial Crops and Products journal, volume 139, 2019.

3.1.1 SCIENTIFIC RELEVANCE

Among the fossil fuels, crude oil derived fuels are the most used, mainly in the transport sector, industry and agriculture [1, 2]; the transport sector alone requires 63 – 65% of the total amount of oil consumed in the world [2, 3]. By gradually replacing of this fuel by others more environmentally friendly, such as biodiesel, it is possible to reduce the emissions of greenhouse gases (GHG) from 19 to 56%, depending on the raw material used (Directive 2009/28/CE). It is forecasted for 2030 that carbon dioxide, the most reported GHG, will increase its concentration in the atmosphere in 80% compared to the values found in 2007 [2]. Nowadays, 22% of GHG emissions are produced by the transport sector and this sector is also responsible for the deterioration of air quality due to the emission of other pollutants such as particulate matter, carbon monoxide and nitrogen oxides [3]. Because most of the vehicular fleets are diesel engines, biodiesel gains special relevance as an alternative fuel [4]. Among the virgin vegetable oils, rapeseed oil, soybean oil, palm oil and sunflower oil are the most used raw materials for biodiesel production [5]. The advantages of using these oils as feedstock are related to the simple chemical conversion in biodiesel, the availability of such oilseed crops and the quality of the resultant product. The fact that these oils are also suitable for human consumption represents the major drawback of such first generation feedstocks for biodiesel production [2]. Non-edible oils arise, therefore, as an alternative and more social and economically friendly feedstock for biodiesel production (advanced biofuels), since: (i) they do not compete with the food market; (ii) they are cheaper, reducing production costs; and (iii) some might be cultivated on non-agricultural or marginal land [2, 6]. Among more than 300 oilseed plant species, only 40 have the potential for biofuel production, based on the oil content of the seeds, and a relevant example is crambe (*Crambe abyssinica*) [7]. Crambe is a non-edible oilseed crop which belongs to the cruciferous family, originally from the Mediterranean region [8]. According to the literature, the cultivation of this crop requires low inputs when compared with other oilseed crops [9] and it is characterized by a short annual cycle, approximately 90 – 100 days, reaching 1 m of height [8, 9]. The short annual cycle makes possible to sow the crop in rotation with other crops, preserving the quality of the soils, improving seasonal nutrient availability and chemical and biological properties of the soil [10]. In Europe, crambe growth as spring crop, showing great tolerance to drought (the roots can reach 15 cm of length) and frost. The seeds are usually very small (0.8 to 2.6 mm) and the weight of 1000 seeds is reported in the range 6 – 10 g, evidencing a high oil content, around 35 wt.% [11]. Erucic acid is the most common acid present in the oil (around 60%); and due to its toxicity,

cannot be used for human consumption [8, 9]. Concerning the bioclimatic requirements, literature shows that crambe can grow in rainfed conditions between 350 and 1200 mm with an annual average temperature between 5.7 °C and 16.2 °C [8]. The emergence is the most important stage for the success of the crop with the best results being found in the spring with enough rainfall and a mean optimal temperature in the range 10 °C – 14 °C [8]. Beside the high seed's oil content, the remaining fraction after oil extraction might have value, namely for animal feed, due to the high protein content (after detoxification) or as a natural fertilizer, due to the high nutrient content [12]. Despite the several benefits associated to the crambe crop, the environmental impacts of its production should be evaluated, because all agricultural activities have impacts related in particular with water consumption and air emissions [13]. Life cycle assessment (LCA) is the most used methodology to appraise environmental impacts associated to a product, service or system taking into account the resources consumed and the resultant emissions [14]. This methodology quantifies the materials and energy flows and identifies several environmental impact categories (climate change, eutrophication, acidification, etc.) associated to the functional unit established [15]. The impacts associated to the background system for material and energy flows related to the production or processing of the functional unit established should also be assessed. The data for environmental impacts of the background system could be provided by databases such as ELCD (European reference Life Cycle Database), USDA (United States Department of Agriculture) or Agribalyse, among others [13].

In Europe, few studies exist concerning the cultivation of crambe. This is reflected in the fact that no commercial seeds are available in this region.

3.1.2 SPECIFIC OBJECTIVES

The main objective of this specific study was to increase the knowledge associated with the cultivation of this crop as well as the related environmental impacts. Accordingly, the study evaluated:

- the viability of *Crambe abyssinica* cultivation in the bioclimatic conditions of Northern Portugal for energy production purposes (namely for biodiesel), using commercial certified seeds (cultivar FMS Brilhante);
- the potential environmental consequences of such agriculture practices by employing the LCA methodology.

3.1.3 MATERIALS AND METHODS

3.1.3.1 Plant materials

The seeds of *Crambe abyssinica* were collected at Brazil and belong to the commercial cultivar FMS Brilhante, provided by Fundação Mato Grosso do Sul. The germination rate for these seeds was assessed as being 84% (% of seeds germinated from the sample with one hundred seeds, during 25 days at 22 – 24 °C in a room with temperature control). This parameter was determined with the objective to ensure the sow density established (1000×10^3 plants.ha⁻¹).

3.1.3.2 Agronomic conditions

The experiments were conducted at Northern Portugal (40°59'07.2"N, 8°32'43.6"W) under field conditions during the years of 2016 and 2017, corresponding to four crop growing seasons.

Because the culture can grow at low temperatures (> 5 °C) it was sowed during the spring, on 28th of April 2016 and 30th of March 2017 (one month earlier with the objective to compare the effect of weather conditions in crop growth). Sowing the crop during the summer was also conducted on the 1st of August of 2016 and 2017 (last trial), respectively with the objective to evaluate the viability of sowing crambe twice a year. The meteorological conditions (temperature and precipitation) were determined using a weather station (Froggit® WH1080) with real time data acquisition located close to the experimental field (Figure 3.1).



Figure 3.1. Weather station used for assessing meteorological conditions.

The cumulative growing degree days (GDD) parameter was calculated for each phenological phase of the crop. This parameter allows to understand the degree of crop's development taking into

account the effect of the temperature, estimating the length of each phenological event considering the mean temperatures of the region, and predicting the best area for crop's growing (regions where the GDD could be achieved considering the duration of the growth cycle) [16]. The GDD were calculated according to equation 3.1 [17]:

$$GDD = \sum \left[\frac{T_{max} - T_{min}}{2} \right] - T_{base} \quad (3.1)$$

where T_{max} and T_{min} represent the maximum and minimum air temperature on the daily basis, respectively, and T_{base} is the base temperature (constant value), which depends on the crop. For crambe, T_{base} takes the value of 5 °C [18].

To assess the quality of the soil, the following parameters were determined: (i) pH; (ii) phosphorus; (iii) organic matter; (iv) calcium; (v) potassium; (vi) magnesium; (vii) iron; (viii) copper; and (ix) zinc, at an external laboratory (Department of Sciences and Engineering of Biosystems – School of Agriculture of Lisbon). Considering the results obtained (see section 3.1.4), a natural fertilizer from organic manure (Fertigranu[®]), stabilized by composting, was used. No chemicals were applied to the crop during the experiments.

Before the sowing, 50 m² of soil were prepared with a plough at 25 cm depth (tillage preparation) and organic fertilization was immediately performed (2000 kg.ha⁻¹). Then, the sowing was executed in rows by hand with seeds at low deep (approximately 3 cm). The distance between rows was 0.35 m with 35 seeds per each meter of row corresponding to a density of plants of 1000x10³ plants.ha⁻¹. During the development of the crop, no diseases were detected and for this fact any fungicide was used. The presence of weeds were controlled by hand without the application of chemical herbicide.

For industrial production, the harvest is performed automatically by heavy machines, consequently, in this study, irrigation of the field was done by sprinkler, avoiding the subsurface irrigation. The irrigation was executed when required with the objective to prevent hydric stress.

The biomass was manually gathered and threshed when the crop showed a brown color indicating the end of the cycle. All the seeds produced in each plant were collected.

In order to evaluate if significant differences existed between the results of the growing seasons (crop yields and characteristics of seeds), Analysis of variance (ANOVA) was performed with the support of the Excel[®] software (Analysis ToolPak). For the parameters/properties with significant

difference ($p \leq 0.05$), Tukey test was performed with objective to identify, for the same property, the groups of results (growing seasons) among which differences were verified.

3.1.3.3 *Life cycle assessment*

- *Scope of the study and system boundaries*

The environmental impacts associated to the cultivation of crambe were evaluated according to ISO 14040:2006 standard, whose methodology has four steps: (i) goal and scope definition with the delimitation of the system boundaries; (ii) inventory – the inputs and outputs were defined, collected and analyzed; (iii) impact assessment – the emissions for soil, water, air and the raw materials/energy consumptions flows were converted into environmental effects; and, (iv) interpretation of the results.

The environmental performance assessment considers the environmental impacts related to the input resources/materials, to the energy use and a “cradle to gate” approach was adopted. This corresponds to an assessment of a partial product life cycle (1 ton of seeds produced), between the extraction of raw materials and the moment when the product leaves the agricultural system (before storage, transport and consumption). The end of life of the functional unit was therefore not considered, since the focus was the agronomic step.

To assess the environmental impacts associated to the production of 1 ton of crambe’s seeds, the system was divided in two levels: (i) background level, considering the impacts caused by the production/supply of diesel, electricity and organic fertilizer; and (ii) the foreground level, which includes the environmental impacts caused by the direct application/use of organic fertilizer, fresh water consumption, diesel combustion (consumption) and by land use/occupation. The system boundary includes the resources used in the system and all the emissions/discharges that potentially can cause environmental effects and which are associated to the seeds production (Figure 3.2).

For each level, sixteen categories of environmental impacts were analyzed and the characterization factors (CF) for each elementary flow were established.

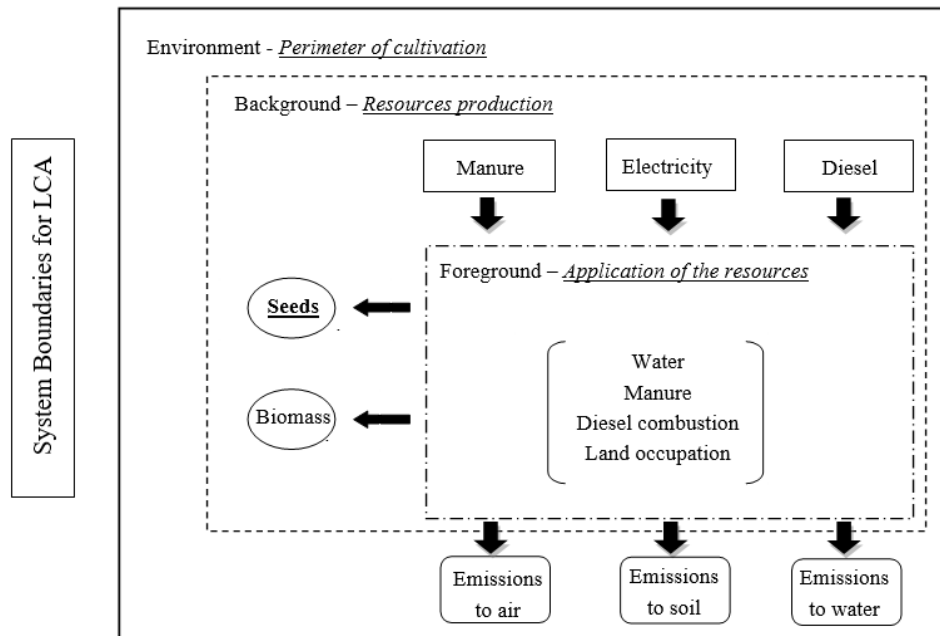


Figure 3.2. Definition of the system boundaries for Life Cycle Assessment (LCA) and respective flows.

The characterization factors (CF) are presented in Table 3.1, being obtained from different bibliographic sources. For electricity and diesel production, the characterization factors used were according to those developed in the study of Maia *et al.* [13]. The electricity characterization factors consider the different sources (renewable and fossil sources) for electricity production in Portugal. The characterization factors for diesel production considered the European process of refining. For the foreground level, diesel consumption generates the emission of carbon dioxide, methane and nitrous oxide. These emissions represent around 2.70 kg CO₂eq for each liter of diesel consumed [19]. Organic fertilizer (manure) characterization factors were calculated using the software OpenLCA®. For this process the main inputs considered were energy consumption of electricity/heat (kWh) and land transport (kg.km⁻¹) of the organic fertilizer [20]. The environmental impacts associated to electricity consumption (6.0x10⁻⁵ kWh per kg of organic fertilizer) consider the Portuguese energy mix scenario. The consumption of electricity was assumed as that established for the production of organic fertilizer from horse manure [20]. For land transport, it was considered that 400 km per kg of organic fertilizer was the maximum distance that the organic fertilizer could reach in the Portuguese territory.

One environmental impact from the application of organic fertilizer to the soil is the eutrophication phenomena because the nutrients present in the organic fertilizer might be leached from the soil or

be transported by runoff. For humid regions or dryland with irrigation, the Intergovernmental Panel on Climate Change (IPCC) considers that 30% of nitrogen applied to the soil is lost by leaching or runoff [21]. For crambe cultivation, according the weather conditions of the region, slope of the area, irrigation technique and type of culture, it was assumed that 20% of nitrogen and phosphorus was leached. The irrigation for crambe growth leads to a loss of water by drainage or infiltration out of the perimeter of irrigation and, in this case, it was considered that 11.5% of the water supply was lost by infiltration/drainage [22].

For the flows presented in Figure 3.2 (electricity, diesel production, organic fertilizer, water consumption, diesel consumption and land occupation) three different environmental impact assessment methods/database were used to determine CF: (i) CML database; (ii) ReCiPe method; and, (iii) tool for the reduction and assessment of chemicals and other environmental impacts (Traci).

Table 3.1. Characterization Factors (CF) for elementary flows associated with crambe cultivation

Impact Category	Unit	Electricity mix (per kWh)	Diesel production (per kg)	Organic fertilizer (per kg)	Land use for agriculture (per ha)	N Fertilizer to water (per kg)	N Fertilizer to soil (per kg)	P Fertilizer to water (per kg)	P Fertilizer to soil (per kg)	H ₂ O (per m ³)	Diesel combustion (per L)	CF source
Climate change (C.change)	kgCO _{2,eq}	0.80139	0.38199	0.02642	-	-	-	-	-	-	2.90	CML
Eutrophication (Eutr.)	kgPO ₄ ^{3-,eq}	0.00030	0.00018	0.00003	-	0.42	0.42	3.06	3.06	-	-	CML
Acidification (Acid.)	kgSO ₂ ^{-,eq}	0.00606	0.00257	0.00012	-	-	-	-	-	-	-	CML
Human toxicity (H.toxic.)	kg 1,4-DB eq	0.06648	0.03782	0.00085	-	-	-	-	-	-	-	CML
Respiratory inorganics (R.inorg.)	kgPM _{10,e} q/	0.00095	0.00035	0.00001	-	-	-	-	-	-	-	Traci 2
Fresh water aquatic ecotox (F. water aqu.)	kg DB	0.00311	0.00296	0.00001	-	-	-	-	-	-	-	CML
Terrestrial ecotoxicity (E.terrest.)	kg DB	0.00154	0.00101	0.00001	-	-	-	-	-	-	-	CML
Photochemical ozone formation (P. ozone form.)	kgC ₂ H _{2,e} q	0.00025	0.00023	0.00001	-	-	-	-	-	-	-	CML
Minerals depletion (M.deplet.)	kg Fe _{eq}	0.00018	0.00084	0.00000	-	-	-	-	-	-	-	ReCiPe
Fossil fuels depletion (F. fuels depl.)	Kg oil _{eq}	0.20155	1.19438	0.00146	-	-	-	-	-	-	-	ReCiPe
Freshwater depletion (F.depl.)	m ³	-	-	-	-	-	-	-	-	0.15	-	ReCiPe
SOC loss mass due to erosion (SOC)	kg SOC	-	-	-	144	-	-	-	-	-	-	Antón et al. 2014
Ecosystem quality (E.Ecosystem Q.)	NPPD	-	-	-	2.66	-	-	-	-	-	-	Antón et al. 2014
Biodiversity, mammals (B.mammals)	PLNE	-	-	-	3.38x10 ⁻⁶	-	-	-	-	-	-	Antón et al. 2014
Biodiversity, birds (B.birds)	PLNE	-	-	-	1.19x10 ⁻⁵	-	-	-	-	-	-	Antón et al. 2014
Biodiversity, plants (B.plants)	PLNE	-	-	-	1.20x10 ⁻⁴	-	-	-	-	-	-	Antón et al. 2014

*PLNE – potentially loss of non-endemic species; *NPPD – net primary production depletion; *SOC – soil organic carbon;

The characterization factors related to land occupation for agriculture practices were according to those referred in the study by Antón *et al.* [23] who developed methodologies to assess the impact of extensive agricultural practices (without application of chemical products) in the region of Spain due to the conversion and occupation of natural areas for agriculture purposes. The weather conditions and land use practices verified in that study were considered similar to the conditions of crambe cultivation at the present study. The production of crambe seeds in Portugal only considers the impacts caused by the occupation of arable areas. The impacts caused by the transformation/conversion of land were not applicable since the area considered in this study was already arable.

Land occupation for agricultural purposes promotes several environmental damages, namely, for the soil and for the natural biodiversity. The erosion of soil can be measured by two different impact categories, i.e., soil loss as soil organic carbon (SOC) and decrease of biomass productivity due to the losses of SOC represented by net primary production depletion (NPPD) [23]. For biodiversity assessment, the number of regional and non-endemic species lost (mammals, birds, plants, etc.) should be quantified according to the different land use practices and the region [23, 24]. This characterization factor is expressed in terms of potential loss of non-endemic species.

- *Data collection and numerical calculation*

The inventory of the most relevant inputs and outputs (life cycle inventory) allows to determine the environmental impact according to the different impact categories. The data used for the calculation of the different environmental impacts results from the measures that were applied in the field during crambe cultivation. In agreement, the data for the production of 1 ton of seeds was obtained by extrapolation considering an arithmetic progression.

For each flow, the environmental impact is calculated by multiplying the amount of resource/raw material used for producing 1 ton of seeds by the respective characterization factor (equation 3.2).

$$EIC_i = \sum_j E_j \cdot CF_{ij} \quad (3.2)$$

EIC_i represents one of the sixteen environmental impact categories; E_j the amount of raw-material/resource/emission (j) used or released per functional unit; and, CF_{ij} is the characterization factor for the raw-material/resource/emission j that causes environmental impacts [25].

3.1.3.4 *Crambe abyssinica* yields, seed characterization and oil properties

Because this crop was established for oil production, the yield of the harvested seeds was determined, together with the yield of the remaining biomass. The seeds were collected by hand and, before weighting, they were sieved (6.37 mm and 4.50 mm sieves) with the objective of removing residual earth and other impurities. The biomass was crushed before weighting. The seed's moisture content was determined after drying the seeds in an oven at 105 °C, overnight. The weight of one thousand seeds was determined (TSW) and the amount of seeds per square of soil seeded was calculated considering the seed weight and the density of plants determined in the field.

Concerning the energy use/production, the oil content of the seeds was evaluated according to standard NP 659:2002 through Soxhlet extraction using petroleum ether as solvent. The quality of the oil was assessed considering the acid value (NP EN ISO 660) and the fatty acid methyl ester (FAME) profile. FAME was produced by alkaline transesterification using a temperature of 65 °C during 1 h, with a methanol:oil molar ratio of 6:1 and 1 wt.% of catalyst [26]. The FAME profile was obtained by gas chromatography analysis (EN 14103) using a Dani Master GC with a DN-WAX capillary column of 30 m, 0.25 mm internal diameter and 0.25 µm of film thickness. The following temperature program used was: 120 °C as the starting temperature, rising at 4 °C per minute, up to 220 °C, with a final holding time of 10 min.

3.1.4 RESULTS AND DISCUSSION

3.1.4.1 *Agronomic conditions*

The weather of the experimental region is temperate with a rainy winter and a dry and not too hot summer (Csb climate according to the Köppen classification). Figure 3.3 shows the weather conditions (rainfall and temperature) verified during the trials, on a monthly basis (mean values).

The mean long-term climatic characteristics between 1971 and 2000 for the region of Porto (near to the experimental field) were: 1862 mm annual rainfall, 13.6 °C mean annual air temperature. The comparison of the results for 2016 and 2017 with the long-term values showed that the year of 2016 was dry, with only 1209 mm of annual rainfall and the year of 2017 was very dry with 793 mm. The mean temperature recorded for 2016 (16.5 °C) was higher than that of the long-term average, suggesting a hot year. Similar conclusions can be assumed for the year of 2017 whose mean temperature was 16.0 °C.

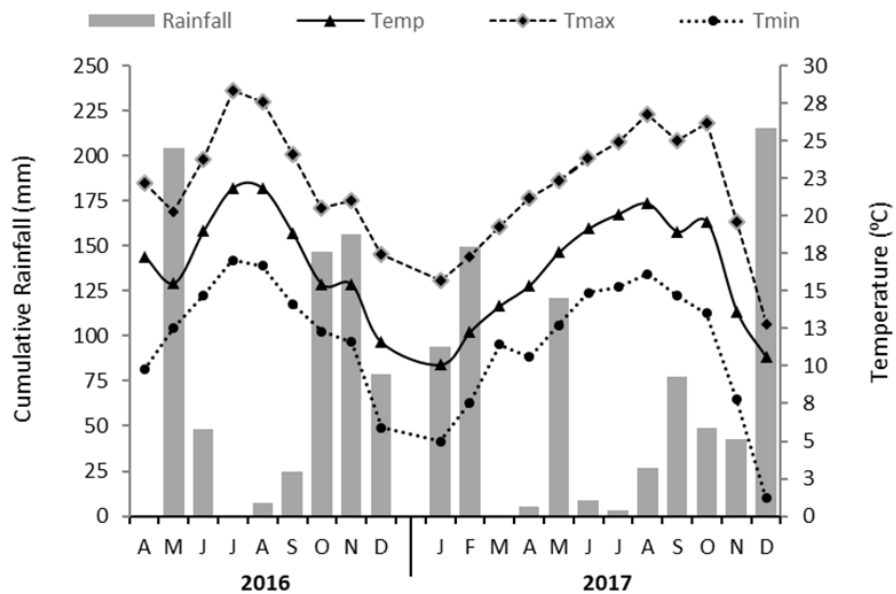


Figure 3.3. Maximum, minimum and mean temperatures and cumulative rainfall (monthly) verified in 2016 and 2017 (1st, 2nd and 3th crop installation).

The results of the soil's analysis were the following: (i) pH = 7.1; (ii) phosphorus >200 mg.kg⁻¹; (iii) 2.91 % of organic matter; (iv) 1737 mg.kg⁻¹ of calcium; (v) potassium >200 mg.kg⁻¹; (vi) 52 mg.kg⁻¹ of magnesium; (vii) 12 mg.kg⁻¹ of iron; (viii) 12 mg.kg⁻¹ of copper; and, (ix) 14 mg.kg⁻¹ of zinc. Based on the results, the soil is considered as having a good fertility with high or very high concentrations for most nutrients. Among the macro nutrients, only the magnesium evidenced low content. The levels of phosphorus, potassium and calcium in the soil were very high, therefore indicating high fertility. Also, micronutrients such as iron, copper and zinc were found in high quantities. Looking to these results, it became evident that this soil presented good quality/fertility for crambe growth, since the concentration of the most important macro and micronutrients was very high. Also, the pH of the soil was suitable for crambe growth [8].

Despite this, with the objective of increase the content of organic matter and magnesium in the soil, a natural fertilizer from organic manure, stabilized by composting, was used.

3.1.4.2 Agronomic observations

In Portugal, crambe showed a good adaptation to spring sowing with a relatively short phenological cycle which allows crop rotation with others crops. The sowing during the summer was considered very difficult due to the low moisture content of the soil. Also, the low temperatures achieved during the autumn did not allowed the germination or growth of crambe.

- *First growing season of Crambe*

Table 3.2 shows the length of phenological phases of the four growing seasons. The first sowing (on 28th April 2016) of *Crambe abyssinica* at the North of Portugal was carried out after a long period of rainfall (soil with high moisture content). This fact, associated to an increase of the mean temperature in the first twenty days, has favored the germination of the first seeds after only nineteen days.

Table 3.2. Length of main phenological phases (days) for the four growing seasons and respective weather conditions verified.

							Until Flowering			After flowering		
	Phenologic phases	S-E	E-F	F-G	G-H	Cycle (d)	Rainfall (mm)	Temperature (°C)	GDD (°Cd)	Rainfall (mm)	Temperature (°C)	GDD (°Cd)
Sowing date	28/04/16	19	36	19	21	95	252.6	16.5	685	0.0	21.8	483
	1/08/16	33	66	27	116	242	183.0	18.8	1423	474.5	13.0	1126
	30/03/17	43	38	19	24	124	127.6	17.3	1100	10.4	19.6	559
	1/08/17	42	-	-	-	-	-	-	-	-	-	-

S – sowing time; E – emergence phase; F – flowering phase; G – seed filling; H – harvest time;

For this first growing season, the length of phenological phase between the sowing and flowering was mainly related with the temperature being flowering achieved for a GDD of 685 °Cd. In Figure 3.4 is depicted the flowering stage for *Crambe abyssinica* crop. The remaining two phases (seed filling and maturation) were less dependent of the temperature. The moisture of the soils proved to be essential for the development of the germination stage considering the low cumulative GDD verified (only 207 °Cd, not shown in Table 3.2). The cumulative GDD registered for this first growing season until flowering was in the range found in others studies developed in Mediterranean region [6].

Irrigation was not performed during the first phenological phase (Sowing – Emergence). During the crop growth until flowering, some extreme events of rainfall occurred (20 mm/day), guaranteeing the moisture in the soil required for germination and flowering. May of 2016 (Figure 3.2) was the month with the highest deviations of the year (around > 70 mm rainfall) when compared with the data between 1971 and 2000. The first irrigation, with the objective of avoiding water stress, was applied only twenty-five days after the sowing, twice a week (100 L), until the beginning of the flowering phase.



Figure 3.4. Flowering stage for crambe crop after 95 days.

Phenological observations during the growth of the crop suggests that no diseases occurred. Only weeds were present, being properly removed mostly in the first two months, to avoid the competition for nutrients and sunlight.

During the crop growing period (Figure 3.5), the total amount of rainfall was 250 mm, a very low value when compared with that required by crambe (350 mm) and it occurred mainly in one week which is not very appropriate for crambe development [11]. The high moisture present in the soil when the sowing was performed allowed a quick germination of the seeds and the flowering stage started approximately 55 days after the sowing. Irrigation was performed during the following stages on a daily basis (50 L), mainly due to the absence of rainfall and to the increase of average temperature. In June of 2016 (seed filling stage) the mean temperature was very high (around 22 °C for an optimum temperature between 8 °C and 14 °C [11]) (Figure 3.5 C).



Figure 3.5. Different stages of crambe's phenological cycle: A – emergence phase; B – germination; C – flowering; D – harvest time;

At the end of 55 days the crop started to bloom being harvested 95 days after sowing (Figure 3.5 – D), when the plants showed a browned color indicating a complete development (end of cycle). After harvesting, the soil was immediately prepared for a second sowing, in a similar way as made for the first period. In Figure 3.6 is present the biomass with seeds collected at the end of the crambe's cycle.



Figure 3.6. Biomass collected (A) at the end of crambe growth cycle and seed's collection (B).

For this first crop installation, the duration of the phenological cycle was very similar to that referred in the literature (60 – 105 days) [8, 27]. After harvesting, 3710 g of seeds, corresponding to a productivity of $742 \text{ kg} \cdot \text{ha}^{-1}$, were obtained; seeds presented $26 \pm 1 \text{ wt.}\%$ of oil content. According Pitol *et al.* [28], the same cultivar in Brazil showed higher productivity, between $989 \text{ kg} \cdot \text{ha}^{-1}$ and $1347 \text{ kg} \cdot \text{ha}^{-1}$, using chemical fertilization (Table 3.3). For the seed number parameter, the variability verified between growing seasons should be related with the different characteristics of the seeds, namely, seed's weight and moisture.

According to the literature, seeds yields should be above $800 \text{ kg} \cdot \text{ha}^{-1}$ for the cultivation of this crop to be economically viable aiming biodiesel production [28].

The biomass yields obtained were $1275 \pm 3 \text{ kg} \cdot \text{ha}^{-1}$. Due to plant's characteristics, the biomass of crambe is usually crushed and added to the soil surface (no-tillage system) supporting the installation of future crops and allowing the maintenance/recovery of soil's quality and productivity with the nutrients recycling.

Table 3.3. Yield of crambe cultivar (MS brilhante) in four seasons in Portugal and comparison with the literature

Cultivar	Seasons	Sowing Date	Seed yield (kg/ha)	Biomass yield (kg/ha)	Seed number (m ⁻²)
<i>MS Brilhante</i> <i>PT</i> ¹	Spring	28/04/16	742 ^a	1275 ^a	12 576
	Summer	1/08/16	119 ^b	411 ^b	1948
	Spring	30/03/17	95 ^c	221 ^c	1671
	Summer	1/08/17	-	-	-
<i>MS Brilhante</i> <i>BR</i> ²	Spring	-	989 – 1347 ^[28]	815 – 2639 ^[29]	-

¹ Cultivated in Portugal; ² Cultivated in Brazil; ^{a,b,c} Mean values with different letters in the same column vary significantly ($p \leq 0.05$, Tukey test). Results between all seasons significantly different $p \leq 0.001$ (ANOVA).

Note: Statistic analysis was not performed to the seeds number parameter because it is a single result.

- *Second growing season of Crambe*

The second cycle of cultivation of crambe (1st August of 2016) was performed to evaluate the hypothesis of sowing twice a year with high seed's yields; due to the time of the year (summer), sowing was done after a long period without rainfall (low soil's moisture) and high temperatures. Such facts made irrigation essential for crambe germination. The first month after sowing (August) was extremely hot and dry (21.8 °C in average) and the rainfall was only 30% of the usual for this month. Irrigation was 125 L of water per day since the beginning of sowing but did not bring visible benefits for germination due to the high temperatures verified. Only one month later the seeds started to germinate. The climatic instability verified influenced crop development and resulted in variable yields. After that, already in September, there was a significant decrease on temperature leading to a reduction of the plant's growing rate. The beginning of the flowering stage (Table 3.2) was not very visible and uniform among the plants. Until flowering, the cumulative GDD was 1423 °Cd (for 99 days), a very high value when compared with the first growing season, which justifies the low growing rate of crambe. The following phenological stages occurred during autumn and winter, when the weather was cold, delaying even more the growth of the crop. The harvest was performed after 242 days (8 months) in March of 2017, therefore, the last stage of the cycle (growing and filling of seeds) was very long. After harvesting, 594.0 g of seeds were obtained which corresponds to a productivity of 118.8 kg.ha⁻¹ (Table 3.3). The mean temperature verified during this season was 15.4 °C and the total amount of rainfall was 657 mm. Despite the high levels of rainfall, the low yields (biomass and seeds) obtained suggest that high temperatures during

germination stage are not advisable. The total amount of biomass collected was $411 \pm 5 \text{ kg.ha}^{-1}$ and seeds had an oil content of $34 \pm 1 \text{ wt.}\%$. Seed and biomass yields significantly differed between the first and the second growing seasons ($p > 0.001$, Tukey test). The results obtained for the oil content parameter also significantly differed between the first and the second seasons ($p \leq 0.05$, Tukey test). Overall, the results indicate that it is possible to sow crambe twice a year, although under the climatic conditions verified, the second season appears to be unviable from an economic point of view. The slightly higher oil content found in the seeds harvested in this second season might be justified by the larger duration of the growth period, i.e., the longer time of seed's filling and maturation (last phenological phases of cycle). According Falasca *et al.* [8], crambe can grow effectively with high yields in regions with temperatures higher than 8°C (autumn/winter), although the results of this second season showed poor yields of biomass/seeds and a very long crop cycle. For that reason, additional studies with the objective to increase crambe yields during the second growing season in Portugal (development of the crop during cold weather) could be justified in the future. Although the sowing, for the second growing season, be performed in the summer, the final stages of crop cycle occurs during the autumn/winter, leading to inefficient and slow development of the crop.

- *Third growing season of Crambe*

In the following year, the sowing months were adjusted in order to evaluate the effect of that change on the yields obtained by doing two growing seasons, at warm and cold climate conditions, taking into account the results previously obtained. Thus, at the end of March (2017) crambe was sown for the third time, one month earlier than the first growing season, and the harvest time forecast for this third growing season was at the beginning of July. The preparation of soil was the same as for the other sowings. However, at the moment of the sowing, the soil was very dry due to the long period without rainfall (Figure 3.3) and therefore the irrigation was applied immediately after sowing, twice a week (100 L per irrigation), in order to increase soil's moisture content. April was very dry being the fifth hotter month since 1945 and the rainfall was only 15% of the normal for this month, corresponding to the driest April since 1931. In spite of all the efforts, the germination of seeds started only after 43 days, when it rained (May of 2017). In some parts of the cultivated area the germination did not occur which affected the plant density and the intended objective of $1000 \times 10^3 \text{ plants.ha}^{-1}$. The anticipation of sowing one month earlier did not allow the harvest of the crop one month earlier, as it was forecasted, mainly due to the late germination; the

cumulative GDD (Table 3.2) of this single phase (454 °Cd) was similar to that found for the first growing season until the flowering stage (two stages). The biomass was only collected at the end of July on the fourth month of the cycle. The seeds and biomass obtained were 95.3 kg.ha^{-1} and $221 \pm 9 \text{ kg.ha}^{-1}$ respectively (Table 3.3), values clearly different from those obtained at the first crop growing season on the same period of the year ($p \leq 0.001$). The reasons for these differences are attributed to the amount of rainfall and moisture on the soil, parameters which confirmed to be essential for the germination stage. In fact, adequate weather conditions revealed to be an essential premise for the success of crambe crop although atypical climatic conditions tend to be more frequent representing a high challenge towards ensuring crop viability. On the first growing season, rainfall occurred mostly before sowing and after germination (Figure 3.3), and on the third growing season the rainfall events did not occur in the same schedule. The temperature range during the crop development was very similar at the two trials ($15.5 \text{ °C} - 21.8 \text{ °C}$) suggesting that seeds and biomass yields were influenced mostly by the absence of rainfall. For this growing season seed oil content was $29.9 \pm 0.2 \text{ wt.}\%$ with a GDD until flowering of 1100 °Cd. The oil content of the seeds did not significantly differ between the first and the third growing seasons ($p > 0.05$).

- *Fourth growing season of Crambe*

The fourth sowing started at August of 2017 taking into account the results and knowledge acquired with the previous growing seasons. Thus, every day, during the first month, 200 L of water were added with the objective of increasing soil moisture. In spite of these efforts, only after forty-two days the seeds started to germinate. This delay on germination, which is related to the cold and dry weather verified during the following months, led to the inefficient development of the crop. Most of the plants died after the germination stage and for this reason no biomass was collected.

The seeds and biomass yields for crambe cultivated in Portugal and those obtained in Brazil (Table 3.3) were quite different mainly due to the characteristics of the weather and time of sowing. crambe is an earlier spring crop, although the results suggest as more advisable to sow crambe later (on middle of April) in Portugal, mainly due to the rainy season which could delay the germination of the crop. The first growing season led to the best results on seeds and biomass yields, making clear how the weather conditions (high levels of soil moisture and mild temperature) influence productivity. In fact, the climatic conditions verified at Brazil are considerable different of those obtained in Portugal and this fact should be considered as one of main reasons for the difference of

yields verified between the two regions. The higher yields found for the cultivation of crambe in Brazil can also be justified by the application of chemical fertilizers. Further studies contemplating additional fertilization should be considered in order to increase seed's productivity, to justify its cultivation in Portugal.

Due to the variation in weather conditions verified in Portugal during this study more research shall be developed regarding the stabilization of crop's yields to scale the process. Besides the economic value of crambe's oil, a multi-use approach should be considered, such as the use of the protein fraction (i.e. animal feed) to make viable its use [12].

Before the first sowing, the soil was analyzed to evaluate its fertility level. Overall, the soil showed very good levels of fertility for most of the parameters assessed (section 3.1.4.1). Considering that the *Brassicaceae* plants (such as crambe) have a small length of the root system (15 cm), organic fertilization was considered a good option for soil amendment, being performed before each sowing. In addition, and based on the acquired knowledge, this type of fertilization is considered sufficient for the well development of the crop, since this crop has a good adaptability to poor sandy soils [30]. Besides that, the preparation of the soil with a plough at 25 cm of depth was considered enough to ensure the replacement of nutrients in the soil's surface. Also, the rest period of the field between the summer and the spring campaigns contributes to recycle soil's nutrients with crops residues. In agreement, there is the conviction that the sown of crambe in two consecutive years (four growing seasons) was not enough to exhaust the nutrient levels of the soil and consequently to affect the crop's yield for that reason.

Rainfall amount and soil moisture appeared to be however preponderant for the growth and development of crambe crop. Therefore, taking into account the obtained results, the sensibility and perspective of the authors by following the growth of the crops during the different seasons, the different yields obtained among the growing seasons were mainly attributed to the variations in the weather conditions.

3.1.4.3 Characterization of *Crambe abyssinica* seeds

The main results from the characterization of *Crambe abyssinica* seeds harvested in Portugal and reference values (seeds harvested in Brazil) are presented in Table 3.4.

Table 3.4. Seeds oil content, yield, thousand seeds weight (TSW) and acid value obtained for *Crambe abyssinica* seeds harvested in Portugal and reference values

Cultivar	Seasons	Sowing date	Oil content (%)	Oil yield (kg/ha)	Oil acid value (mgKOH.g ⁻¹)	Seeds moisture (%)	1000 seeds weight (g)
<i>MS Brilhante PT</i> ¹	Spring	28/04/16	26 ± 1 ^a	194	0.61 ± 0.01 ^a	-	5.4
	Summer	1/08/16	34 ± 1 ^b	40	1.48 ± 0.08 ^b	7.00 ± 0.20 ^a	6.1
	Spring	30/03/17	29.9 ± 0.2 ^a	29	0.63 ± 0.03 ^a	7.20 ± 0.06 ^a	5.7
	Summer	1/08/17	-	-	-	-	-
<i>MS Brilhante BR</i> ²	Spring	-	39.1 ± 0.5	387	2.38 ± 0.09	6.7 ± 0.2	5.9
Growing seasons Factor			x		xx	ns	-

¹ Cultivated in Portugal; ² Cultivated in Brazil; ^{a,b} Mean values with different letters in the same column vary significantly ($p \leq 0.05$, Tukey test). Results between all seasons significantly different * $p \leq 0.05$; ** $p \leq 0.01$ (ANOVA). ns – Not significant.

Note: Statistic analysis was not performed to the thousand seeds weight because it is a single result and for the oil yield (results from oil content).

Regarding the oil content, the most important parameter for biodiesel production, the results ($p \leq 0.05$) showed a high content (> 26 wt.%) which seems to make this specie a good alternative to the edible crops used for biodiesel production in Portugal, namely soybean (oil content between 16 – 20%), being however lower than that of rapeseed (oil content $> 40\%$) [31, 32]. The highest seeds oil content obtained in this work for *Crambe abyssinica* corresponded to the seeds harvested at the second growing season (33.5%), although both results are close to 30%. Seeds oil content may be correlated with the GDD value accumulated until flowering [6] which justifies the highest oil content found at the second growing season (1423 °Cd) followed by the third growing season (1100 °Cd), as previously discussed.

Because seeds from Brazil showed higher oil content (close to 40%), the yields of oil per hectare were much lower in the Portuguese case. Less different were the values of TSW and moisture from the seeds harvested in Portugal and Brazil. In spite of these results, we hope to achieve higher oil

contents on crambe cultivated in Portugal, by maximizing the value of GDD accumulated until flowering (directly dependent on the date of sowing and environmental conditions). For the acid value parameter, the results obtained for the oil extracted from the seeds harvested in the first and third growing seasons are similar ($\sim 0.6 \text{ mgKOH.g}^{-1}$, $p \leq 0.05$, Tukey test). Although in the summer season the acid value was higher (probably due to the hot weather conditions during seed's processing), all extracted oils evidenced an acid value lower than 2 mgKOH.g^{-1} . Thus, the use of this oil for biodiesel production through alkaline transesterification reaction is possible (maximum advisable is 2 mgKOH.g^{-1}) [26]. The low acid value found for this oil is according to the literature, as suggested by Costa *et al.* [26], study which developed a full characterization of crambe oil was performed. Table 3.5 presents the fatty acids methyl ester profile and respective comparison between the different growing seasons and published values. Statistical data is also presented. The composition verified between growing seasons was statistically different for some FAME (methyl esters from the followed fatty acids: linoleic; linolenic; gadoleic, $p \leq 0.001$). However, FAME profile is in the range found in the literature and such differences are not preponderant for the final oil quality [26, 33].

Table 3.5. Fatty acid methyl esters profile for biodiesel obtained from *Crambe abyssinica* raw oil (wt.%) from seeds harvested in the different growing seasons

Methyl Ester of the following fatty acids	Palmitic (C16:0)	Oleic (C18:1)	Linoleic (C18:2)	Linolenic (C18:3)	Gadoleic (C20:1)	Behenic (C22:0)	Erucic (C22:1)	Others (< 2wt.% each)
Season 1 (wt.%)	1.83 ^a	17.24 ^a	8.17 ^a	4.74 ^a	3.45 ^a	2.26 ^a	56.59 ^a	5.72 ^a
Season 2 (wt.%)	1.80 ^a	16.21 ^a	8.44 ^a	5.09 ^b	3.69 ^b	2.24 ^a	55.83 ^a	6.70 ^b
Season 3 (wt.%)	2.19 ^b	16.58 ^a	10.05 ^b	6.29 ^c	5.65 ^c	1.41 ^b	52.43 ^b	5.41 ^a
Silveira <i>et al.</i> [33]	2.5	21.7	8.4	4.6	3.4	1	54.5	3.9
Growing Seasons Factor	x	ns	xxx	xxx	xxx	xx	xx	xxx

^{a,b,c} Mean values with different letters in the same column vary significantly among growing seasons ($p \leq 0.05$, Tukey test). Results between all seasons significantly different x $p \leq 0.05$; xx $p \leq 0.01$; xxx $p \leq 0.001$ (ANOVA). ns – Not significant.

3.1.4.4 Life Cycle Assessment of Crambe cultivation

The application of the methodology described in ISO 14040:2006 standard had as main goal the identification of some particularities of crambe cultivation, expressed in terms of numerical values, which can be further compared with other studies. In the present study, the results reflect a case study of non-intensive small-scale agricultural production, in an arable land. A summary of the raw materials associated to the production of the functional unit are present in Table 3.6.

Table 3.6. Life cycle inventory data to produce 1 ton of crambe seeds

Inputs	Total
Fresh water (m ³)	377
Electricity (kWh)	103
Manure fertilizer (kg)	1348
Land (ha)	1.35
Diesel (kg)	1078

Cultivation of *Crambe abyssinica* in the field used fresh water for irrigation of the crop. Electricity consumption was mainly related with the pump (0.75 kW) used to transport water to the sprinkler irrigation system. The fertilizer used in this study was from animal manure stabilized by composting and the amount applied was according the dosage suggested by the producer. Mineral diesel was spent during the preparation of the soil for sowing, using automatic ploughing (7 kW). Land occupation was calculated take into consider the seed's yields obtained for this crop in Portugal.

The outputs of the system comprise seeds production (main product), biomass production, emissions of nutrients to the surface and groundwater, due to the application of organic fertilizer, and water losses.

Table 3.7 presents the life cycle inventory outputs. The environmental impacts associated to the outputs of crambe seeds and crambe biomass are considered null because they are the final products of the system.

Table 3.7. Life cycle inventory – outputs flows associated to the production of 1 ton of crambe seeds

Outputs	Total
Crambe seeds (t)	1.0
Crambe biomass (t)	1.7
Nitrogen - Surface water (kg)	67
Nitrogen – Groundwater (kg)	202
Phosphorus - Surface water (kg)	67
Phosphorus – Groundwater (kg)	202
Water losses (m ³)	43

Taking into account the amount of resources used and emissions generated for crambe seeds production it was possible to calculate the environmental impacts for the different sixteen categories using Equation 3.2, as previously explained. Table 3.8 shows the results for the environmental impact categories resulting from the life cycle assessment of crambe crop cultivation.

Table 3.8. Results for the environmental impact categories concerning crambe cultivation (1 ton of crambe seeds)

	Foreground	Background	Total
<i>Climate change (kg CO₂ eq)</i>	313	160	473
<i>Eutrophication (kg PO₄)</i>	938	0.09	938
<i>Acidification (kg SO₂)</i>	0.00	1.07	1.07
<i>Human toxicity (t DB)</i>	0.01	12.1	12.1
<i>Respiratory inorganics (kg PM₁₀)</i>	0.00	0.15	0.15
<i>Freshwater aquatic ecotoxicity (kg DB)</i>	0.00	0.66	0.66
<i>Terrestrial ecotoxicity (kg DB)</i>	0.00	0.28	0.28
<i>Photochemical ozone formation (kg C₂H₄)</i>	0.00	0.06	0.06
<i>Minerals depletion (kg Fe)</i>	0.00	0.11	0.11
<i>Fossil fuels depletion (kg oil)</i>	0.00	152	152
<i>Freshwater depletion (m³)</i>	56.6	0.00	56.6
<i>SOC loss mass due to erosion (kg SOC.ha⁻¹)</i>	1.94x10 ²	0.00	1.94x10 ²
<i>Erosion ecosystem quality (NPPD)</i>	3.58x10 ⁰	0.00	3.58x10 ⁰
<i>Species lost - Mammals (PLNE)</i>	4.56x10 ⁻⁶	0.00	4.56x10 ⁻⁶
<i>Species lost - Birds (PLNE)</i>	1.60x10 ⁻⁵	0.00	1.60x10 ⁻⁵
<i>Species lost - Plants (PLNE)</i>	1.62x10 ⁻⁴	0.00	1.62x10 ⁻⁴

Figure 3.7 describes the relative contribution of each resource and energy source to the environmental impact categories associated with crambe growth. The impacts at the foreground level are due to the: (i) diesel consumption (climate change and human toxicity); (ii) use of organic manure as fertilizer (eutrophication of water ecosystem); (iii) irrigation of the crop (freshwater depletion); and (iv) land occupation for agronomic practices (SOC loss mass due to erosion, erosion ecosystem quality and biodiversity losses). For the background level, the environmental impacts of the resources required for crambe's seeds production were also evaluated. Climate change, acidification, human toxicity, respiratory inorganics, freshwater aquatic toxicity, terrestrial ecotoxicity, photochemical ozone formation, minerals depletion, fossil fuels depletion are the impacts categories mostly affected by the production of the resources (diesel, organic fertilizer and electricity) required in the system. Fossil fuels depletion impact is only associated to the background level and a major contribution was that resulting from diesel production. On the remaining categories, the environmental impacts are mainly due to the production of electricity from fossil fuels sources and diesel refining. The production of organic manure was the flow which less contributed for the background environmental impacts, due to its origin. When compared with conventional fertilizer, organic fertilizer (poultry manure) is less harmful than the conventional fertilizers that require extraction of high amounts of natural resources, causing very impact for climate change category [34].

The climate change impact for foreground level is very high compared to background level mainly due to the high emissions released during diesel combustion. According to Antón *et al.* [23], 1 ha of land used for agriculture production in an extensive mode (without application of chemical products) contributes to climate change (foreground level) with near to 1730 kg CO₂ eq.

The SOC loss mass due to erosion is directly related to the type of land use, type of soil and crop management. For crambe's cultivation, SOC loss mass was high because the preparation of the soil by conventional tillage increases the losses of carbon from the soil.

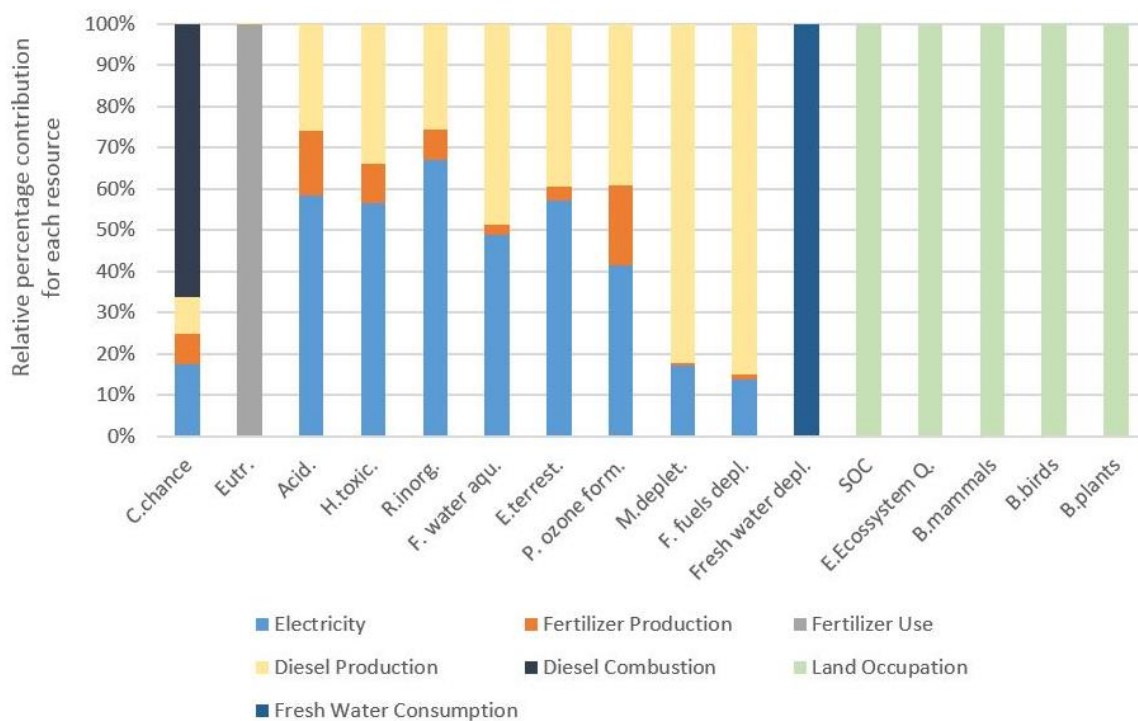


Figure 3.7. Relative contribution (%) of each resource and energy source for environmental impacts according the different impact categories.

According to Nachimuthu and Hulugalle [35,38], the cultivation of the edible crop soybean with preparation of the soil by mechanical agitation (tillage) causes a SOC loss of 114 kg C.ha⁻¹. This impact on crambe cultivation (194 kg C.ha⁻¹) can be reduced by adopting non-tillage practices which protect the soil and reduce its mobilization [36].

The net primary production depletion represents the decrease of productivity resulting from the erosion of the soil (Table 3.8). For comparison, the production of one hectare of tomato in Spain lead to a decrease of productivity (net primary production depletion) between 4.67 and 7.98 NPPD; for crambe, the NPPD obtained was only 2.65 (per ha) [37, 38].

Taking into account the impacts from land resource occupation of 1.35 ha with crambe crop, the values found are in the range predicted for the agriculture practices in European region [37, 38]. The overall losses of biodiversity associated to cultivation of crambe were low because the land that was used in this study was already arable.

Looking to the results presented in Figure 3.7, the high impact of diesel consumption on climate change category is evident (above 60%). On all the environmental impact categories (except climate change), the electricity production (background level) step was the one that impacted the most. For climate change, diesel combustion (foreground level) had the major contribute. Mineral depletion and fossil fuels depletion are mainly caused by the production of electricity and diesel (background level). In Europe, the electricity produced from renewable sources represents 28.3%, being the remain produced from fossil fuels (natural resources depletion); for this reason, biodiesel should be taken into consideration as complementary alternative energy source [39]. Organic manure production had a small contribution for fossil fuel depletion (not visible in the figure). With the objective of minimizing the environmental impacts caused by the production of 1 ton of crambe's seeds, using less diesel is an option that should be considered, for the reasons presented before (high impact for foreground and background level). The integration of biodiesel fuel in alternative to mineral diesel is suggested to reduce the environmental impacts of agriculture labors because biodiesel consumption lead to a reduction of greenhouse gases between 19 to 56% [40].

The impact due to crambe cultivation under the conditions of this work is low, for most of the parameters, when compared with other types of crop cultivation, such as canola or maize [23]. For example, the production of 1 ton of canola edible oil (packaged) caused, only at farm level, an impact for climate change of 1588 kg CO₂ eq, higher than that verified for crambe (473 kg CO₂ eq) [41]. For maize crop the impact on climate change (1730 kg CO₂ eq) from cultivation was higher than those verified for crambe crop (473 kg CO₂ eq). For the impact on erosion ecosystem quality, maize crop had associated an impact of 2.66 NPPD, similar to the found for crambe crop (2.65 NPPD, respectively) [23].

3.1.5 CONCLUSIONS

This study showed that *Crambe abyssinica* might be cultivated in Portugal resulting in seeds with relatively high oil content (> 26 wt.%) and acceptable yields (742 kg.ha⁻¹), but that viability of scaling production in Portugal should also depend upon integrated use of all the biomass resource. Under

the period of two years studied, crambe cultivation was clearly affected by Portuguese weather conditions, namely some extreme climate events, which tend to be unfortunately more frequent. In spite of the unstable weather conditions (atypical periods of high rainfall and temperatures), the results from the four growing seasons indicate that high soil's moisture and mild temperatures until flowering are necessary to achieve good emergence rates on short periods of time (between 19 – 43 days). Taking into account the progressive climatic change, more studies regarding the adaptation of this culture to Portuguese edapho-climatic should be developed in the future. In addition, seeds oil content seems to be slightly influenced by the GDD value accumulated until flowering (33.5 wt.% of oil obtained from seeds when GDD was 1420 °Cd and 26.1 wt.% when GDD was 685 °Cd).

Besides the relatively low seed yields obtained, crambe non-edible crop can be considered an alternative source of oil namely for use as raw material for biodiesel production. The plants density, distance between rows, stem branching and amount of fertilizers used can contribute to increase and stabilize the yields of the crop under different weather conditions.

Despite the small scale of the agronomic study carried out, the example of application of the life cycle assessment showed the main origin of the environmental impacts associated to crambe cultivation. The impacts found are mainly related to the consumption of diesel and electricity used in the preparation of field for sowing and its irrigation. The substitution of mineral diesel for biodiesel might be a way to improve the environmental performance of the system decreasing impacts on climate change, eutrophication, human toxicity, freshwater depletion and fossil fuel depletion (background production). Application of organic manure decreases the impacts caused by the cultivation of crambe when compared with intensive agriculture practices, being also a positive aspect of the system employed. Overall, crambe cultivation showed lower impact for climate change than that verified for others agriculture systems (canola and maize); although, the impact on SOC loss by cultivation of crambe is higher than expected.

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3.2 *CRAMBE ABYSSINICA* OIL REFINING BY DEGUMMING PROCESS

ABSTRACT

In the present study, the effect of different degumming processes on the phosphorus content of *Crambe abyssinica* oil and resultant biodiesel was evaluated. The non-edible oil was submitted to water degumming and chemical degumming with different concentrations of phosphoric acid and varying the acid to oil volume percentages. Phosphorus content of the products was measured by UV spectroscopy after sample calcination, according to NP 1994:2000. Biodiesel was produced by transesterification using a 6:1 methanol to oil molar ratio and 1 wt.% sodium hydroxide as catalyst at 65 °C, during 1 h. The biodiesel produced directly from the raw oil presented high phosphorus concentration (>20 ppm); consequently, a degumming process was required to fulfil the quality standard (<4 ppm according to EN 14214). Water degumming was not effective, leading to biodiesel with a phosphorus concentration of 12.2 ppm. Among the acid degumming processes evaluated and taking into account technical and operational variables, the best established conditions, which allowed the fulfilment of the quality standard regarding phosphorus concentration, was considered to be 25 wt.% acid concentration and 0.80 vol.% of acid to oil.

This chapter was based on:

Emanuel C., Manuel F. Almeida, Maria C.M. Alvim-Ferraz, Joana M. Dias, “*Effect of Crambe abyssinica oil degumming in phosphorus concentration of refined oil and derived biodiesel*”, Renewable energy journal, volume 124, 27-33, 2018.

3.2.1 SCIENTIFIC RELEVANCE

The direct use of non-edible oils for biodiesel production is not usually possible because typically they contain free fatty acids (FFA) and phospholipids as well as other undesirable compounds. Such impurities might, in particular: i) react or inactivate the transesterification (most used reaction) catalyst [1] and affect mass transfer; ii) difficult the separation of the biodiesel and glycerol products [2]; and iii) make more demanding the water washing purification step (conventionally used). In agreement, the degumming pre-treatment (to remove phospholipids, waxes, among others) together with the neutralization or esterification should be applied before biodiesel production. These procedures will therefore avoid problems concerning the biodiesel synthesis and purification aiming a proper product quality.

Since each oil might present different proportion of the mentioned contaminants, degumming should be studied for different raw materials, contributing to the economic viability of their use.

The most common impurities in crude plant oils are phospholipids, sugars, acylglycerols, free fatty acids, steroids and trace metals [3-5]. Phospholipids are molecules constituted by glycerol with two fatty acids attached and a phosphoric molecule [1]. The presence of phosphorus in biodiesel is directly related with the degree of oil refining and it is regulated by the standard EN 14214 (< 4 ppm). The phospholipids which can be found in the crude plants oil are mainly of two types: hydratable (HP) and non-hydratable (NHP).

The presence of phosphorus in biodiesel affects the catalytic conversion at the exhaust systems in diesel engines and increases the generation of several pollutants in the exhaust gases, such as particulate matter, carbon monoxide and sulfur dioxide [6, 7].

The HP can be removed from the oil by adding hot water, which forms an insoluble water-phospholipid complex (hydrated the phospholipid), easily separated from the remaining phase by centrifugation [4, 8]. The HP present in the crude oil are of two types: phosphatidyl choline (PC) and phosphatidyl inositol (PI). PC is easily hydrated because it contains a quaternary ammonium salt with positive charge for all range of pH and good affinity for water. PI charge balance depends on the pH but the presence of five hydroxyl groups (OH^-) makes it extremely hydratable by water for all pH range, presenting therefore a hydrophilic nature. After de degumming process it is possible to recover products with commercial value as a food supplement like lecithin phospholipid or phosphatidic acid [9].

In order to separate the NHP, chemical degumming is performed using mostly phosphoric acid (H_3PO_4), sulphuric acid (H_2SO_4), hydrochloric acid (HCl) and citric acid ($\text{C}_6\text{H}_8\text{O}_7$) [1, 9-11]. These reagents promote their conversion into the hydratable form which can be more easily further removed [11]. The chemical degumming allows the removal of the two phospholipids types because usually combines the addition of acid and water. The main NHP found in vegetable oils are phosphatidyl ethanolamine (PE) and phosphatidic acid (PA). The PE shows a balance of charges equal to zero; and, therefore, it has no affinity with the polar water molecule, this characteristic is evidenced when the pH is close to neutral. For pH smaller than 3 or higher than 9, the phospholipid molecule can be hydratable. Sometimes it is possible to find PA in the form of calcium salts in the vegetable oils, which remain stable without charges regardless of the pH. For this reason, some degumming procedures include the addition of an alkali with objective to convert calcium salts in other salts (sodium salt or potassium salt) with higher water solubility [8, 11].

From the literature review performed, few studies were founded regarding crude oil degumming, with focus in phosphorus content reduction [3, 9-14] and none existed for crambe oil pre-treatment towards bioenergy production; from those found, although it is consistent the use of phosphoric acid and acid degumming as the best option, the conditions vary considerably making difficult the selection of the best route [10]. In addition, although studies mention the importance of degumming raw oil before biodiesel production [12, 15], no studies existed on the effect of the pre-treatment on the biodiesel quality, as well as the combined effect of the crude oil pre-treatment and biodiesel post-purification procedures [8, 16].

3.2.2 SPECIFIC OBJECTIVES

The main goal of the present work was to evaluate different refining processes to decrease phosphorus content present in the raw oil extracted from crambe commercial seeds. Accordingly, the study evaluated:

- the viability of degumming to decrease phosphorus concentration in crambe oil;
- different degumming procedures, assessing their efficiency;
- the impact of oil degumming on the phosphorus concentration of the biodiesel obtained.

3.2.3 MATERIALS AND METHODS

3.2.3.1 Materials

Crambe abyssinica seeds were provided by “B & T World Seeds” (Czech Republic), and “Fundação MS” (Brazil). The use of two different seed suppliers was justified by the fact that the amount of seeds available for oil extraction from the European source (from which initial studies were developed) was not enough to complete the studies and no additional material was available. It should be highlighted that for the study of the degumming process both seeds were used, whereas for the biodiesel production using the refined oil only the oil obtained from the Brazilian seeds was used (Figure 3.8).



Figure 3.8. *Crambe abyssinica* seeds used for oil's extraction.

Petroleum ether (LabChem) was used for chemical extraction (soxhlet). Methanol 99.9% (analytical grade, Fischer Chemical) was used in the transesterification reaction and NaOH 97% powder (analytical grade, Aldrich) was used for transesterification and degumming processes. Phosphoric acid was prepared from concentrated acid (85%) supplied by Merck.

3.2.3.2 Preparation and extraction of *Crambe abyssinica* seed oil

The oil extraction was performed using a 1 L Soxhlet extractor. The procedure adopted was based in NP EN ISO 659 (2002). In a first phase, the seeds were crushed in a mortar and placed inside the thimble. Then the thimble was dipped in petroleum ether solvent for 6 h (equivalent to 14 turns of the solvent in the extractor). At the end of the extraction, the solvent was removed in a rotary evaporator at 73 °C. Several extraction cycles were conducted until the amount of oil necessary for the study was obtained.

3.2.3.3 Crude oil properties

Crude oil was characterized in terms of phosphorus content, acid value, oxidation stability, density and fatty acid profile. The methodology used for phosphorus determination is described in Section 3.2.3.5. The acid value was determined according to EN 14104 (titrimetric method). The oxidation stability was measured in agreement with EN 14112 by accelerated oxidation using a Rancimat equipment (Metrohm) and the density was determined at 15 °C using a pycnometer (standard NP 938 1988). The fatty acid profile was assessed through the fatty acid methyl esters content of *Crambe abyssinica* biodiesel using gas chromatography and following the standard EN 14103.

3.2.3.4 Oil degumming

- Water degumming

Water degumming experiments were conducted at different ratios of oil to water and at different temperatures (Figure 3.9), in order to evaluate its impact on phosphorus content as well as to establish the best refining conditions.

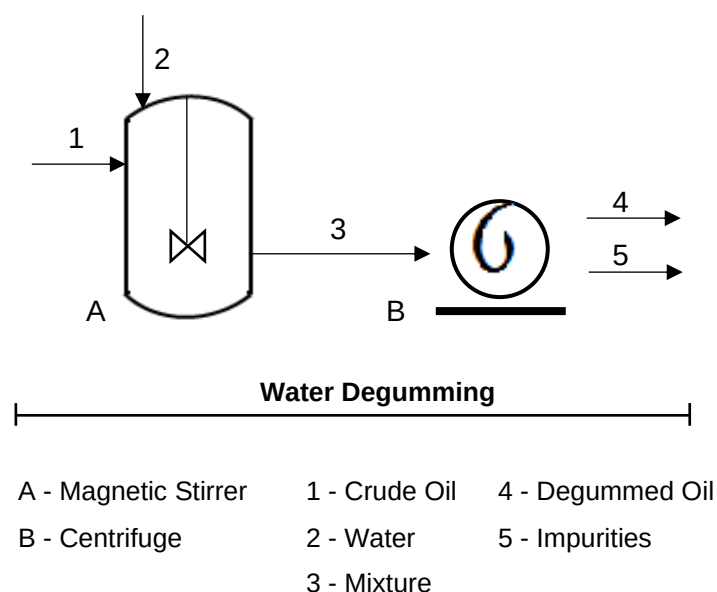


Figure 3.9. Diagram adopted for water degumming using *Crambe abyssinica* crude oil.

The experiments were carried out by mixing raw oil (30 g per experiment) with water (3 – 5 wt.%) at different temperatures (22 °C (ambient T), 35 °C, 40 °C, 45 °C and 50 °C) and stirring the mixture with magnetic stirrer (slow mix) during 30 min [11, 16, 17]. Finally, the mixture was submitted to centrifugation during 10 min at 3500 rpm.

- *Chemical Degumming*

To remove the maximum amount of phospholipids, chemical degumming is proposed by different authors [10, 11, 15] in order to convert NHP into HP and remove both. The most common acids employed are phosphoric acid or citric acid at doses between 0.05 and 2 wt.%, relative to oil, depending on the acid concentration [9, 18]. The addition of acid allows pH reduction and consequently the conversion of NHP into HP; the further use of an alkaline solution is sometimes required to neutralize the excess acid. The amount of alkaline solution should be the minimum possible to reduce the amount of soaps produced. The neutralization in the industrial oil's sector is usually made with sodium hydroxide (4 – 8 wt.%) at a dose of 2 vol.%, relative to oil [9, 15, 16]. The addition of water to the mixture is justified to remove the hydratable phospholipids fraction; amounts of water between 2 and 15 vol.%, relative to oil, are usually employed [11, 15].

The order of addition of the reactants previously referred varies significantly in the revised studies [11, 15]. To reduce mass transfer limitations, the heating of the oil and an efficient mixing is advised [9]. Based on the literature review, two methodologies were studied, according to the following procedures:

Methodology A1 (25 g oil per experiment)

Heating the oil till 60 °C; add acid solution with concentration of 85 wt.% varying the ratios between 0.05 and 2 wt.% (relative to mass of oil); mix during 5 min with magnetic stirrer; add 2 wt.% NaOH (relative to oil mass, with concentration of 4 wt.%); mix during 60 min with magnetic stirrer; add 5 wt.% of water (relative to oil mass); mix during 30 min with magnetic stirrer; and, centrifuge during 10 min at 3500 rpm (Figure 3.10).

Methodology A2 (25 g oil per experiment)

This methodology was similar to the A1 except the fact that the acid to oil mass percentage varied between 0.1 and 0.2 wt.% and the addition of water was performed before the addition of the alkali (NaOH). To be applicable to this methodology, please read in Figure 3.10 input 4 as the addition of water and input 6 as the addition of NaOH.

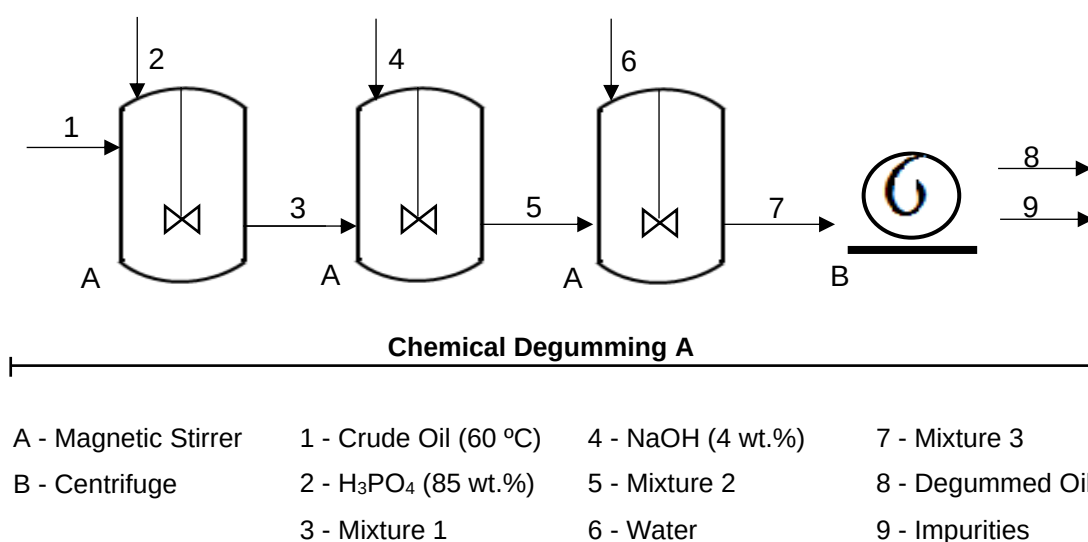


Figure 3.10. Diagram adopted for chemical degumming through methodology A1 using *Crambe abyssinica* crude oil.

Methodology B (30 – 40 g per experiment)

Prepare different acid solutions: 85%, 75% and 25 wt.%, heat the oil till 75 °C; mix different amounts of acid solution with the oil, namely, 0.10 vol.% (85%) (meaning a dose of 0.10 vol.% relative to oil, considering an acid solution of 85 wt.% concentration), 0.18 vol.% (85%), 0.18 vol.% (75%) and 0.80 vol.% (25%) with 0.6 wt.% water (relative to mass of oil); add this mixture to the oil; shake intensely during 1 min with a vibrator stirrer; stir the mixture during 5 min using magnetic stirring; add 2 vol.% water (relative to oil); shake the mixture for 20 min; and, centrifuge during 10 min at 3500 rpm (Figure 3.11).

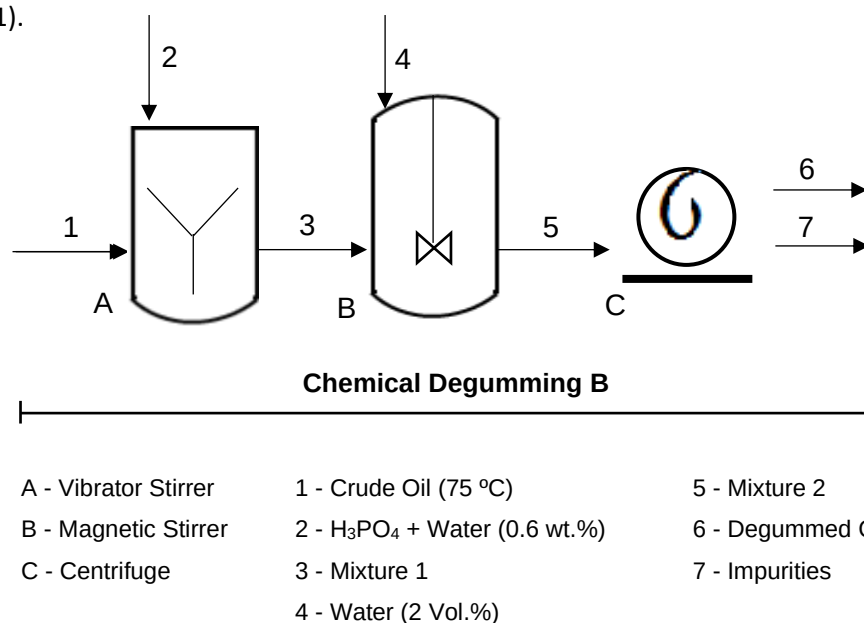
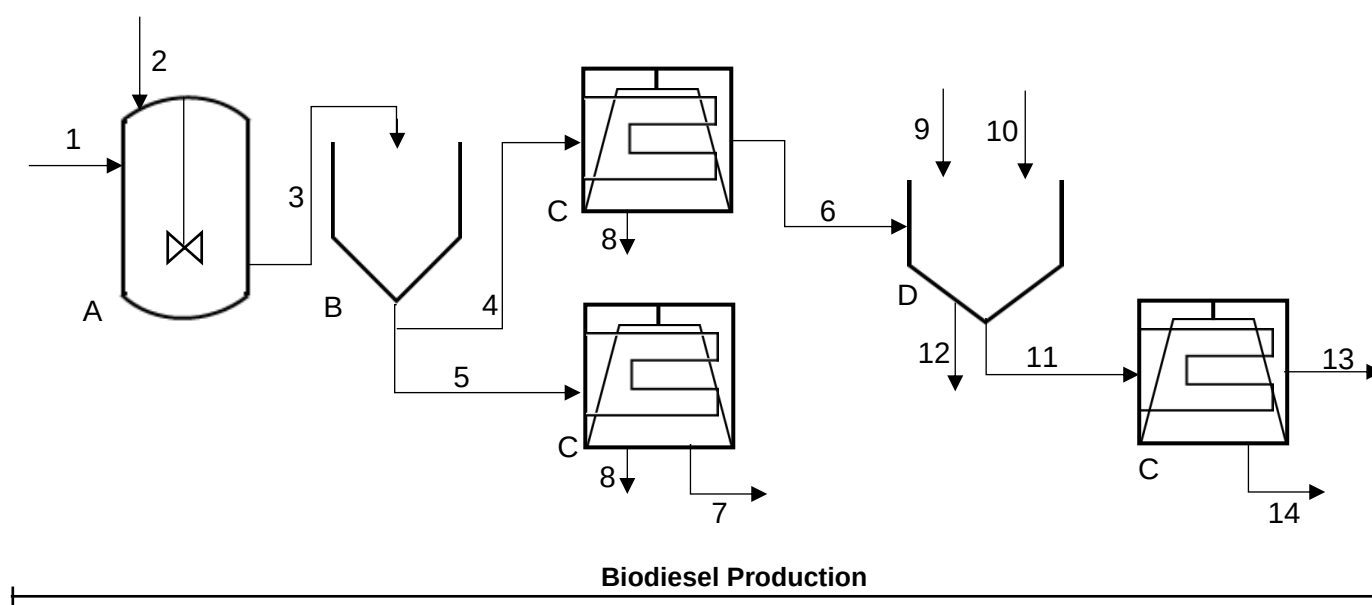


Figure 3.11. Diagram adopted for chemical degumming through methodology B using *Crambe abyssinica* crude oil.

3.2.3.5 Biodiesel transesterification – alkaline transesterification

Biodiesel was produced by transesterification (Fig. 3.12). The oil (30 – 160 g) was added to a batch reactor consisting of a round-bottom flask equipped with a water-cooled condenser to allow methanol reflux and magnetic stirring, immersed in an acrylic thermostatic bath [19]. The temperature was 65 °C, the methanol to oil molar ratio was 6:1 and the catalyst concentration was 1 wt.% of NaOH (relative to mass of oil) for a reaction time of 1 h in agreement with previous studies [19]. At the end of the reaction, the products were left to settle under gravity for 1 h in a separating funnel (decanter), originating two phases: biodiesel (upper layer) and glycerol (lower layer). After separation, the biodiesel phase was first washed with 50 vol.% of hydrochloric acid (0.5 wt.%) and after repeatedly with 50 vol.% of distilled water. The washing steps ended when the pH of the washing water was close to that of the distilled water. Finally, the biodiesel was dried using a rotary evaporator.



A - Magnetic Stirrer
B - Decanter
C - Rotary Evaporator
D - Decanter/Washer

1 - Crude Oil
2 - Methanolic Solution
3 - Products from the reaction
4 - Biodiesel + methanol + impurities
5 - Glycerol + methanol + impurities
6 - Biodiesel + impurities
7 - Glycerol + impurities

8 - Methanol
9 - Distilled water
10 - HCl 0.5 % V/V
11 - Biodiesel + water
12 - Wastewater
13 - Biodiesel
14 - Water

Figure 3.12. Diagram adopted for biodiesel production for *Crambe abyssinica* oil.

3.2.3.5 *Determination of phosphorus content*

Phosphorus was determined in the oil and in the biodiesel product according to NP 1994:2000 standard. The procedure included calcination of the sample to eliminate organic matter followed by phosphorus determination by UV spectroscopy (UVmini-1240 Shimadzu). The determination of phosphorus content by standard EN 14107 (inductively coupled plasma emission spectrometry) was not possible due to technical constraints.

Considering the nature of the degumming process it is expected that it will affect mainly the following biodiesel properties: i) phosphorus content; ii) acid value; and iii) oxidation stability. Total contamination and viscosity might also be affected by the presence of impurities. From those, there is no doubt that the most relevant is the phosphorus content, since the main purpose of degumming is to remove HP and NHP [7]. Due to the low free fatty acid content of the oil (further presented in Section 3.2.4.1) and the conventional use of antioxidants by the biodiesel industry, in the present work the influence on product quality was measured only considering the phosphorus content.

3.2.4 RESULTS AND DISCUSSION

3.2.4.1 *Crude oil physico-chemical properties and biodiesel production*

The raw oil showed the following properties: i) acid value of 2.38 ± 0.09 mg KOH.g⁻¹; ii) oxidation stability of 4.04 ± 0.03 h; iii) oil density of 0.890 g/cm³; and iv) phosphorus content of 86.30 ± 15.45 ppm. In Figure 3.13 is possible see the aspect of crambe crude oil.



Figure 3.13. Crambe crude oil extracted for laboratorial studies.

The acid value of the oil indicates the amount of free fatty acids present in the raw material, parameter very important for the biodiesel production with good quality, given that its presence decreases the yield of the transesterification reaction, if done using the homogeneous alkali catalyzed route, as previously referred. The acid value obtained is in the limit to allow the direct application of homogeneous alkali transesterification without the need of pre-treatment [20]. The stability of crude oils is expected not to be very high, namely due to the presence of FFA and it will vary depending on the natural antioxidants' composition, among other factors. The value determined was lower than that verified in a similar study for *Jatropha curcas* crude oil (7.92 h) [15] and it is in the range of values found in different crude oil extracts (3.91–6.62 h) [21]. The oil density agrees with values found for other raw oils [11, 15]. The phosphorus content is lower than that reported for several raw oils, namely that found for coconut raw oil (600 ppm) or *Jatropha curcas* raw oil (1200 ppm) [10, 11]. For sunflower oil, according to Carelli *et al.* [22], phosphorus concentration on raw sunflower oil extracts can vary between 342 and 657 ppm.

The composition of both oils is not expected to differ, since both seed suppliers ensured quality, i.e., seeds of *Crambe abyssinica*. Fatty acid composition, evaluated through the methyl ester profile (Table 3.9) was assessed for the seeds with origin in Brazil, and it is in agreement with the range reported in the literature [6, 23], showing the predominance of monounsaturated acids (represent 77.8 wt.%) and more specifically of the erucic fatty acid (C22:1). The parameters evaluated were fairly constant for oil obtained from both seeds; the variation found in the phosphorus content was attributed to the use of different seed suppliers and the agronomic conditions applied for crop development.

Table 3.9. Fatty acid methyl esters content of *Crambe abyssinica* biodiesel and reference values

Methyl Ester of the following fatty acids	wt.%	[23]	[7]
Palmitic (C16:0)	2.0	2.5	2
Oleic (C18:1)	16.8	21.7	17–19
Linoleic (C18:2)	8.4	8.4	9
Linolenic (C18:3)	4.8	4.6	6–7
Gadoleic (C20:1)	3.4	3.4	4
Behenic (C22:0)	2.3	1.0	1–2
Erucic (C22:1)	56.1	54.5	56–59
Others (<2 wt.% each)	6.2	3.9	3

In order to verify if the degumming process was not required for the production of biodiesel with good quality in terms of phosphorus content, the production of biodiesel from raw crambe oil was

performed. Table 3.10 presents the results obtained, showing a product with phosphorus content around 21 ppm (75.4% of phosphorus concentration reduction), much higher than the threshold value of 4 ppm imposed by standard EN 14214. Thus, the degumming process is required before using this oil for the production of biodiesel with appropriate quality regarding this parameter.

Table 3.10. Phosphorus content of *Crambe abyssinica* crude oil and resultant biodiesel

Phosphorus in crude oil (ppm)	Phosphorus in biodiesel (ppm)
86 ± 16	21 ± 2

2.2.4.2 Effect of the water degumming process on oil and biodiesel phosphorus content

The first experiments of water degumming were conducted at 22 °C (ambient T) with 3 wt.% of distilled water. It was possible to achieve a reduction in phosphorus content of 44%; however, the phosphorus content of the refined oil was still high (55.4 ppm). For the additional experiments, in agreement with the literature, a higher dosage of water (5 wt.%) and higher temperatures (35 °C – 55 °C) were evaluated [9, 15, 24]. Figure 3.14 presents the results of the water degumming at different temperatures.

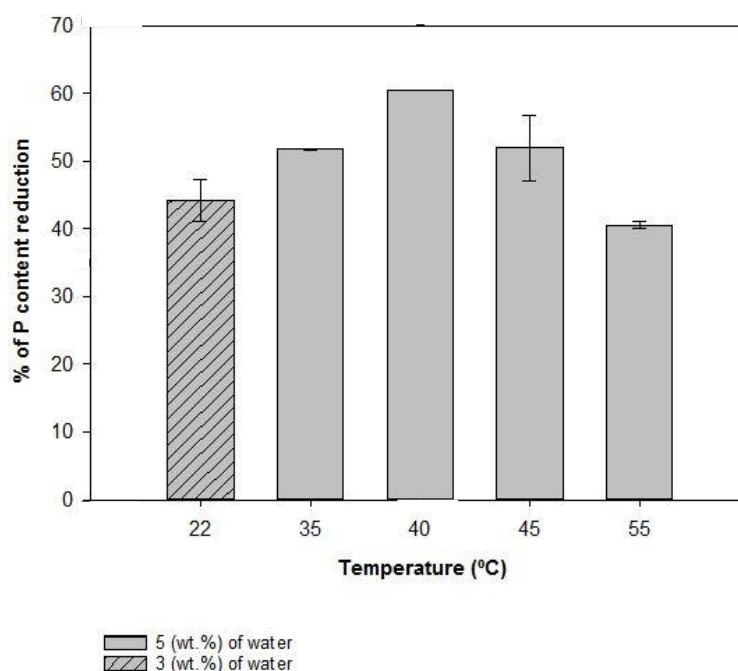


Figure 3.14. Reduction of phosphorus content during water degumming at different temperatures.

A higher temperature reduces oil viscosity which might enhance the hydration of the phospholipids; however, a temperature increase can also have a detrimental effect since it might increase HP solubility in the oil [8]. In agreement, it could be seen that by increasing the temperature up to 40 °C a beneficial effect was observed and a higher efficiency of the degumming was achieved, whereas for temperatures higher than 40 °C, the efficiency of the water degumming decreased. After water degumming at 40 °C (the best condition established), the oil presented a phosphorus content of 39.4 ppm ($\pm 0.02\%$), which corresponded to a reduction of phosphorus concentration around 60%.

In order to evaluate if the process would be adequate to obtain biodiesel with good quality in terms of phosphorus content, transesterification of the refined oil was conducted for the best condition found (40 °C). The lowest phosphorus content in biodiesel determined was 12.21 ± 0.65 ppm, more than 3 times the standard limit. The high reduction of phosphorus concentration after the biodiesel production process (which was around 70% of the concentration in the degummed oil), is associated with the purification steps of the process which drags out phosphorus components. Although this value was lower than that obtained from raw oil transesterification, it does not enable the production of biodiesel with good quality in terms of phosphorus concentration (<4 ppm). In agreement, the chemical degumming of *Crambe abyssinica* oil was further studied aiming a higher reduction of phosphorus content in biodiesel. In Figure 3.15 is depicted the aspect of crambe oil pre-treated after centrifugation where it is visible some settling of phospholipids.

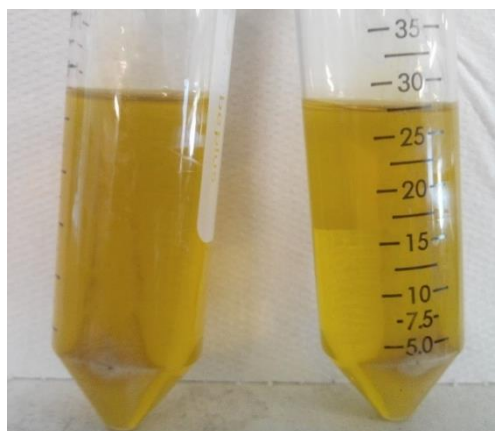


Figure 3.15. Crambe oil at the end of water degumming.

2.2.4.3 Effect of the chemical degumming process on oil and biodiesel phosphorus content

- Methodology A

Figure 3.16 shows the results obtained using the acid degumming methodology A.

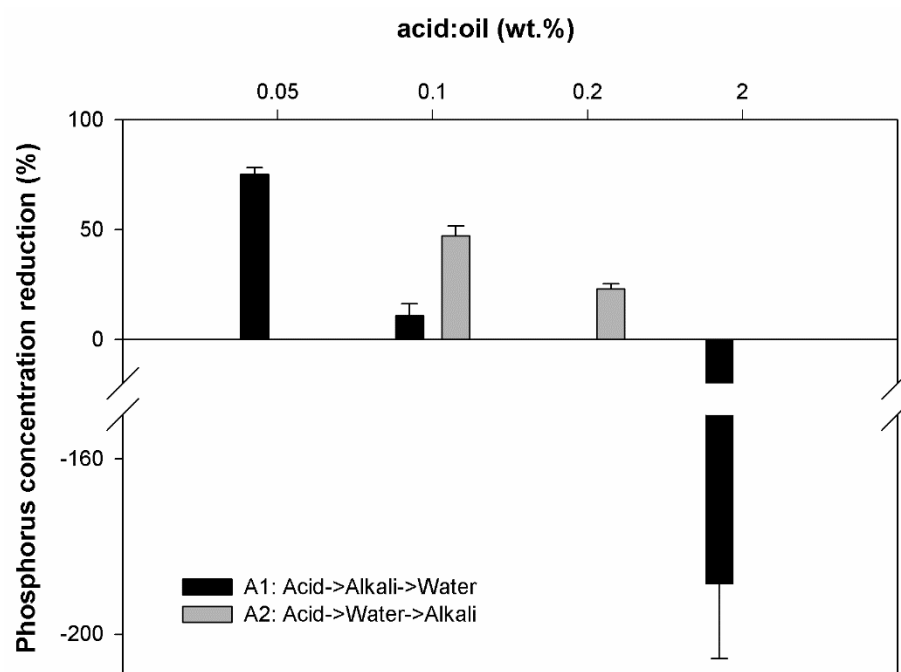


Figure 3.16. Phosphorus concentration reduction (%) after degumming using methodology A.

For the acid degumming using methodology A1, different amounts of acid relative to oil were used (0.05 – 2 wt.%). The highest (2 wt.%), gave a final phosphorus content higher than the original found in the oil (249 ppm compared to 86.3 ppm). Thus, the use of a higher dosage of H_3PO_4 did not bring any benefit for the degumming process (a high amount of inorganic phosphorus still remained in the oil and, despite the neutralization step, this excess was not completely removed). Using 0.10 wt.%, the reduction of phosphorus content was approximately 11% and a high phosphorus concentration was still found in the product (77.1 ppm). The lowest concentration, of 0.05 wt.%, led to the highest phosphorus concentration reduction, of 75% (final concentration 21.4 ppm). In Figure 3.17 it is possible to observe the crambe oil after chemical degumming being very visible the presence of two phases, namely, crambe oil and impurities (mostly phospholipids). The methodology A2 at 0.10 wt.% H_3PO_4 reduced phosphorus content in 47%, showing better results than the previous methodology (A1), whereas with 0.20 wt.% H_3PO_4 , the reduction achieved was

23%. Biodiesel produced using the degummed oils from methodology A2 showed, on average, a phosphorus concentration of about 20 ppm, meaning an overall reduction of 74%. However, such value was still above the limit established by the Standard EN 14214 (4 ppm) with no significant advantages related to that obtained directly from crude oil, as shown in Section 3.2.4.1.

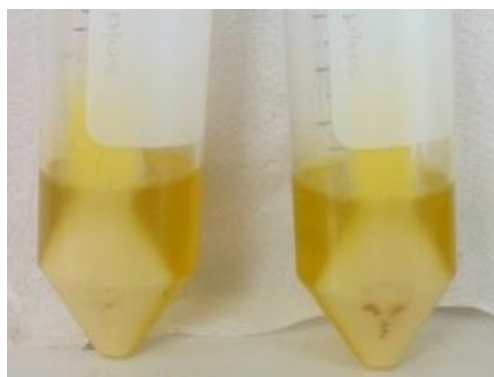


Figure 3.17. Crambe oil at the end of chemical degumming.

Several industrial processes add NaOH to the oil aiming to remove FFA and other undesired matter like soaps and gums. For the same load of acid, the methodology A2 led to better results than A1, possibly due to the formation of soapstocks with high consistency and thickness, by adding NaOH in the first step of the methodology (which does not occur in methodology A1), becoming easier to remove phosphorus and salts [9].

The methodology A1 was applied at a larger scale, using 160 g of crude oil, and biodiesel was produced under the best conditions found (Figure 3.18).



Figure 3.18. Biodiesel and glycerol obtained using refined crambe oil.

Table 3.11 shows the results obtained for the reduction of phosphorus concentration in the degummed oil and biodiesel product. Under such experimental conditions, the reduction achieved was around 29%. After biodiesel synthesis and purification, the final content of phosphorus in biodiesel was approximately 24.2 ppm (reduction of 71.95%), also with no advantages relatively to the direct use of the raw oil for biodiesel production which showed a final phosphorus content of 21.19 ppm.

Table 3.11. Phosphorus concentration in raw oil, after degumming with methodology A1 using 0.05 wt.% H_3PO_4 , and in the biodiesel product

Phosphorus concentration in raw oil (ppm)	86 ± 16
Phosphorus concentration in refined oil (ppm)	61 ± 3
Phosphorus concentration in biodiesel (ppm)	24 ± 1

Note: 160 g of crude oil;

The results suggest that the methodologies A1 and A2 were not effective to produce a biodiesel with good quality, despite allowing a high reduction of phosphorus content. It seems that such conditions were not effective, in particular, to remove NHP given that phosphorus content is similar both after biodiesel production followed by washing and after acid degumming of the raw oil followed by biodiesel production.

Also, results show that the degumming process was more efficient at this scale than at the smaller one before tested (< 40 g per experiment, see 3.2.3.4 – chemical degumming), which may be explained by issues related with the mixture of the reagents and centrifugation efficiency, difficulties that should be considered for the upscaling of such processes. Considering the results and further industrial application of such methodologies it is advised to ensure vigorous stirring and avoid vortex during reactants mixture through reactor design and type of mechanical stirring equipment. Also, it is recommended to strictly control temperature, using the highest possible, with the objective to maximize mass transfer but avoiding the chance of increased solubility of gums on the oil. The possibility to add phosphoric acid to the crude oil by drip system might in addition allow the gradual hydration of NHP, contributing to a higher phosphorus removal efficiency [9]. Due to the high content of phosphorus after the degumming procedure it was necessary to evaluate other methodology to reduce biodiesel phosphorus content to acceptable levels.

- *Methodology B*

These experiments intended to assess the effect of using different vol.% ($V_{\text{acid solution}}/V_{\text{degummed oil}}$) of acid solution (with different concentrations) mixed with 0.6 wt. % of water and also intensive mix. Intensive mix is expected to be more effective than the mixture provided by magnetic stirring leading to a better contact between the acid and the oil avoiding the formation of a vortex, very common with magnetic stirring. In agreement, it is expected that hydratation occurs almost instantly, reason why the time of mixing can be very short (1 min.) in comparison to that of the methodology A, to achieve the desired degree of NHP decomposition into hydratable phospholipids [9]. Results obtained using a 0.18 vol.% acid solution allowed a phosphorus content reduction of 53%. Following, the biodiesel produced using the degummed oil had 3.5 ppm of phosphorus, meaning a concentration reduction of 96% and allowing a product complying with the biodiesel quality standard. With a lower loading dose, of 0.10 vol.%, the reduction achieved was 48.2% and biodiesel had 6.4 ppm of phosphorus (91% of overall reduction), higher than that allowed by EN 14214.

Using a dose of 0.18 vol.% with a less concentrated acid solution (75%), a 53% reduction of phosphorus at the degumming step and 95% overall reduction after biodiesel synthesis (4.4 ppm) were obtained, very close to the standard limit. For an acid dose of 0.80 vol.% with a concentration of 25%, a 63% phosphorus reduction was achieved, the highest for all experiments. Following, biodiesel obtained gave an overall similar phosphorus reduction, of 95%. In these two cases, the mass of acid used was the same. Fig. 3.19 depicts all the results obtained for phosphorus reduction in the degummed oil and biodiesel after using methodology B.

The major mean overall reduction of phosphorus, for this methodology, was obtained by acid degumming at a dose of 0.18 vol.% (around 96%, using a solution with 85% of concentration), giving 3.5 ppm of phosphorus in the biodiesel. The same was obtained with a dose of 0.80 vol.% (solution with 25% of concentration), resulting in a concentration of 3.8 ppm in the biodiesel (95% of phosphorus reduction).

The value obtained was lower than the upper limit of EN 14214 (4 ppm) and therefore such operational conditions were considered effective for phosphorus removal. All the results showed similar final phosphorus content for biodiesel produced, related to biodiesel's washing step, which contributes to the removal of phosphorus, with different efficiencies of NHP for the initial degumming step.

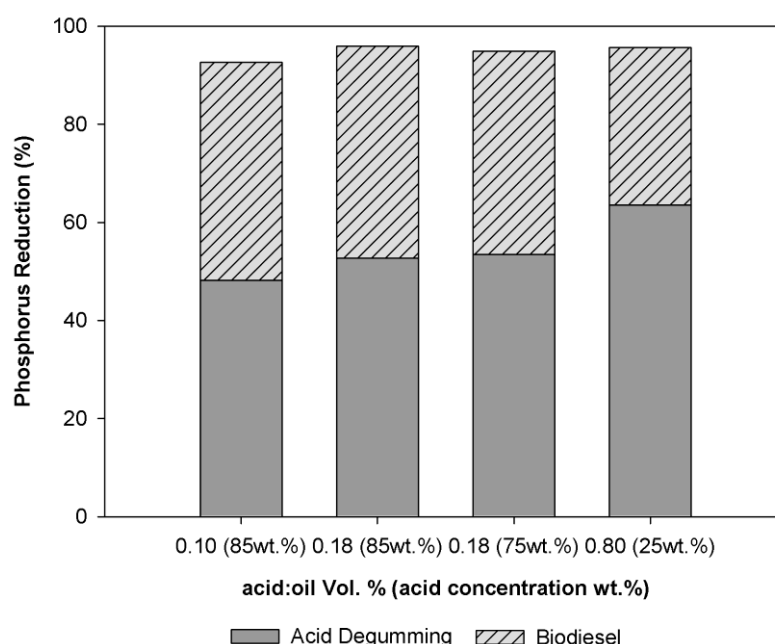


Figure 3.19. Phosphorus reduction (%) by using methodology B at different volume percentages of acid to oil (vol.%) and acid concentrations (wt.%).

However, it seems to be clear that this methodology allows a more effective conversion of NHP into HP. As handling a less concentrated acid solution (25 wt.%) is preferable than a more concentrated one in terms of operational security, the use of such solution at a dose of 0.80 vol.% was established as the best conditions for acid degumming of raw oil between the operational conditions studied. The best conditions were replicated using 60 g of oil and the results are presented in Table 3.12, showing consistency with the results previously obtained.

Table 3.12. Results of phosphorus reduction after degumming according to methodology B using a dose of 0.80% vol.% (acid/oil) using a phosphoric acid concentration of 25 wt.%, followed by biodiesel synthesis (60 g of raw oil used)

Reduction of Phosphorus concentration (%) after degumming	59 ± 12
Reduction of Phosphorus concentration (%) after biodiesel synthesis	97 ± 7

For industrial application, it is clear the need for the acid degumming to obtain a product conforming biodiesel quality standard regarding phosphorus concentration, since biodiesel production using water washing purification of the product allows a significant reduction of the HP but only using acid degumming the remaining NHP might be removed. The results show however that for an effective removal of NHP, the acid concentration and dose as well as the mixing step are critical.

Using this methodology, it was therefore possible to establish a degumming procedure and operational conditions that allowed to reduce phosphorus content until acceptable levels (< 4 ppm) using *Crambe abyssinica* oil.

3.2.5 CONCLUSIONS

Using raw *Crambe abyssinica* oil it is not possible to obtain biodiesel with a good quality regarding phosphorus concentration (< 4 ppm). Acid degumming using phosphoric acid was found to be a feasible process towards phosphorus removal to levels admitted for a biodiesel product with quality, whereas water degumming was unfeasible, due to the presence of non-hydratable phospholipids. Similar reductions in the chemical degumming processes employed led to different final phosphorus content after biodiesel production using the refined oils, showing different efficiencies concerning the removal of non-hydratable phospholipids.

It was evident that the effectiveness of phosphorous removal from the crude oil was linked to the methodology employed and that the concentration, dose of the phosphoric acid solution and the type of mixing were the critical parameters. Overall, the biodiesel obtained from degummed oils using the best range of conditions established had a phosphorus concentration from 3.5 to 6.4 ppm, meaning an overall reduction from 91 to 95% of phosphorus concentration in raw oil.

Considering technical and operational variables, the best conditions selected were the use of 0.80 vol.% of a 25 wt.% phosphoric acid solution and vigorous stirring (avoiding vortex), which allowed to obtain a final biodiesel with 3.8 ppm of phosphorus.

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3.3 THE CYCLE OF BIODIESEL PRODUCTION FROM *CRAMBE ABYSSINICA* IN PORTUGAL

ABSTRACT

Biodiesel is usually produced from edible oils which compete with the food market. *Crambe abyssinica* is a non-food crop referred as having high potential for biodiesel production. An entire cycle of seeding, harvesting, oil extraction and biodiesel production was performed with crambe; probably, the first study carried out with this specie in Portugal aiming to evaluate its full viability. Biodiesel was produced from crude and refined oil of crambe seeds cultivated (oil content of 26 ± 4 wt.%) and the results were compared to those obtained using a commercial oil originated from Brazil, where most studies were found. Biodiesel produced from the oil extracted from the Portuguese seeds showed good quality fulfilling almost all the requirements settled by EN 14214. The viscosity ($5.80 \text{ mm}^2.\text{s}^{-1}$) and the cold filter plugging point (7°C), which are mostly associated with the oil's nature, were close to the limits, indicating the need for blending at an industrial scale. Anyway, the biodiesel produced with the refined oil showed the highest quality, due to the lower level of contaminants. The high oxidation stability (16 h) of the biodiesel produced from seeds cultivated in Portugal can be argued as an advantage for this product. The results show that crambe oil might be a promising complementary alternative to the conventional edible oils used for biodiesel production.

This chapter was based on:

Emanuel C., Manuel F. Almeida, Maria C.M. Alvim-Ferraz, Joana M. Dias, “*The cycle of biodiesel production from Crambe abyssinica in Portugal*”, Industrial Crops and Products journal, volume 129, 51-58, 2019.

3.3.1 SCIENTIFIC RELEVANCE

In Europe (the biggest biodiesel producer in the world, followed by the United States and Brazil), biodiesel is the most produced biofuel, representing eighty percent of the total consumption of transport biofuel [1]. In this region, biofuels are considered essential to promote changes in the energy sector, namely for the transport and agriculture sectors and also for heating purposes. According to the Directive 2009/28/EC, all the member states, including Portugal, should incorporate 10% (energy basis) of biofuels in the transport sector until 2020.

For 2018, the forecast for the percentage of biofuel penetration in the Portuguese transport sector is around 7.5% (energy basis), lower than the 9% originally intended for this year. In 2016, the most used biodiesel was produced mainly from waste frying oil (38.9%), rapeseed oil (37.7%) and soybean oil (15.6%) [3].

Taking into account the concerns over quality and the scarcity of waste materials and the use of edible oil for energy purposes, it becomes relevant to have non-edible oilseed crops for biodiesel production, too; if possible, they should present high oil seed content, high biomass yields and be easily adapted to the country's edaphoclimatic conditions.

Crambe non-edible oilseed crop is described in literature as having high potential for biodiesel production. By comparison between several oilseed crops (canola, sunflower and soybean), the production costs associated to crambe oil were the lowest [4]. Results of around R\$ 2 per liter (0.46 €/L) or less are referred in the literature [4, 5]. No exact prices for commercial crambe oil could be found, but informal information might be encountered concerning values ranging from slightly higher than the production cost up to margins of around 30%. These advantages made the study of the crambe crop very attractive; accordingly, it is the second most researched oilseed crop referenced in scientific journals up to 2010 and the second most studied and cited in 2011 [6, 7]. Some studies show that it is possible to cultivate crambe in marginal lands or contaminated soils due to its tolerance to heavy metals and salinity. Although more studies should be developed to maximize the yields of this crop [8-10]. These characteristics can avoid the occupation of arable land essential for food production, making it a real and relevant alternative to food crops. Because the oils produced in Portugal (mostly sunflower oil) are used mainly for human consumption, biodiesel is mostly produced from imported raw materials. Such fact makes Portugal very dependent from international markets and shows how important is to find new and sustainable alternative oilseed crops for biodiesel production. For non-edible crops, few developments have been made; *Jatropha*

curcas and *Cynara cardunculus* are the only crops reported in Portuguese studies for bioenergy purposes although without further developments [11, 12]. For *Jatropha curcas*, studies on cultivation of this crop were conducted; however, without further developments [11]. The cold weather typical of Portuguese winter is one of the main reasons for the lack success of the crop. *Cynara cardunculus* crop was installed in large scale for the production of aboveground biomass for energy production. The results showed the potential of the plant for biomass production since the crop survived and developed well under Portuguese climatic conditions of Portugal, including in years with extreme drought [12]; however, no information was found concerning further developments. Nowadays, in Portugal, it is already possible to find some developments/investments for the production of rapeseed [13].

It should be mentioned that some edible crops used for biodiesel production are also important to produce animal feed due to the high protein content of the fraction remaining after oil extraction.

3.3.2 SPECIFIC OBJECTIVES

The main objective of the work was to produce biodiesel from *Crambe abyssinica* oil. Accordingly, the study evaluated:

- the viability of *Crambe abyssinica* oil as an alternative raw material to conventional edible oils usually used in Europe;
- all the processes steps, from seeding and harvesting to oil extraction and its use (raw and refined) for biodiesel production through alkaline transesterification;
- quality of biodiesel obtained using a commercial crambe oil (from Brazil) with the raw and refined oils, considering the applicable European biodiesel quality standards.

3.3.3 MATERIALS AND METHODS

3.3.3.1 *Materials*

The Portuguese seeds of *Crambe abyssinica* came from an experimental field study at Northern Portugal (40°59'07.2" N; 8°32'43.6" W) with agronomic control, conducted between April and July of 2016. The seeds sown belong to the “FMS Brilhante” variety and were donated by “Fundação MS”, Brazil. Crambe was sown in rows spaced 0.35 m with a density of 100 plants/m² (Figure 3.20).



Figure 3.20. Crambe growth after 15 days (left side) and 55 days (right side) of sowing.

Table 3.13 presents the chemical composition of the soil where Crambe was sown. This analysis led to the conclusion that the soil had high levels of fertility taking into account the most important macronutrients; in agreement, an organic fertilizer (Fertigranu®) was added to the soil only for minor adjustments (e.g. organic matter content). Sprinkler irrigation was performed twenty-five days after the sowing, twice times per week (100 L) until the beginning of flowering stage. The weather conditions were assessed using a local weather station (Froggit® WH1080). An average temperature of 18.5 °C and a total amount of precipitation of approximately 250 mm was recorded during that period.

Table 3.13. Chemical composition of the soil used for crambe cultivation

pH	P (mg/kg)	Organic Matter (wt.%)	Ca (mg/kg)	K (mg/kg)	Mg (mg/kg)
7.1	>200	2.91	1737	> 200	52

The seeds were harvested when the plants aged 120 days and they were stored in a shelter, at room temperature, until oil extraction. After that, the seeds (around 3 mm) were sieved (< 4.75 mm) with objective to remove impurities such as leaves, earth, small stones and other particles which could affect the following steps.

The commercial oil of crambe used in this work was also from Brazilian seeds of cultivar “FMS-Brilhante” and it was donated by a Brazilian local producer (State of Mato Grosso do Sul).

For the chemical extraction of the oil from the seeds cultivated in Portugal (by Soxhlet), petroleum ether (LabChem) was used. Methanol 99.9% (analytical grade, Fischer Chemical) and NaOH 97% powder (analytical grade, Aldrich) were used in the transesterification reaction (biodiesel

production). Phosphoric acid applied during the chemical degumming procedure was prepared from concentrated acid (85%) supplied by Merck. All the reagents required for analytical procedures were of analytical grade.

3.3.3.2 *Oil extraction*

The oil content of the seeds was determined using a 100 mL Soxhlet extractor, following NP EN ISO 659 (2002). Taking into account the amount of oil required for the experimental studies, it was extracted using a mechanical press (Komet Vegetable Oil Expeller CA 59 1-H), whereby the seeds were loaded and pressed under heating using a nozzle with 5 mm of diameter. At the final of this procedure, it was possible to determine the extraction yield. Following extraction, the oil was subjected to centrifugation with the objective to remove impurities (e.g. residual solids). Figure 3.21 shows the Soxhlet extractor and oil presser (mechanical system) used.

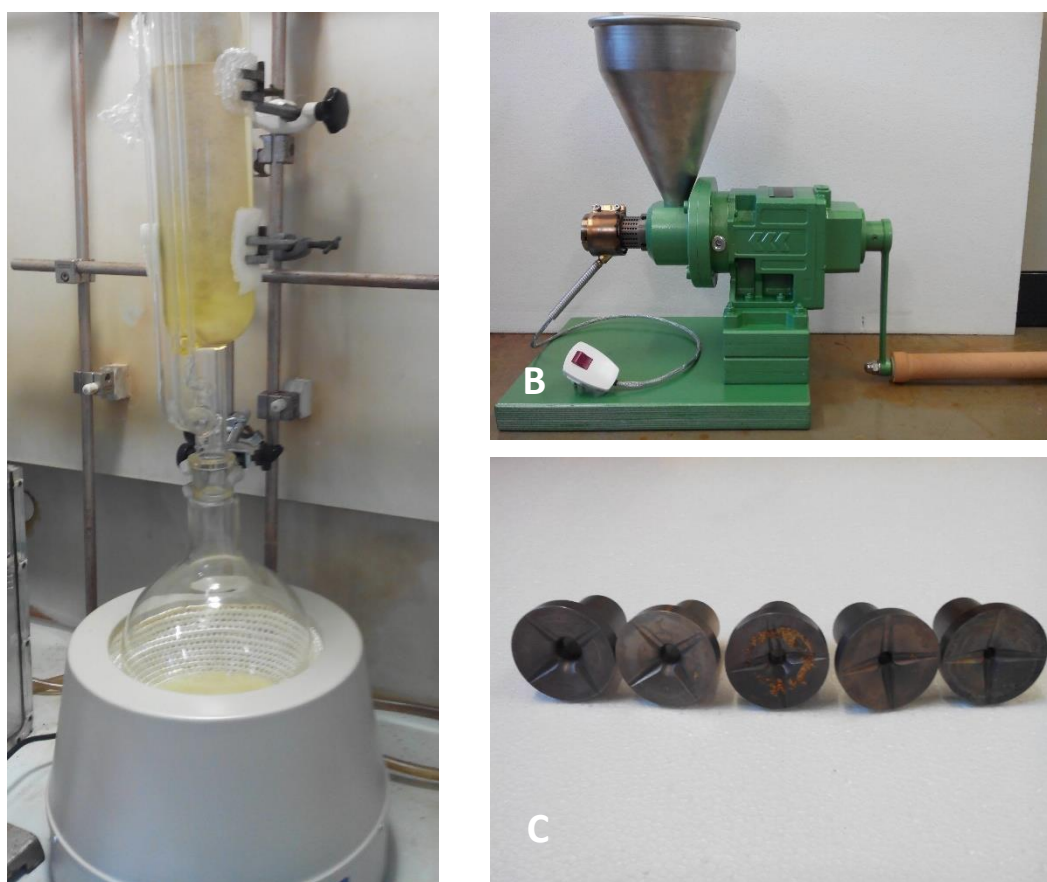


Figure 3.21. Equipment used for oil's extraction: A – soxhlet extractor; B – mechanical extractor; C – nozzles of oil presser.

For mechanical extraction, to maximize the yields of the oil's extraction, the diameter of the nozzle (Figure 3.21 – C) should be chosen according to the seed's size.

3.3.3.3 *Analytical methods*

Considering the expected influence of oil quality towards the biodiesel production process and the product quality (further discussed in the results section), both the raw material and the biodiesel were characterized on several parameters.

With respect to the raw oil, the following parameters were evaluated: (i) oxidation stability, by accelerated oxidation using a 873 Biodiesel Rancimat equipment from Metrohm; (ii) density, by the pycnometer method, according NP 938:1988 (iii) viscosity at 40 °C, measured according to ISO 3104 using capillary viscometers immersed in a thermostatic bath; (iv) acid value, by volumetric titration according to NP EN ISO 660; and, (v) phosphorus content, by calcination followed by phosphorous determination through UV spectroscopy, according to NP 1994:2000 standard.

For the produced biodiesel, apart from the previous parameters, measured according to the applicable standards (oxidation stability – EN 14122; density – ISO 3675; viscosity – ISO 3104; acid value – NP EN ISO 660 and phosphorus content – NP 1994:2000), the following parameters were also evaluated: (vi) iodine value, from the methyl esters composition obtained by GC according to annex B of EN 14214; (vii) cold filter plugging point using an appropriate equipment (Stanhope Seta® 99000-3) according to EN 116; (viii) insoluble impurities content, according ISO 663; (ix) water content, following NP EN 12937 (2003) using a Karl Fischer coulometric titrator; (x) flash point, according to ISO 3679 using a closed cup tester (Stanhope Seta® 30000-U.); (xi) linolenic acid methyl ester content from the methyl esters composition obtained by GC; and, (xii) fatty acid methyl esters content, by gas chromatography according to EN 14103. GC analyses were conducted using a Dani Master GC with a DN-WAX capillary column of 30 m, 0.25 mm internal diameter and 0.25 µm of film thickness. The temperature program was as follows: 120 °C as the starting temperature, rising at 4 °C per minute up to 220 °C, held for 10 min.

It should be highlighted that when the degumming procedure was evaluated, the study focused on its influence on the biodiesel quality, in agreement with a previous study [14]; accordingly, in such case the characterization was made only on the biodiesel and no additional characterization was performed on the degummed oil.

3.3.3.4 Oil refining

The direct use of non-edible oil for biodiesel production is discouraged due to the presence of free fatty acids and phospholipids that create problems during biodiesel synthesis and purification [15]. In addition, the probable high content of phosphorous might lead to a final product that does not agree with the quality specifications required by standard EN 14214 (phosphorus content < 4 ppm), namely because it affects the exhaust systems (catalytic conversion) in diesel engines, increasing the resultant emissions [14]. Costa *et al.* [14] showed the need to apply chemical degumming (Figure 3.22) of raw oil in order to obtain biodiesel complying with the biodiesel standard in terms of phosphorous concentration [14]. In agreement, a pre-treatment of chemical degumming was performed on the extracted oil from the *Crambe abyssinica* seeds harvested in Portugal, before the transesterification reaction. The oil was heated at 75 °C and a 25 wt.% of phosphoric acid solution was added to the oil (around 200 g) with an acid to oil volume of 0.80 % (step I). Following, 0.6 wt.% of water (relative to mass of oil) was added and the mixture was shaken intensely during 1 min in a vibrator stirrer (step II). Then, the mixture was stirred under magnetic stirring for 5 min and water was added (2 vol.%, relative to oil); this mixture was further stirred for 20 min (magnetic stirring). Finally, the product was centrifuged during 10 min. at 3500 rpm (step III) and the oil was separated from the remaining non-oil fraction (gums) [14].

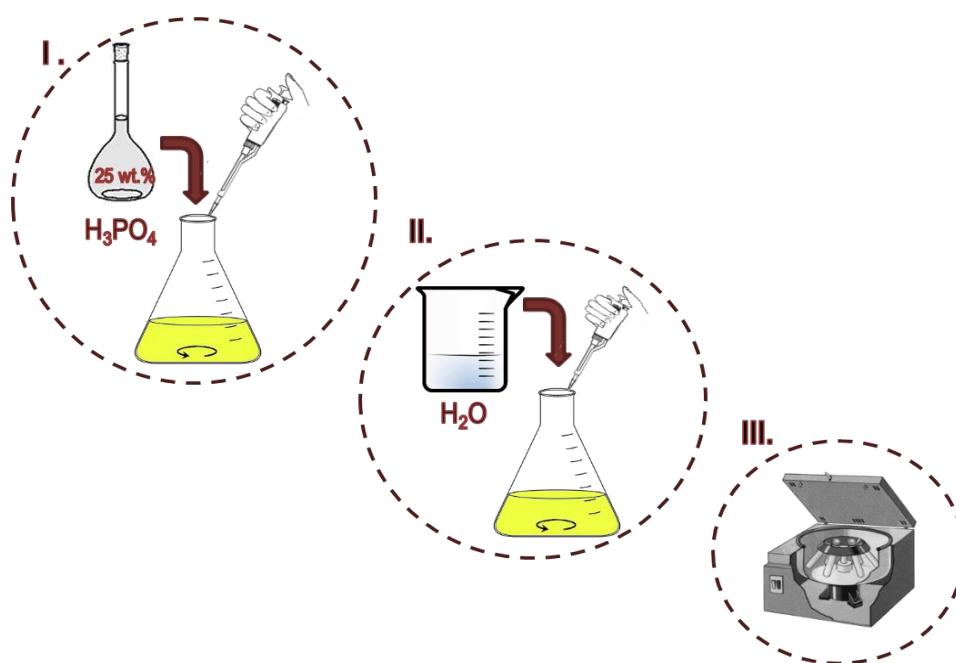


Figure 3.22. Schematic procedure of chemical degumming of crambe oil.

3.3.3.5 Biodiesel production

Biodiesel was produced following the well-known two major steps, namely: (i) transesterification reaction; and, (ii) purification step. The same procedure was applied to both the raw oil and the refined oil from seeds cultivated in Portugal as well as to the commercial oil from Brazil.

For the transesterification, the oil (170 g) was added to a batch reactor consisting of a round-bottom flask equipped with a water-cooled condenser (to allow methanol reflux) and magnetic stirring, immersed in thermostatic bath (Figure 3.23) [16].

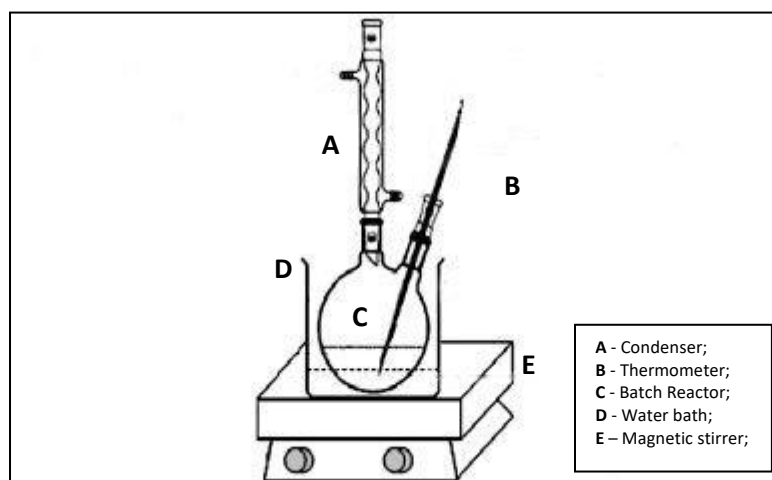


Figure 3.23. Equipment used for biodiesel production: round-bottom flask (batch reactor); condenser; magnetic stirring; thermometer and water bath temperature control.

The methanol:oil molar ratio was 6:1, the catalyst concentration was 1 wt.% of NaOH (relative to mass of oil) and the reaction time was 1 h, in agreement with previous studies [16]. After reaction, the products were left to settle under gravity for 1 h in a separator funnel, originating two phases: biodiesel (upper layer) and glycerol (lower layer). After separation, the methanol was recovered for each phase in a rotary evaporator ($T = 65\text{ }^{\circ}\text{C}$; 200 mbar, 30 min). The biodiesel phase was washed with 50 vol.% of hydrochloric acid (0.5 wt.%) and after, repeatedly, with 50 vol.% of distilled water up to the pH of the washing water was very close to pH of the distilled water. The last step of purification consisted on removal of residual moisture by drying the biodiesel in a rotary evaporator ($T = 85\text{ }^{\circ}\text{C}$; 200 mbar, 1 h).

All the steps and methods used from the extraction of crambe oil up to the final biodiesel are summarized in Figure 3.24.

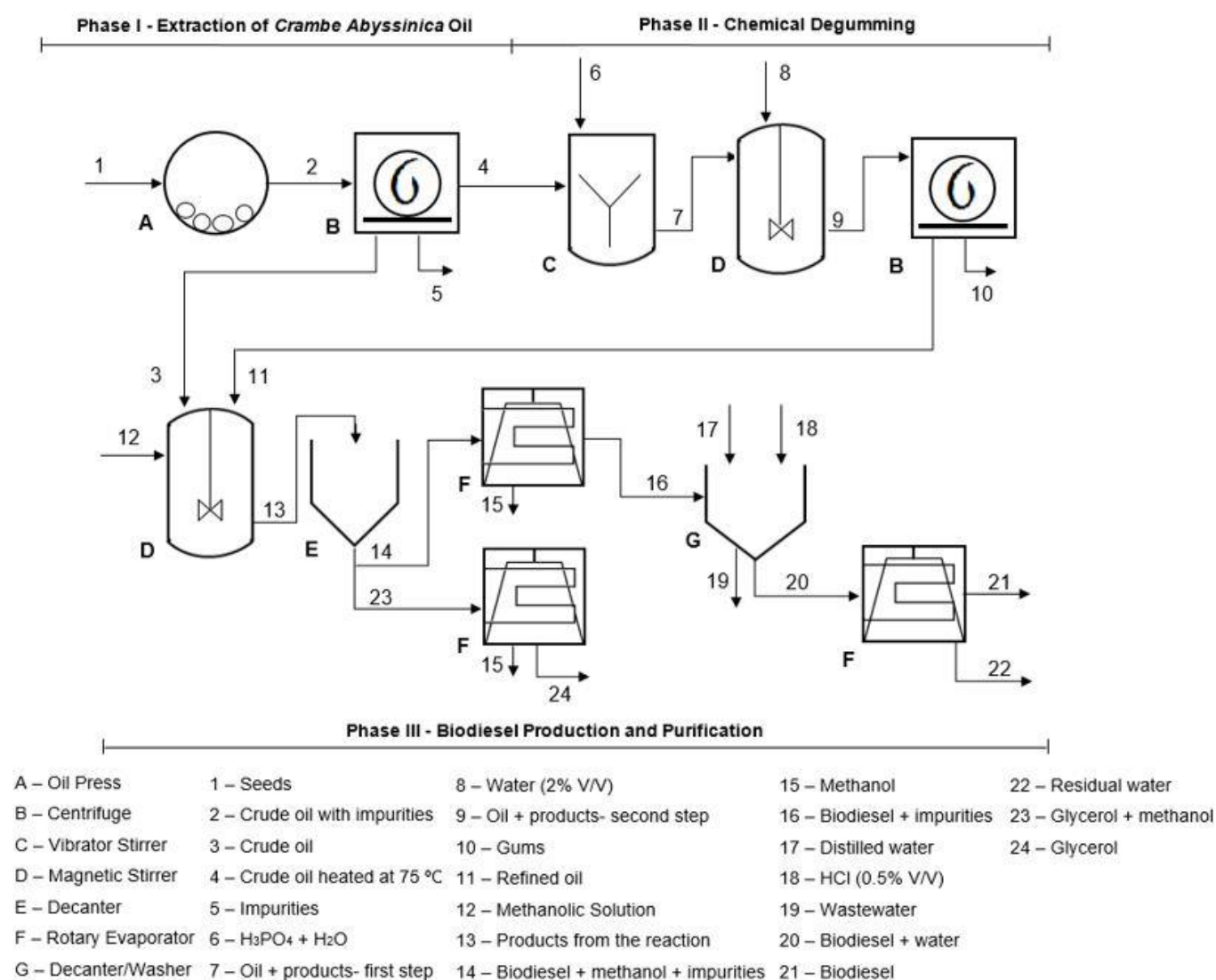


Figure 3.24. Flow sheet for crambe oil extraction, chemical degumming as well as biodiesel production and purification.

3.3.4. RESULTS AND DISCUSSION

3.3.4.1 Oil extraction and Yields

The crambe seeds harvested in Portugal presented an oil content of 26 ± 4 wt.% (chemical extraction). The same variety of seeds grown in Brazil showed a maximum oil yield around 38% [10]. The lower oil content obtained may be attributed to the lower water availability during the first two months of the vegetative cycle (probably irrigation done was insufficient), perhaps generating hydric stress in the plants and also to the temperature range verified during crop growth (between 16°C and 22°C). The effect of temperature can be evaluated using the parameter Cumulative Growing Degree Days (GDD) that allows to understand the degree of crop's development along the time. For

the last stages of crambe's vegetative cycle, the GDD verified (483°Cd, where °Cd refers to cumulative degrees) was lower than the value found in other studies with seed's oil content near to 31% (782°Cd). Lower GDD indicated that the seeds harvested in Portugal were subjected to less temperature which then affected the rate of growth, seed's oil content and harvest time [17]. Taking into account the seeds yields and oil content as well as the cultivated area, the oil yields corresponded to 194 kg.ha⁻¹. Future studies will take into account a stricter control of water irrigation.

The soil composition and the harvesting time (taking into account the complete maturation of the plant) could also have influenced the oil content of the seeds [18]. Phosphorus nutrient stimulates flowering, seed formation and physiological maturation, being accumulated in the youngest tissues of the plant, e.g. leafs and seeds [19]. Table 3.13 shows a soil with a relatively high phosphorus concentration (> 200 mg/kg); accordingly, organic fertilization was made to ensure appropriate levels of organic matter but with low content of phosphorus not to influence even more the seed's oil content in comparison to other studies [10].

The mechanical extraction of the oil was carried out in two steps, the second with the biomass resulting from the first, leading to an average yield of 24.5 wt.%. Taking into account the result obtained with chemical extraction (only 2% above those obtained with mechanical extraction), although lower oil yields were obtained in the seeds, independently of the method used, in comparison to the literature [20, 21], mechanical extraction might be an appealing process for recovering the oil from the crambe seeds, as suggested by Bondioli *et al.* [22].

For *Crambe abyssinica* seeds, the diameter of the nozzle which allow the highest extraction of oils was the nozzle with 6 mm. In Figure 3.25 are present the different yields obtained among the different sizes.

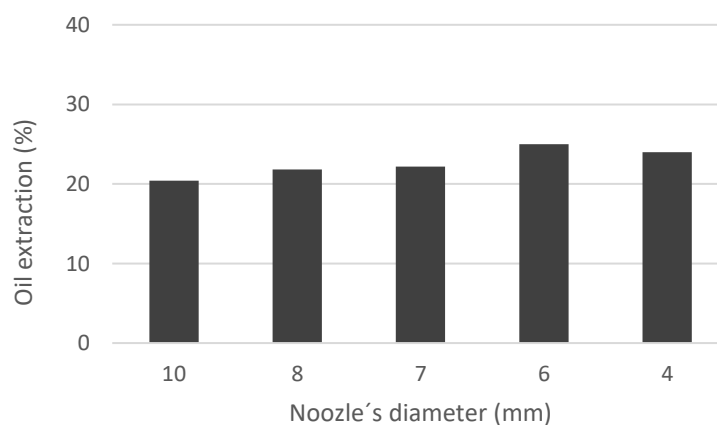


Figure 3.25. Yields of oil extracted with different sizes of nozzles.

The amount of oil (25 wt.%) obtained by mechanical pressing with a nozzle of 6 mm is very similar to the obtained by chemical extraction (26 ± 4 wt.%). For this reason, mechanical extraction can be consider a good solution for seed oil extraction, shortening the duration of the extraction.

3.3.4.2 Fatty acid profile

The fatty acid profile of the oils is usually predicted from the resultant methyl esters profile of biodiesel (Table 3.14) and it is directly related with the oil composition.

Table 3.14. Fatty acid methyl esters profile for biodiesel obtained from *Crambe abyssinica* raw oil and commercial oils, wt.%

Methyl esters of the fatty acids	Palmitic (C16:0)	Oleic (C18:1)	Linoleic (C18:2)	Linolenic (C18:3)	Gadoleic (C20:1)	Behenic (C22:0)	Erucic (C22:1)	Others (<2 wt.% each)
PT raw oil ^{a)}	-	17.1	9	5.3	4.2	2.0	54.7	7.8
BR commercial oil ^{b)}	-	18.8	7.7	4.4	6.3	1.7	54.2	7.0
Referred in [23]	2.0	17.0 – 19.0	9.0	6.0 – 7.0	4.0	1.0 – 2.0	56.0 – 59.0	3.0

^{a)} Biodiesel of raw oil, from seeds harvested in Portugal.

^{b)} Biodiesel of Commercial oil, obtained from Brazil.

Such characteristics agree with those reported in the literature [23] and make possible to determine the molecular weight of the crambe oil taking into account the weighted average of the triglycerides of the fatty acids present. The result was 989.7 g.mol^{-1} .

As expected, composition does not differ significantly in the two analyzed fatty acids methyl esters (FAME), since both were obtained from oil extracted from *Crambe abyssinica* seeds. Most of methyl esters are from monounsaturated acids and erucic acid (more than 54% in both cases).

The small amount of polyunsaturated fatty acids (< 15 wt.% for all biodiesel types) should be responsible for the high stability of the oils and the respective biodiesel (as seen in the following sections) because polyunsaturated esters have double bonds particularly susceptible to radical attack, leading to oxidation [23]. The FAME composition of the refined biodiesel obtained from seeds sown in Portugal was similar to that of the raw oil, with maximum differences of 13%.

Composition profile of biodiesel make possible defining the molecular weight of the crambe oil. This value is important for biodiesel reaction because allows more accurately calculating the amount of methanol needed for the transesterification reaction. The molecular weight is calculated from the

molecular weight of each fatty acids for Portuguese crambe's oil (Table 3.15) [24]. The procedure was applied also for other two samples of oil (degummed oil and Brazil crambe oil).

The equation 3.3 presents the procedure for calculate the molecular weight of triglyceride:

$$M(TG) = \sum_{i=1}^n [3 \times M(FFA_i)] + M(C_3H_5) - 3 \times M(H) \times P_{ME}$$

$$\Leftrightarrow M(TG) = \sum_{i=1}^n ([3 \times M(FFA_i)] + 41,03 - 3,0) \times P_{ME} \quad (\text{eq.3.3})$$

where M (TG), M (FFA) and M (H) correspond to the molecular weight of triglyceride, free fatty acid and hydrogen. P_{ME} corresponds to the percentage of methyl ester, by weight.

The triglyceride (TG) is constituted by three molecules of monoglycerides that when linked to the glycerol (C₃H₈O₃) promote the liberation of three groups OH (remain C₃H₅). For instance, the monoglyceride (MG), generally represented by COOH – R, loses one hydrogen (three in the total for each TG produce), leading to the formation of water (H₂O).

Table 3.15. Fatty acid composition of Portuguese crambe oil and molecular weight of Free fatty acids and triglycerides

Fatty acid	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	C20:2	C22:0	C22:1	C22:2	C24:0	C24:1
<i>M(FFA)</i> [23]	254.4	284.5	282.5	280.5	278.4	312.5	310.5	304.5	340.6	338.6	336.6	368.6	366.6
%wt.	2.1	1.0	17.1	9.0	5.3	1.0	4.2	0.8	2.0	54.7	0.7	0.8	1.4
<i>M(TG)</i>	801.3	891.5	885.5	879.4	873.3	975.6	969.6	951.4	1059.9	1053.7	1047.7	1143.9	1137.9

According to equation 3.3, the molecular weight of the triglycerides (crambe oil) was 989.7 g.mol⁻¹. For degummed oil and Brazil crambe oil the molecular weight were 993.14 and 998.69 g.mol⁻¹, respectively. The results were very similar because the fatty acid composition for the three biodiesels did not vary considerably as showed in Table 3.14. When compared with the values present in the bibliography for other raw-materials like rapeseed or waste cooking oil it is verified that crambe oil has a molecular weight 10% higher [25]. In literature it is possible to find a study which refers that the molecular weight of crambe is around 983.0 g.mol⁻¹ [26].

Analyzing the composition profile, it is notorious the presence of erucic acid (C22:1) in great amounts. This acid can cause adverse effects to human health when ingested [23, 27]. For public safety, only 5 wt.% of erucic acid can be present in fatty acid oils [27].

When compared the molecular weight of crambe oil with other non-edible oils, like *Ricinus communis* oil, the differences are small, because *Ricinus communis* oil show high content of C20:1 (high molecular weight) [28].

3.3.4.3 Physicochemical properties

The properties of the raw oil from seeds collected in Portugal and the commercial oil from Brazil are presented in Table 3.16.

The results agree in general with the information found in the literature for this kind of oil [14, 29, 30]. A slight difference was found for the viscosity of the oil obtained from the Portuguese seeds, compared to that found in the literature ($49 \text{ mm}^2 \cdot \text{s}^{-1}$) [30].

Table 3.16. Main physicochemical properties of *Crambe abyssinica* oils

Origin	Density ($\text{g} \cdot \text{cm}^{-3}$)	Viscosity@40°C ($\text{mm}^2 \cdot \text{s}^{-1}$)	Oxidation stability (h)	Acid value ($\text{mg KOH} \cdot \text{g}^{-1}$)	Phosphorus content (ppm)
Portugal ^{a)}	0.906	45 ± 3	27.4 ± 0.0	0.61 ± 0.01	72 ± 4
Brazil ^{b)}	0.912	53.5 ± 0.5	18 ± 2	7.2 ± 0.2	4.6 ± 0.4

^{a)} Biodiesel of raw oil, from seeds harvested in Portugal.

^{b)} Biodiesel of Commercial oil, obtained from Brazil.

The presence of phosphorus in the raw oil agrees with the normal seed content that can range 86 to 102 ppm, being associated with its formation and maturation [14]. The raw oil from Portuguese seeds presented a high phosphorus content particularly when compared to the oil from Brazil, only with residual phosphorus. The higher amount of phosphorus in the raw oil is expected to be due to the presence of phospholipids; despite that value, phosphorus is very low when compared with values found in other raw oils like rapeseed oil (864 ppm), sunflower oil (294 ppm), coconut oil (600 ppm) or *Jatropha* oil (1200 ppm) [14, 31]. The phosphorus content is directly related with the composition of the soil where crambe was sowed, the amount of fertilizer used and, of course, whether or not a refining step (removal of hydratable and non-hydratable phospholipids) was applied. The significant difference found between phosphorus on the oils from seeds cultivated in Portugal and Brazil is attributed mainly to a refining step [14].

The high oil viscosity is typical of the triglycerides and it is caused by intermolecular forces associated with saturated and unsaturated fatty acids [23, 32]. The viscosity of the crambe oil obtained in Portugal was close to the range of the values found in other studies (around $49 \text{ mm}^2 \cdot \text{s}^{-1}$) [30].

The high oxidation stability found for both raw-materials is related with the fatty acid composition of the oil which comprises mostly the monounsaturated acid – erucic acid – and a small content of polyunsaturated fatty acids which are less stable to oxidation, as previously referred. The presence of natural antioxidants such as tocopherols and carotenes also lead to a higher oxidation stability [33]; with this respect, the lower stability found in the Brazilian oil might be attributed to the removal of the natural antioxidants during a probable refining step of the raw oil, as previously discussed.

The high oxidation stability of the Portuguese raw oil shows it as an interesting raw-material for biodiesel production, as a source of natural antioxidants that can replace chemical additives usually used in the commercial biodiesel [34]. Although the commercial Brazilian crambe oil presented less oxidation stability, both oils have higher oxidation stability than that reported in literature for this type of oil (around 9 h) [33]; factors such as soil composition, climate, irrigation, degree of seed's maturation and its interaction with the plant might be responsible for such results [35].

The acid value reflects the free fatty acid (FFA) content of the oil and it is a critical property aiming at biodiesel production, particularly to enable homogeneous alkaline transesterification. The maximum FFA value advisable in the oil is 2 mgKOH.g⁻¹ (around 1 wt.%) [36]. Higher values of FFA lead to: (i) difficult separation of the biodiesel and glycerol phases; (ii) formation of soaps by reaction with the catalyst; and (iv) decrease of the product yield [37]. Only the raw crambe oil from seeds collected in Portugal had an acid value lower than 2 mg KOH.g⁻¹. As known, the low content of FFA is related with the nature of the oils as well as with the processing and preservation procedures, being possible to consider such raw oil as suitable for biodiesel production by direct homogeneous alkaline transesterification [38]. The higher FFA content on Brazilian oil, above 2 mg KOH.g⁻¹, was possibly due to some natural process of degradation which might have occurred during the storage at the origin.

3.3.4.2 Biodiesel production and quality

For homogenous alkali transesterification reaction, the acidity of the raw-material should be lower than 2 wt.% [39]. Despite the fact that the commercial oil from Brazil had an acidity higher than 2 wt.%, the alkali transesterification reaction was also evaluated to produce FAME from this oil (Figure 3.26). The idea was to evaluate the quality of the product obtained for comparison to that of the other oils, although a less yield would be expected.

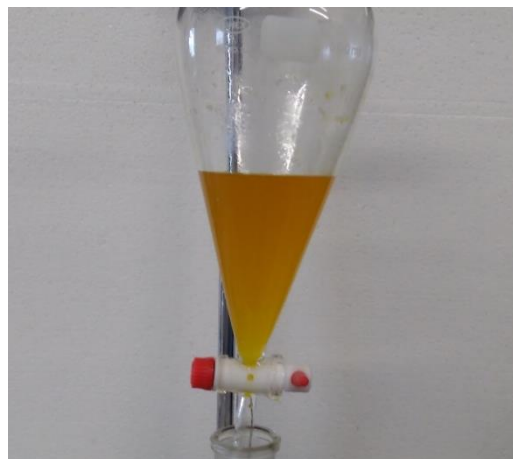


Figure 3.26. Biodiesel produced from crambe oil produced in Portugal.

Table 3.17 gives the characteristics of the biodiesel obtained. The values in bold correspond to those not in agreement with EN 14214 (the European quality standard for biodiesel). The values underlined assign properties very close to those required by the standard. All the remaining values and properties fulfil the requirements of the European standard.

After the transesterification reaction, the density of biodiesel is lower than 0.88 g.cm^{-3} , representing a slight decrease compared to the density of the respective oils (Table 3.17), a result due to the differences in densities between triglycerides and the respective FAME. The densities were very similar to those found in other studies: $0.874 - 0.900 \text{ g.cm}^{-3}$ for biodiesel of crambe oil [30, 33] and agrees with the biodiesel quality standard requirements (EN 14214).

Although the viscosities found for the biodiesel were according to the values usually verified for this raw-material ($5.92 - 7.69 \text{ mm}^2.\text{s}^{-1}$) [18, 30, 40, 41], they do not fit the range required by the standard.

The viscosity was slightly higher than $5.0 \text{ mm}^2.\text{s}^{-1}$ for all three products, particularly in the case of Brazilian one. The viscosity parameter is linked to the fatty acid methyl ester composition and the rate of conversion of the oil to esters; this is highly relevant for fuel admission to the engine and also for the behavior of the fuel in cold weather [16].

The presence of saturated acids contributes to higher viscosities of the respective biodiesel which may lead to clogging in injectors and deposition of carbon in the combustion chamber [42]. Viscosity also affects the quality of atomization inside the engine and might cause incomplete combustion [43].

Table 3.17. Properties of *Crambe abyssinica* biodiesel obtained using raw oil and degummed oil from seeds harvested in Portugal and commercial oil from Brazil

Property	Raw Oil, Portugal ^{a)}	Degummed oil , Portugal ^{b)}	Commercial oil, Brazil ^{c)}	EN 14214
Density (g.cm ⁻³)	0.874	0.878	0.881	0.86 – 0.90
Viscosity (mm ² .s ⁻¹)	5.80 ± 0.04	5.96 ± 0.05	7.56 ± 0.04	3.5 – 5.0
Oxidation Stability (h)	16 ± 1	9 ± 1	0	> 8
Acid value (mgKOH.g ⁻¹)	0.48 ± 0.01	0.41 ± 0.03	0.68 ± 0.03	< 0.50
Phosphorus Content (ppm)	2.9 ± 0.2	2.65 ± 0.02	1.1 ± 0.2	< 4
Moisture (ppm)	507 ± 17	228 ± 49	365 ± 62	< 500
FAME content (%)	<u>96 ± 1</u>	<u>96 ± 1</u>	88 ± 1	> 96.5
Linolenic Ester content (%)	5 ± 1	5 ± 1	4 ± 1	< 12.0
Iodine Value (gl ₂ .100g ⁻¹)	89	87	87	< 120
CFPP (°C)	7	9	4	-
Flash Point (°C)	179	175	171	> 101
Total Contamination (ppm)	42 ± 4	24 ± 6	35 ± 5	< 24

Note: 170 g of oil used per experiment.

a) Raw oil from seeds harvested in Portugal.

b) Degummed oil obtained from seeds harvested in Portugal.

c) Commercial oil obtained from Brazil.

Despite the high purity values obtained, the agreement with the values in literature makes evident that this parameter cannot be fulfilled by using this oil alone, due to its chemical composition [23], since crambe oil is constituted mostly by erucic acid (> 50%), which has a long chain mono-unsaturated fatty acid; and, overall, mono-unsaturated fatty acids prevail (> 75%) (Table 3.12). The decrease of biodiesel viscosity until acceptable levels can be done by blending with other type of biodiesel or aking a biodiesel from a less viscous oils blend [14, 44]. This is a common practice in the industry, for example, when palm oil is used as raw-material for biodiesel production to compensate the high content of unsaturated fatty acids of other oils (e.g. soybean oil) [23].

The oxidation stability of biodiesel must be high (> 8 h) because low values result in fast deterioration of the product, particularly by contact with the oxygen in the air. The susceptibility to

oxidation is therefore very important regarding the transport and storage of biodiesel. As previously indicated, unsaturated FAME are more prone to oxidation [44]; however, this parameter does not depend only on the fatty acid composition but also on the presence of natural antioxidants in the oil.

The differences between this property in the Portuguese biodiesel and that from the commercial oil of Brazil are evident, the second being highly unstable and immediately started to oxidize. In Brazil, this oil is mostly used for biodiesel production; this instability was not expected since previous studies found on this subject showed that this type of oil is more stable than, for example, soybean oil (one of the most important food oils used worldwide for biodiesel production) [44, 45]; it might be related with storage conditions (section 3.3.4) as well as the conditions of the refining process. Biodiesel produced from the Portuguese raw and degummed oils presented high oxidation stabilities, fulfilling the EN 14214 requirements (> 8 h). Taking into account the low stability conventionally found for biodiesel obtained from other oils [46, 47], these results suggest that crambe biodiesel might be used in blends with other types of biodiesel with the objective of improving the stability, thus reducing the use of synthetics antioxidants [23, 48]. The decrease of oxidation stability verified after biodiesel production was mainly attributed to the biodiesel purification step which promotes the “washing out” of natural antioxidants [44]. In case of the biodiesel produced from the commercial crambe oil the decrease on oxidation stability was more pronounced. It was not possible to determine the natural antioxidants present at the oil; however, due to its relevance, such evaluation should be considered in the future. No studies were found with the characterization of oxidation stability using Standard EN 14122 (10 L/h at 110 °C) for crambe oil; however, the indications regarding the stability of the oil, previously presented, suggest high stability for biodiesel produced.

The acid value is closely associated with the chemical composition of the oil and the instability of the product; high acidities should be avoided because acids lead to corrosion and deposits at the engine [49]. Its value decreases from oil to respective biodiesel due to the purification steps, but might further increase due to the product instability. Thus, the higher acid value found for the commercial oil, which causes this parameter to be out of the standard (although by a small difference) may result from its hydrolysis. Such characteristic might be further improved with a stricter control of the moisture content and storage conditions. In the study developed by Jasper *et al.* [30] a biodiesel from crambe oil was obtained with 0.06 mg KOH.g⁻¹ of acid value.

Due to the phosphorus content on the raw oil, a degumming procedure was evaluated in order to reduce the phosphorus on the final biodiesel, in agreement with previous studies [14]. Phosphorous is usual found in the oil as hydratable phospholipids, which are easily removed by water, and as non-hydratable phospholipids, only removable by chemical degumming.

Phosphorus in the biodiesel produced from raw oil and that on degummed oil had small differences, contrary to what was observed in the study of Costa *et al.* [14]. In all cases the phosphorus content was below the threshold value from EN 14214 standard, which is 4 ppm. Therefore, the results obtained show the degumming pre-treatment not bringing a relevant benefit in the reduction of phosphorus; also, they lead to consider that most of the phosphorus present in the Portuguese crambe raw oil was in the hydratable form, thus being removed with water during the biodiesel purification step.

Water in biodiesel can promote several problems, namely: (i) on fuel-injection system, (ii) corrosion on the storage tanks, (iii) microorganisms proliferation; and (iv) decreasing of biodiesel oxidation stability [50].

The presence of water in biodiesel is mostly due to the wet purification step (more specifically the number of washing cycles and the ease of phase separation). The slight hygroscopic nature of fatty acids methyl esters might also lead to an increase of water content in the product. For this reason, and depending upon the humidity at the storage place, increase of water content might be observed during storage [50].

The water content of biodiesel should be lower than 500 ppm (standard EN14214). Among the three biodiesel products only the Portuguese biodiesel had a moisture higher than 500 ppm. Despite that, there is the conviction of being possible to solve easily this issue and decrease the moisture until acceptable levels by either adjusting the drying procedure or using a chemical desiccant [16].

The fatty acid methyl esters (FAME) content means the purity degree of the biodiesel obtained and it is extremely relevant to control the incorporation of illegal substances in the admixtures of diesel with biodiesel offered in the market. Products like glycerol, residual alcohol and unsaponifiable matter also decrease the quality of biodiesel, and, consequently, the content of FAME in the product. The FAME content must be higher than 96.5 wt.% in agreement with EN 14214 standard. As shown in Table 3.16, none of the three types of biodiesel achieved such high purity. Anyway, the values found were high (> 88%) and very close to the limit in case of the biodiesel produced from the Portuguese seeds. An operational aspect that must be emphasized is the easy separation achieved between biodiesel and glycerol phases; also, there was any evidence on formation of soaps

and gums during washing step when biodiesel is produced from degummed oil. The purity of the biodiesel produced was in the range of values reported in the literature (76 – 99%) [26]

The content of linolenic acid methyl ester (C18:3) indicates the degree of instability of the biodiesel since it is a polyunsaturated fatty acid very oxidizable when exposed to the light or oxygen. The results obtained suggest that crambe biodiesel had a slightly less linolenic acid (around 4.5 wt.%) when compared with the values referred in the literature (6 – 7 wt.%) [23]. This parameter agrees with the maximum admissible content (12%) according to EN 14214 standard, too.

The iodine value is another parameter closely related to the oil composition, particularly the functionalized hydrocarbons unsaturation. Biodiesel should have a limited degree of unsaturation to have quality as an alternative fuel [42]. In particular, double bonds can lead to polymerization of glycerides by heating (usual inside of the engine), causing the formation of gums [42]. The results in the Table 3.17 show a biodiesel with some level of unsaturation ($87 - 89 \text{ gI}_2.100\text{g}^{-1}$) but still below the maximum limit ($120 \text{ gI}_2.100\text{g}^{-1}$) of EN 14214 standard. The contribution for the degree of unsaturation is mainly from the erucic acid in the composition, as already discussed.

The cold filter plugging point (CFPP) is a very important parameter which allows to understand the behavior of the biodiesel at low temperatures in the engines, mainly due to waxes (long chain fatty acid esters) settling, which can cause the clogging of filters [51]. Low temperatures also lead to biodiesel solidification causing blockages of fuel lines, clogging of filters and problems during engine start-up [23]. It is also possible to predict the performance of the biodiesel in the engine at low temperatures analyzing the fatty acid composition. The higher the carbon chain, the bigger the problems of performance during biodiesel combustion [52]. The presence of a higher content of saturated fatty acids also negatively influences the CFPP, leading to biodiesel crystallization [53].

According to the literature, CFPP of crambe biodiesel varies between 7 to 10 °C [23]. The results obtained varied from 4 to 9 °C, agreeing with the referenced studies. The result for the biodiesel from seeds cultivated in Brazil (4 °C) allows to include it in the Grade A for temperate climates (max. 5 °C); the others were not within the indicated range/scale.

The results suggest that crambe biodiesel might present limitations when used pure, even in temperate climate. Because biodiesel is usually commercialized blended with mineral diesel, this problem might be overcome [54].

The flash point corresponds to the temperature of fuel ignition when exposed to an external flame. One relevant advantage of using biodiesel holds with the fact that it has much higher flash point than the mineral diesel (around 170 °C compared to 70°C) being more safe to handle [23, 41].

Improper purification, namely during methanol removal, will be reflected in the flash point, which tends to be small [23]. Only 1% of methanol on biodiesel can decrease the flash point from 170 °C to less than 40 °C. According to the EN 14214 standard, the flash point should be higher than 101 °C, characteristic easily fulfilled by all the three products whose flash points were from 171 to 179 °C.

The extraction of oil by using the mechanical press of seeds can lead to the increase of impurities on the oil which might cause contamination on the biodiesel, and, consequently, promoting clogging problems at the engine. These impurities mainly include pieces of seed's husk or pigments. For this reason, the standards of quality demand to determine the amount of non-lipidic matter present on the biodiesel.

With respect to this parameter, it is notorious the difference of content between the biodiesel from degummed oil (lowest content) and the other two types of biodiesel. Anyway, the results showed the need of additional biodiesel purification steps for removing impurities since all biodiesel types had impurities above the threshold value of 24 ppm. As expected, the refined oils had the lowest content of impurities and the biodiesel from Portuguese seeds was close to the limit (< 24 ppm). The differences found between the content of impurities in both types of biodiesel produced from refined oils is probably related with the lower quality of the Brazilian oil, reflected by its high acidity.

3.3.5 CONCLUSIONS

From the cultivation of crambe in Portugal, seeds with an oil content of 26 ± 4 wt.% were obtained after 90 days of plant growth. Such oil yield is lower than that obtained in Brazil using the same variety (38%) and differences were attributed to the weather conditions verified in the growth season, namely the mild temperatures (16 °C – 22 °C) and the low rainfall (250 mm).

Portuguese crude oil had much higher phosphorous content than Brazilian commercial oil (72 ppm in average compared to 4.6 ppm) attributed to the refining of the last. Although with high content of phospholipids, those were mostly hydratable and fortunately easily removed during biodiesel wet purification, making the crude oil refining unnecessary from this point of view.

Biodiesel produced directly with the oil extracted from the seeds showed good quality, fulfilling almost all properties evaluated according to EN 14214. Based on the properties of the oils used, the following quality rank of the three kinds of biodiesel produced was obtained: Biodiesel from Portuguese Refined oil > Biodiesel from Portuguese Crude oil > Biodiesel from Brazilian commercial oil. In fact, the biodiesel produced from the refined oil presented the highest quality, due to lower

content of contaminants, showing relevance of using a purification step despite not being required for the purpose of phosphorus removal.

The high oxidation stability of the biodiesel obtained with oil from Portuguese seeds, crude or refined (16 h and 9 h in average, respectively), was identified as an important advantage of using this product. Viscosity and cold filter plugging point were not fulfilled in the best products. Because such properties are straightly associated with the oil's chemical nature, such drawback might be overcome by adequate blending.

The work carried out, particularly the quality assessment made to the products obtained, has confirmed that biodiesel from crambe oil is technically viable and constitutes an additional promising complementary alternative to the conventional edible oils generally used for biodiesel production in Portugal.

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3.4 OPTIMIZATION OF *CRAMBE ABYSSINICA* ENZYMATIC TRANSESTERIFICATION USING RESPONSE SURFACE METHODOLOGY

ABSTRACT

An eco-friendly route for biodiesel production from crambe oil by enzymatic transesterification was evaluated in order to maximize fatty acid methyl esters content. Response Surface Methodology and central composite design (two factors, three levels, one central point, two replicas) was used to obtain predictive models considering catalyst concentration and methanol:oil molar ratio as independent variables. Since the reaction was monitored overtime, a predictive model considering three independent variables (three factors, three levels) was also obtained. Enzymatic transesterification is a good alternative process to obtain high FAME yield, with 84 ± 5 wt. % being obtained after 2 h using a methanol:oil molar ratio 6:1 and 8 wt.% of enzyme, a value which predictably can increase to almost 99 wt.% after 24 h. The results showed that the methanol:oil molar ratio and the enzyme's concentration considerably influence the FAME content during enzymatic catalysed reaction. The polynomial regression models obtained can help to balance the concentration of enzyme and alcohol to improve FAME production and to predict the resulting yield.

3.4.1 SCIENTIFIC RELEVANCE

Diesel engines are very common in the industry, agriculture and transport sectors, despite having associated a large emission of pollutants. In the fuel transition, biodiesel can contribute for the reduction of exhaust emissions and to mitigate related climate change impacts [1]. Beside biodiesel's renewable origin, the low content of aromatic hydrocarbons and sulphur, the high flash point, the non-toxicity and the possibility of biological degradation make this fuel an immediate response to meet the current demand of fuel and emissions limits imposed [2]. Biodiesel can also be a good alternative for marine transportation because most transports use diesel engines [3]. The exhaust gases from this activity sector are considered the main cause of air pollution at sea [4, 5]. Shipboard transport is responsible for a large contribution in terms of emissions at global scale, as in case of NO_x and SO_x emissions which correspond to 15% and 13% of the total emissions in the world, respectively [3]. In addition, several studies showed that biodiesel fuel leads to engine performance similar to that of mineral diesel [6].

Crambe non-edible oilseed crop arises as a new feedstock for biodiesel production showing physicochemical characteristics that allow to obtain biodiesel in agreement with the quality requirements imposed by EN14214 [7]. The oil extracted from crambe has additional interest when compared with that obtained from other oilseeds crops due to this particular composition (e.g. sunflower, *Ricinus communis* L., peanut, among others), having several potential applications such as lubricant, corrosion inhibitor, and in the production of adhesives, nylon and synthetic rubber [8]. Usually, biodiesel is produced by the homogeneous alkaline transesterification route due to its low cost and ease of implementation, although at the end of its production, the removal of catalyst is required which generates wastewater requiring further treatment.

The use of enzymatic catalysts can mitigate some problems of the conventional process including the wastewater treatment and the high energy demands [9]. Also, the use of enzymatic catalysts, such as lipases, allow to use low-cost raw materials with a high content of free fatty acids (FFA) for biodiesel production, useful to reduce production costs. At the end of biodiesel production, the glycerol by-product that results from the transesterification reaction is recovered more easily, being purer than that obtained by the homogenous transesterification reaction [9].

Extracellular lipases are the most common type of enzymes used for biodiesel production, although they also can perform hydrolysis reactions in the presence of water or esterification reaction, depending of the experimental conditions and products presents in the mixture.

Some studies report that enzyme inactivation can occur in the presence of alcohol due to the increase in the medium polarity [10], and to overcome this problem, stepwise addition of alcohol is advised.

Regarding the optimization of enzymatic transesterification from crambe oil, Response Surface Methodology (RSM) might be used although no studies have been reported on this matter. This mathematical and statistical method has as main goal to determine design factor settings to improve the performance of a process with high precision, combining design of experiments (such as central composite design) and optimization methods, thus providing a minimum set of experiments to obtain results that allow obtaining a regression model, validated by statistical tests, to optimize, in this case, a chemical reaction, based on a selected response and key independent variables [11, 12].

In the present study, biodiesel production from crambe oil by enzymatic transesterification was optimized to maximize fatty acid methyl esters content (response) by using response surface methodology and central composite design (two / three factors, three levels, one central point, two replicas) to obtain predictive models considering the following independent variables: enzyme concentration, methanol:oil molar ratio and reaction time.

3.4.2 SPECIFIC OBJECTIVES

The main objective of the work developed was to study *Crambe abyssinica* oil conversion into biodiesel by transesterification using an enzymatic catalyst. Accordingly, the study evaluated:

- the viability of enzymatic transesterification using *Crambe abyssinica* oil;
- the yields of the reaction and the purity of the final products;
- the influence of methanol:oil molar ratio, enzyme concentration and reaction time in biodiesel purity.

3.4.3 MATERIALS AND METHODS

3.4.3.1 *Materials*

Crambe abyssinica seeds were harvested in Portugal, in an experimental field study with agronomic control, conducted at Northern Portugal (40°59'07.2" N; 8°32'43.6" W) between May and August of 2018. The seeds sown belong to the "FMS Brilhante" variety. Crambe was sown in rows spaced 0.35

m with a density of 100 plants.m⁻². The soil sown had high levels of fertility (pH of 7.1; phosphorus > 200 mg.kg⁻¹; calcium = 1737 mg.kg⁻¹; potassium > 200 mg.kg⁻¹; magnesium = 52 mg.kg⁻¹) for most macronutrients. Organic fertilizer was used only to adjust the organic matter content.

Sprinkler irrigation was performed, twice a week (100 L), until the beginning of flowering stage. The weather conditions were assessed using a local weather station (Froggit® WH1080) and an average temperature of 18.0 °C and a total amount of precipitation of 219 mm were recorded.

The seeds were harvested when the plants aged 105 days and, following, they were stored at room temperature until oil extraction.

Petroleum ether (LabChem) was used on determination of the seed oil content. In the experiments carried out, methanol 99.9% (analytical grade, Fischer Chemical) was the acyl acceptor. The catalyst was lipase *Thermomyces lanuginosus* (Lipolase 100L, activity ≥ 100,000 U/g) from Sigma-Aldrich. Methyl heptadecanoate 99% was the internal standard (C17:0) for fatty acid methyl esters content (FAME) determination by gas chromatography (GC). All the reagents required on the analytical procedures were of analytical grade.

3.4.3.2 Analytical methods

The fatty acid methyl esters content was determined at different time intervals during the enzymatic transesterification reaction. GC analysis was conducted according to EN 14103, using a Dani Master GC with a DN-WAX capillary column of 30 m, 0.25 mm internal diameter and 0.25 µm of film thickness. The temperature setup was: 120 °C at the start, rising at 4 °C per minute up to 220 °C, held 10 min. All FAME analyses were performed in duplicate and the results are presented as mean values considering the error in terms of the relative percentage difference to the mean (RPD). In all cases, RPD was less than 2%. The seed's moisture content was determined after drying the seeds in an oven at 105 °C, overnight. Oil's acid value was determined by volumetric titration according to NP EN ISO 660.

3.4.3.3 Oil extraction

The oil content of the seeds was determined using a 100 mL Soxhlet extractor, following NP EN ISO 659 (2002). Firstly, the raw material was crushed in a mortar and placed inside the thimble. Then, the thimble was dipped in petroleum ether during 6 h. At the end, the solvent was removed in a rotary evaporator at 70 °C.

To conduct the experimental studies, taking into account the high amount of oil required, a mechanical press was used (Komet Vegetable Oil Expeller CA 59 1-H), where the seeds were loaded and pressed under heating using a nozzle having 5 mm of diameter.

3.4.3.4 Physical refining of the oil

The direct use of raw oil for biodiesel production is discouraged due to the presence of impurities like pieces of seeds (e.g. residual solids) resultant from the process of mechanical extraction under high pressure. Thus, crambe crude oil was submitted to centrifugation at 3500 rpm for 10 minutes and the clean oil obtained was used as the feedstock in the experiments carried out.

3.4.3.5 Experimental design

In order to study the production of biodiesel with enzymatic catalyst, the experimental conditions were established using the Design of Experiments (DOE) methodology, also helping to better understand the relationship between the experimental conditions and the selected process response variable, the FAME content [13, 14]. Using DOE, a parsimonious range of experiments were established (reducing the number of tests), leading to results that can be considered representative of the real phenomena. The experimental design applied in this study was a 3^2 factorial Response Surface Design (two factors, three levels) with central composite design (one central point, two replicas). JMP software was used. From this design, 20 random experiments were performed, to minimize errors [15], and the response was measured at the following reaction times: 30 min, 60 min, 120 min, 240 min, 480 min and 1440 min (24 h).

The definition of factor levels took into consideration the operational limits of the reaction (stoichiometric boundaries) and the information gathered from several studies as indicated in section 2.6. Thus, for each variable, the three levels were coded by -1, 0 and 1. For the central point (zero level) the following conditions were chosen: catalyst concentration (x_1) of 5 wt.% and methanol:oil molar ratio (x_2) of 6. The three levels for the independent variables were in agreement: (i) catalyst concentration (x_1), corresponding to the levels of 2 wt.% (coded level -1), 5 wt.% (coded level 0) and 8 wt.% (coded level 1), relative to oil mass; and (ii) methanol:oil molar ratio (x_2), corresponding to the values of 3:1 coded level -1, 6:1 (coded level 0) and 9:1 (coded level 1). Since the FAME content produced during the enzymatic reaction was monitored at different time periods, an additional variable was further introduced in the analysis: (iii) reaction time (x_3). The main

objective was to obtain an additional predictive model with three independent variables (x_1 – catalyst concentration; x_2 – methanol:oil molar ratio; x_3 – reaction time), thus corresponding to a 3^3 factorial Response Surface Design (three factors, three levels), to compare the performance with the initial model.

High temperature can cause the enzyme deactivation and the temperature selected for the transesterification reaction was 35 °C, as suggested by Cruz *et al.* [16]. The stirring speed should promote a good contact between the enzyme and the oil. Low stirring speed leads to slow reactions rates and high values (high shear stress) can lead to lipase denaturation [17]. Stirring speed was selected at 200 rpm, as referred in several studies [17-19]. Although with high relevance, temperature and stirring speed have a limited range of variation, and for this reason, this study focused on the influence of enzyme concentration and methanol:oil molar ratio in transesterification reaction.

The results of the experiments allowed to express the FAME concentration (y) – the dependent variable in relation to the independent variables, as a polynomial model [20].

The experimental conditions and the design of the experiments obtained from the response surface design are presented in Table 3.18.

Considering the results from each assay, it was possible to build polynomial regression equations for predicting the FAME content of this complex phenomenon, as function of the selected variable, along the reaction time. The adequacy of the model can be assessed by the coefficient of determination, r^2 , and the root mean square error (RMSE). In the present study, the linear, quadratic and interaction terms of the independent variables of the polynomial regression were considered significant for p value < 0.05 (5% level of significance), otherwise excluded, and followed by the reconstruction of the model without that term.

Because FAME content was assessed at six different reaction times, an additional approach was conducted to include this variable (x_3) in one additional model, as previously referred; for this variable three levels of variation were selected: 30 min (coded level -1), 735 min (coded level 0) and 1440 min (coded level 1).

Table 3.18. Experimental design used for the study

Assay	enzyme (wt.%)	Coded level x_1	methanol:oil molar ratio	Coded level x_2
A	8	1	6:1	0
B	2	-1	6:1	0
C	5	0	3:1	-1
D	2	-1	9:1	1
E	8	1	9:1	1
F	2	-1	3:1	-1
G	2	-1	9:1	1
H	8	1	6:1	0
I	8	1	3:1	-1
J	8	1	3:1	-1
K	8	1	9:1	1
L	5	0	6:1	0
M	5	0	3:1	-1
N	5	0	1:6	0
O	5	0	9:1	1
P	5	0	6:1	0
Q	5	0	6:1	0
R	2	-1	6:1	0
S	5	0	9:1	1
T	2	-1	3:1	-1

For the established predictive models with two or three independent variables, the predictive results were compared with additional experimental results obtained for validation. Taking into account this objective, the following experimental conditions were established for models validation: (i) enzyme concentration of 5.70 wt.% and methanol:oil molar ratio of 5.25 (480 min and 1440 min); and (ii) enzyme concentration of 4.46 wt.% and methanol:oil molar ratio of 6:1 (70, 240 and 480 min).

3.4.3.6 Enzymatic biodiesel production

For each assay (experimental condition), after 30 min, 60 min, 120 min, 240 min, 480 min and 24 h, samples of the mixture were collected to determine its FAME content, allowing to obtain the response values further referred as y_1 , y_2 , y_3 , y_4 , y_5 and y_6 , respectively. In each experiment, the total amount of samples collected did not exceed 10% of the initial oil's mass. After the enzymatic transesterification, four different fractions can be found in the reactional mixture, namely oil/biodiesel, unreacted methanol, enzyme and glycerol; therefore, each collected sample was centrifuged with the objective of removing the enzyme and glycerol fractions.

The residual excess of methanol at the lipids fraction was distilled in a rotary evaporator ($T = 65\text{ }^{\circ}\text{C}$; 200 mbar, 30 min) and, following, the product was submitted to GC analysis. All the referred methods, from the extraction of crambe oil up to the biodiesel production and purification are summarized in Figure 3.27.

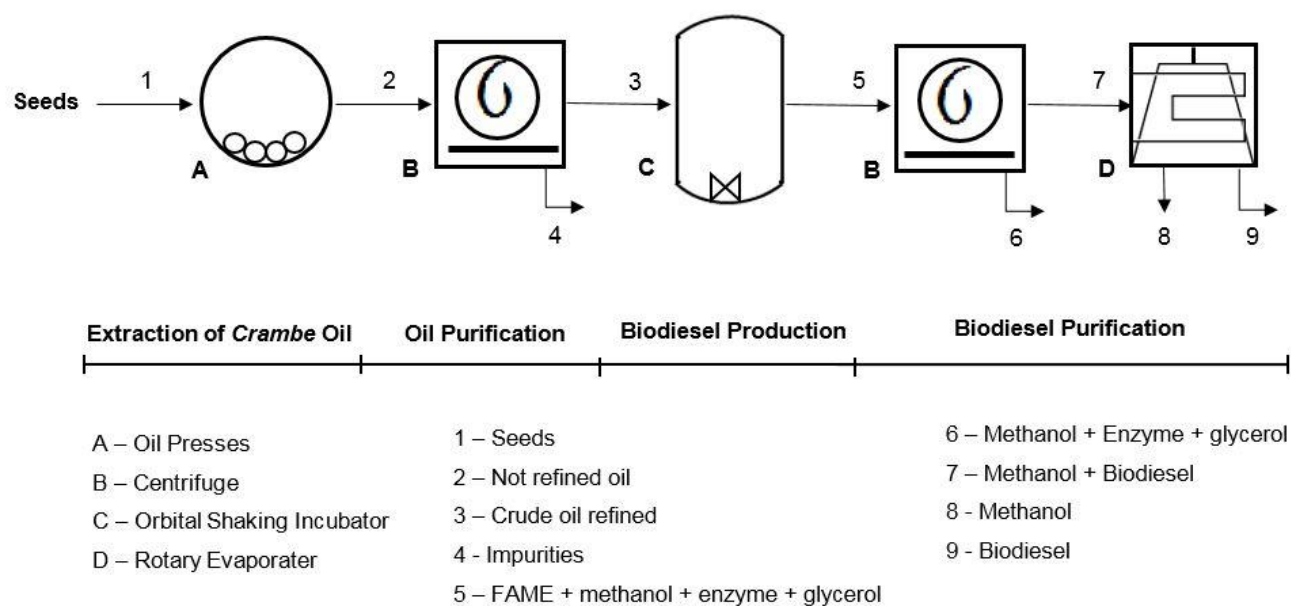


Figure 3.27. Schematic procedure of the experimental steps performed with Crambe oil until obtaining the final product.

At the end of the experiment, i.e., after 24 h, the mixture in the flask was centrifuged during 5 min at 3500 rpm, originating four distinct phases which were separated, i.e., FAME mixture, methanol, glycerol and enzyme.

3.4.1.1 Product yield

The final product yield was calculated taking into consideration the amount of raw material used in the transesterification reaction and the final weight of the organic phase, as described in equation 3.4:

$$\text{Product yield (\%)} = \frac{\text{Weight of product}}{\text{Weight of raw material used}} \times 100 \quad (\text{eq. 3.4})$$

For all the experimental conditions, the product yield after 24 h was calculated to be further

considered together with the product purity (FAME content) in order to support the selection of the best reaction conditions.

3.4.4 RESULTS AND DISCUSSION

3.3.4.1 Raw material characterization

The crambe's seeds from portuguese origin had an oil content of 29 ± 2 wt.% and a moisture of 6.20 ± 0.07 wt.%. The acid value of the oil was below 2 mg KOH.g⁻¹.

The fatty acid profile of crambe oil might be inferred through the analysis of the resultant methyl esters presented in Table 3.19. The FAME profile does not differ significantly from the literature values, except the 2.5 wt.% methyl palmitate content found in the one from Brazilian origin, which was not detected in the present study, as well as a slightly higher methyl oleate content observed in the oil obtained from the seeds from the present study (> 4.6 wt.%). The fatty acid profile was very similar for the biodiesel produced using enzymatic and alkaline catalyst (for oil extracted from Portuguese's seeds). Overall, the profile agrees with that reported, with the majority of oil being represented by erucic acid (55 – 57 wt.%).

Table 3.19. Fatty acid methyl esters profile for the biodiesel obtained from crambe oil during enzymatic transesterification and its comparison with biodiesel obtained using homogenous

Methyl ester of the following fatty acids	Enzymatic biodiesel production (wt.%) ^{a)}	Alkaline biodiesel production (wt.%) ^{a)} [7]	[21] ^{b)}
Oleic (C18:1)	17.2	17.1	21.7
Linoleic (C18:2)	8.2	9.0	8.4
Linolenic (C18:3)	4.7	5.3	4.6
Gadoleic (C20:1)	3.5	4.2	3.4
Erucic (C22:1)	56.6	54.7	54.5
Others (< 3 wt.% each)	9.8	9.7	7.4

a) Biodiesel produced from oil extracted from the Portuguese's seeds; b) Biodiesel produced from oil extracted from the Brazilian's seeds.

3.3.4.1 Model fitting for FAME content prediction

Table 3.20 presents the results of the FAME content in the samples collected in all the assays during crambe oil enzymatic transesterification. The highest content for each time of reaction is highlighted in grey.

Table 3.20. Results for the experiments under different conditions - E: weight of enzyme relative to oil (wt.%); M: molar ratio of methanol:oil; FAME content (wt.%) y (y₁; y₂; y₃; y₄; y₅; y₆ – after 30 min, 60 min, 120 min, 240 min, 480 min and 24 h of reaction, respectively (wt.%))

Assay	E	M	Coded Levels		y ₁	y ₂	y ₃	y ₄	y ₅	y ₆
			x ₁	x ₂						
A	8	6:1	1	0	52.7	62.9	83.3	95.1	97.0	97.4
B	2	6:1	-1	0	10.3	17.6	20.3	25.8	31.5	37.8
C	5	3:1	0	-1	39.3	56.0	66.4	75.8	83.0	87.4
D	2	9:1	-1	1	1.70	2.80	3.70	4.10	6.20	11.6
E	8	9:1	1	1	38.4	56.8	61.6	64.6	75.5	85.9
F	2	3:1	-1	-1	39.3	50.2	58.7	67.6	82.1	90.9
G	2	9:1	-1	1	1.10	2.00	2.70	3.50	5.00	9.80
H	8	6:1	1	0	52.9	65.8	84.9	95.2	95.9	97.6
I	8	3:1	1	-1	45.2	54.7	64.7	74.7	81.9	86.1
J	8	3:1	1	-1	46.8	56.4	66.2	74.8	81.6	87.7
K	8	9:1	1	1	37.7	64.7	76.5	75.5	85.2	87.8
L	5	6:1	0	0	41.8	62.1	72.6	80.8	90.6	97.0
M	5	3:1	0	-1	44.4	62.0	66.5	75.4	85.5	87.4
N	5	1:6	0	0	50.1	59.5	71.0	75.0	88.4	94.8
O	5	9:1	0	1	12.2	21.2	34.7	39.2	47.9	56.0
P	5	6:1	0	0	42.2	53.7	59.5	70.6	79.5	90.1
Q	5	6:1	0	0	36.3	52.4	59.9	65.9	79.6	88.2
R	2	6:1	-1	0	7.50	10.4	15.3	18.1	25.5	33.4
S	5	9:1	0	1	19.6	21.8	41.8	46.3	53.2	58.5
T	2	3:1	-1	-1	42.6	54.1	62.5	71.7	82.7	88.8

Among all the assays, those which were conducted at the highest enzyme concentration (8 wt.% of enzyme) and at the central point in terms of methanol:oil molar ratio (6:1 methanol:oil molar ratio) resulted in the highest product purity / FAME content (assay A and H). In fact, at only 30 min of reaction about 53 wt.% of FAME was obtained, and more than 95% after 240 min, and about 97.5% in both replicates after 24 h, fulfilling biodiesel quality requirements which demand a minimum purity of 96.5 wt.% (EN 14214).

Most of the assays gave products with FAME content above 85 wt.% after 24 h, demonstrating the lipase efficiency in the methanolysis of crambe oil, although using higher reaction times than the traditionally used for the homogeneous process. This performance was similar to that reported for enzymatic catalyzed transesterification using other oils, like cotton seed oil (92.1 wt.% at 24 h) or canola oil (84.4 wt.% at 72 h) [9]. In the study of Costa *et al.* [10], the enzymatic catalyzed process using 15 wt.% of *Thermomyces lanuginosus* allowed the production of biodiesel from soybean oil with a purity of 96 wt.% after 7 h.

The general regression model proposed in equation 3.5 might be used to predict the response variable (FAME content) at each time of reaction and considers the linear and quadratic terms of the independent variables x_1 (catalyst concentration) and x_2 (methanol:oil molar ratio) as well as the interaction effects between these variables; the last term reflects the random error.

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_1^2 + \beta_4 x_2^2 + \beta_5 x_1 x_2 + \varepsilon \quad (\text{eq. 3.5})$$

The values of estimates obtained for the β 's coefficients in this model were considered only when the p probability was lower than 0.05, as previously referred. Table 3.21 presents all the polynomial regressions obtained for the different reaction times, together with the RMSE and the coefficient of determination associated.

The coefficient of the quadratic term associated to the catalyst amount (x_1^2) showed no significance (p value ≥ 0.05) in 3 cases, respectively at 30, 120 and 240 minutes.

The analysis of residuals – difference between each value measured and that estimated by the equation – puts in evidence the less “normal” (and unexplainable) results obtained (outliers), which are generally assumed as consequence from unknown and uncontrolled experimental causes, thus considered unfeasible in normal conditions. For this reason, outliers may be eliminated from the data used for obtaining the β 's estimates, thus improving the model fitting [22, 23].

This was the case of the results obtained with assays B and R (2 wt.% of enzyme and 6:1 methanol:oil molar ratio) that were not considered for fitting the model at the reaction time of 30 minutes. The same happened to the results obtained on assays O, R and S at 60 minutes, the results on assays B and R at 120 minutes, on assays B and R at 240 minutes, on assays B and R at 480 minutes and on assays B, L and R at 24 hours.

For times of reaction longer than 60 min, the tendency expressed by the models presented in Table 3.21 was decreasing RMSE and increasing r^2 .

Table 3.21. Predictive regression models obtained at the different reaction times after enzymatic transesterification reactions (x_1 -catalyst concentration; x_2 - methanol:oil molar ratio)

Reaction time (min)	Polynomial regression equation	RMSE (wt.%)	r^2
30	$42.5 + 10.4 x_1 - 12.3 x_2 + 7.9 x_1 x_2 - 11.8 x_2^2$	3.6	0.96
60	$54.9 + 16.8 x_1 - 9.7 x_2 + 13.7 x_1 x_2 - 11.6 x_1^2$	5.4	0.95
120	$65.9 + 17.8 x_1 - 13.7 x_2 + 15.3 x_1 x_2 - 15.4 x_2^2$	4.9	0.90
240	$74.3 + 18.4 x_1 - 17.2 x_2 + 15.3 x_1 x_2 - 18.2 x_2^2$	4.8	0.97
480	$84.2 + 18.4 x_1 - 18.7 x_2 + 18.9 x_1 x_2 - 5.4 x_1^2 - 16.4 x_2^2$	3.9	0.98
1440	$89.0 + 17.5 x_1 - 18.2 x_2 + 19.8 x_1 x_2 - 6.0 x_1^2 - 15.2 x_2^2$	3.6	0.99

Figure 3.28 depicts the response surface for FAME percentage obtained at the different reaction times during the enzymatic esterification reaction, when varying the studied parameters. With iterative calculations (for example using Microsoft Excel®) it is possible to obtain the reaction conditions corresponding to the maximum FAME yields for each time of reaction (red marker in Figure 3.28). According to this procedure, a maximum FAME content of 53 ± 4 wt.% will be obtainable at 30 min for 8 wt.% of enzyme and 5.25 of methanol:oil molar ratio, well above 33 wt.%, the average for FAME obtained considering all the experimental results at 30 min. After 60 min the equation predicts a FAME content of 65 ± 5 wt.% which will be achievable with an enzyme's amount of 5.75 wt.% and a methanol:oil molar ratio of 3:1, against 44 wt.% for FAME average obtained with all the different experimental conditions at 60 min.

The maximum FAME content predicted at 120 min is 84 ± 5 wt.% when using 8 wt.% of enzyme and methanol:oil molar ratio of 6:1, against 54 wt.% for FAME average obtained considering all the experimental conditions. With these reaction conditions, the predictable values are 93 ± 5 wt.% of FAME after 240 min (well above the 60 wt.% of FAME, on average, with all the experimental conditions after 240 minutes), 97.1 ± 4 wt.% after 480 minutes (against 68 wt.% obtained on average), and almost 100 wt.% of FAME at the end of 24 h of reaction (against 74 wt.% on average of FAME obtained under all the experimental conditions).

Taking into account the economic sustainability of the process whose high costs are associated to the enzyme [24], the model and what is seen in the surface plot depicted in Figure 3.28, becomes possible to identify as a good choice the 3:1 methanol:oil molar ratio where the amount of enzyme is minimal (2 wt.%) and the FAME content is 88.2 wt.% after 24 h (black marker).

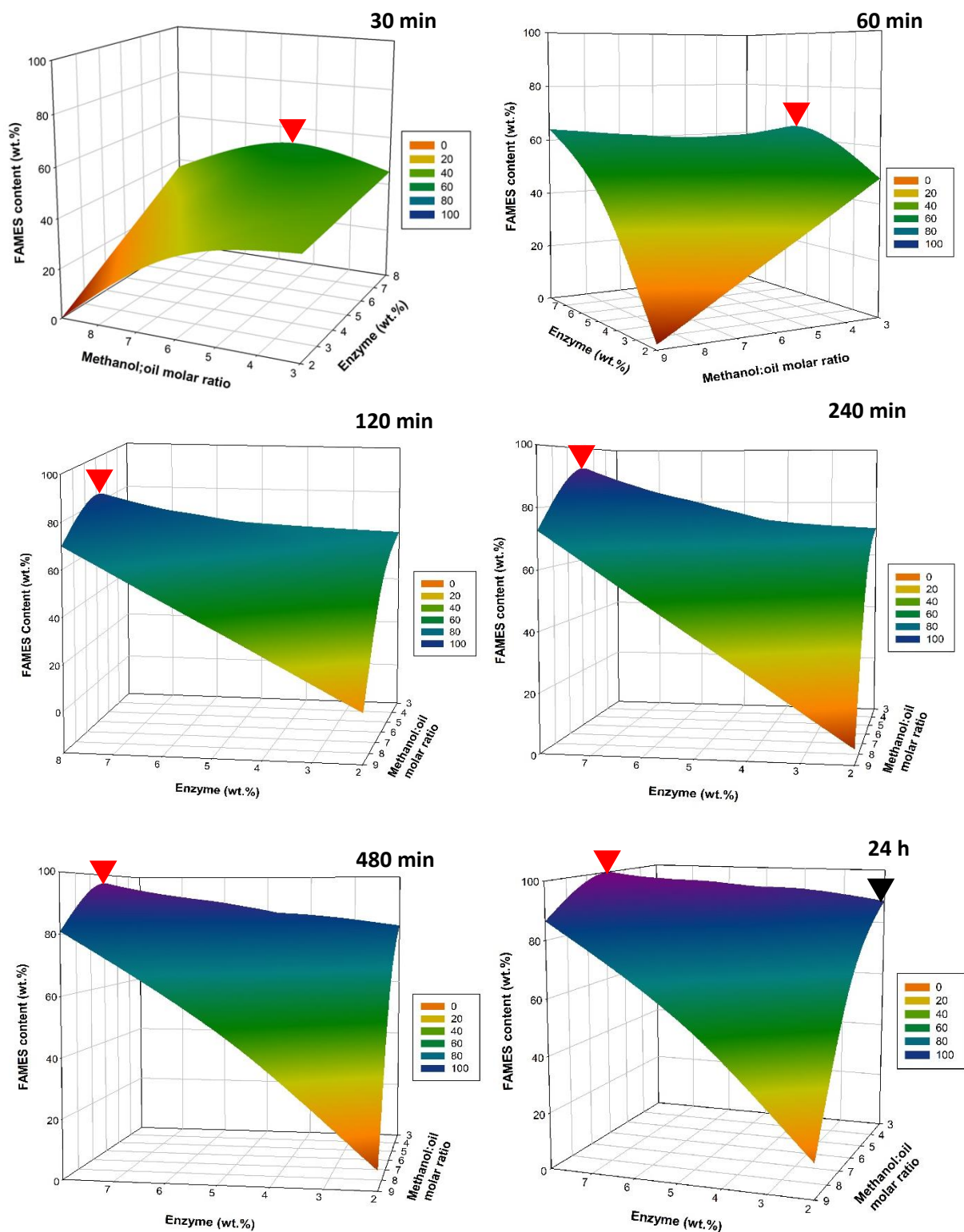


Figure 3.28. Response surface for fatty acid methyl esters percentage at different reaction times (30 min, 60 min, 120 min, 240 min, 480 min and 1440 min) and variable reaction conditions (enzyme concentration – 2 wt.%, 5 wt.%, 8 wt.%; methanol:oil molar ratio – 1:3, 1:6, 1:9).

Despite the reduction of FAME percentage predictable under these reactional conditions (around less 12 wt.% than the maximum), they seem to be reasonable regarding the economic sustainability of the enzymatic process. Increasing the amount of enzyme up to 5 wt.% will give a predictable FAME content of 94.3 wt.% (using a methanol:oil molar ratio of 5.25), close to the minimum limit of 96% imposed by the EN 14214 (European biodiesel quality standard). In order to reach the quality requirements, another alternative is to increase the amount of enzyme up to 5.75 wt.% using a methanol:oil molar ratio of 5.25 (FAME content around 95 wt.%). For the reaction time of 1440 min (24h), according the predictive model, a value around 100 wt.% of FAMES will be obtained using 8 wt.% of enzyme and the methanol:oil molar ratio of 5.25.

For a polynomial regression with three independent variables (considering the general regression model presented in equation 3.6 including the x_3 terms), where x_3 is the reaction time, the equation 3 might be obtained not including the quadratic term of the enzyme concentration ($p > 0.05$). The analysis of the residuals from the first equation discharged showed two outliers, since the results from 2 wt.% of enzyme, 6:1 methanol:oil molar ratio and reaction time of 480 min were not significant and thus were excluded (a representation of the residuals is presented in Figure 3.29).

$$Y = 95.3 + 14.3 x_1 - 16.3 x_2 + 19.1 x_3 + 13.8 x_1 x_2 + 3.9 x_1 x_3 - 3.8 x_2 x_3 - 17.2 x_2^2 - 28.0 x_3^2$$

(eq. 3.6)

Such predictive model presented a RMSE of 6.1 wt.% and a determination coefficient, r^2 , of 0.97.

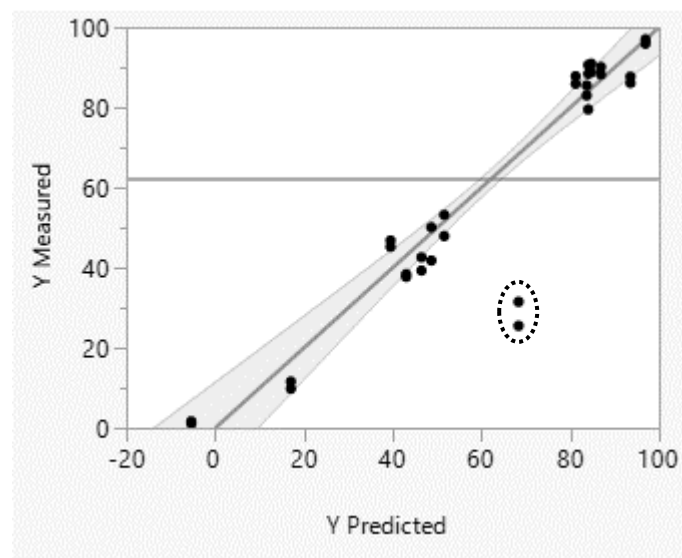


Figure 3.29. Analysis of residuals from the 1st regression equation with outliers shown inside an ellipsoid.

Through the adopted equation an estimate of around 100 wt.% of FAME is obtained at the end of 24 h using 8 wt.% of enzyme and the methanol:oil molar ratio of 5.25. A similar result was obtained using the predictive model with two independent variables for the reaction time of 24 h. Also, with only 480 min of reaction, 80 wt.% of FAME will be achievable with the lower enzyme concentration of 4.25 wt.% using a methanol:oil molar ratio of 6:1. Note that this predictive regression model under some conditions may give a negative response, i.e., FAME < 0 wt.%, what is an unreal result only verified for the first 30 min of the reaction where the reaction rate differs much from the other periods of time that gave most of the values used on the model fitting. Anyway, as a real biodiesel enzymatic process is not expectable to last only 30 min, the model obtained remains useful to predict results for the enzymatic esterification.

With the objective to verify the accuracy of the predictive models including two or three independent variables, two experiments with the following conditions were carried out: (i) enzyme concentration of 5.70 wt.% and methanol:oil molar ratio of 5.25, which would allow obtaining almost the minimum purity required by EN14214; and (ii) enzyme concentration of 4.46 wt.% and methanol:oil molar ratio of 6:1, conditions close to the central point for both variables. In the second case, different times of reaction were monitored to evaluate the accuracy of the model out of the three levels established (30, 735 and 1440 min). Table 3.22 presents the results predicted by the models and on the experiments.

Table 3.22. Comparison between results predicted by the regression models and the experimental results

Experimental conditions	Time of reaction (min)	FAME content (wt.%)		Experimental
		Polynomial regression 2 variables	Polynomial regression 3 variables	
(i)	480	90.7	89.2	92.2
	1440	95.2	93.9	96.0
(ii)	70	N.A	50.4	48.6
	240	71.0	65.8	67.4
	480	80.8	82.1	83.6

N.A – Not applicable;

As seen, the values predicted by the polynomial regressions are not very far from the experimental ones with all the differences being less than 3%. Using an enzyme concentration of 5.70 wt.% and the methanol:oil molar ratio of 5.25 (condition “i”), the minimum limit of purity corresponding to 96 wt.% of FAMES required by the standard EN 14214 for commercial biodiesel was achieved, as predicted.

3.3.4.1 Biodiesel yields

Considering the initial mass of crambe oil used in each assay and the mass of product, the yields obtained at each experimental condition are presented in Table 3.23. The highest FAME content and product yield are highlighted in grey.

Table 3.23. Results obtained in terms of product yield and purity (FAME content) under the different experimental conditions (E – enzyme relative to weight of oil (wt.%); M – methanol:oil molar ratio)

E	M	Yield	FAME content ^{a)}
2	3:1	70	89.9
5	3:1	57	87.4
8	3:1	68	86.9
2	6:1	80	35.6
5	6:1	80	92.5
8	6:1	79	97.5
2	9:1	85	10.7
5	9:1	87	57.3
8	9:1	84	86.9

a) Purity measured in the products of the enzymatic reactions after 24 h;

The highest product yield of 87% was obtained for the 9:1 methanol:oil molar ratio and 5 wt.% of enzyme, although with only 57.3% of FAME. The highest value of 97.5% of FAME obtained with the 6:1 methanol:oil molar ratio and 8 wt.% of enzyme gave the product yield of 79%. In the study developed by Bayramoglu *et al.* [25], a biodiesel yield of 85.7% was obtained from algal oil by enzymatic transesterification (free enzyme system). According to Talukeder *et al.* [26], it is possible to increase the biodiesel product yields up to 96% when a mixture of methanol and methyl acetate was used as acyl acceptors. Despite the purity was not an assumed objective of optimization in the present study, to improve the product yield deserves further studies, since the overall efficiency of process demands high yields as well as high purities.

3.4.5 CONCLUSIONS

Biocatalysed transesterification with one step addition of methanol is a good alternative process to the conventional route of biodiesel production (homogenous alkaline transesterification) using *Crambe abyssinica* oil as raw material. With such process it is possible to obtain 84 ± 5 wt.% of FAME after 2 hours, a value that using the predictive model built can increase to almost 99 wt.% after 24

h using a methanol:oil molar ratio of 6:1 and 8 wt.% of enzyme (experimental result – 97.5 wt.%). The response surface methodology, the central component design and the statistical analysis allowed to indicate potential improvements in the enzymatic process towards more efficiency and economic viability of the biocatalysed biodiesel production process. In particular, these statistical instruments were shown useful to balance the concentration of enzyme and the alcohol:oil molar ratio since the results made clear that: (i) the methanol:oil molar ratio and the enzyme's amount (expensive reagent) considerably influence FAME yield from the enzymatic reaction; (ii) a high amount of alcohol leads to enzyme inactivation; and, (iii) higher enzyme concentrations favor biodiesel conversion.

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CHAPTER 4 *CYNARA CARDUNCULUS* L. CROP – AGRONOMIC ASSESSMENT AND BIOENERGY PRODUCTION

ABSTRACT

Cynara cardunculus L. is a perennial crop referenced in the literature as having high potential for renewable energy production. A comprehensive study regarding cultivation of Cardoon was developed considering agronomic and climatic aspects (plant density, fertilization, weather conditions). This is probably the first study carried out with this specie in the North of Portugal aiming to evaluate its adaptability. Cardoon was cultivated during three consecutive years using seeds of a wild ecotype. The seeds yields were 189 kg ha⁻¹ (2018) and 95.9 kg ha⁻¹ (2019), much lower than that reported in the literature (1 210 – 1 790 kg.ha⁻¹). The low seed yields found can be explained by several factors, namely, the low agronomic inputs (irrigation and fertilization) applied, the wild variety of cardoon used and the low plant density employed (8 600 plants.ha⁻¹). For seed oil content, the results found in 2019 were similar (23 ± 2) to the values obtained in 2018 (25 ± 4). Statistical analysis confirmed that seed oil content in the years of 2018 and 2019 were not significantly different (*p* value > 0.05). It was possible to verify a linear relationship between capitula's weight and seed oil content ($r^2 = 0.58$, inversely proportional) and between seed oil content and the percentage of viable seeds ($r^2 = 0.88$ – directly proportional). Biodiesel produced from *Cynara cardunculus* oil showed high purity (FAME content > 86 wt.%), although lower than the minimum imposed by EN 14214 (96.5 wt.%).

4.1. SCIENTIFIC RELEVANCE

Cynara cardunculus L. (Cardoon) is a native species from Portugal (Asteraceae family) with the ability to grow in arable areas with low fertility (marginal lands) and low water availability [2]. The deposition of leaves on the soil at the end of their yearly life cycle, contributes to the increase of organic carbon levels in the soil. Cardoon has the capacity to develop a deep root's system, allowing the transport of nutrients for soil's surface, in specific, during the development of aboveground biomass, being consequently resistant to drought. For a plant density of 8 plants.m⁻², an increase on the soil's fertility is verified – organic matter (> 6.5%), nitrogen content (> 15.5%) and phosphorus content (> 15.8%) [2]. The dense canopy that results from the aboveground biomass growth acts as soil protector against the erosion [3]. The interest of the scientific community in this crop is notorious, reflected by the huge increase of publications and citations about Cardoon. In 2016, the number of publications was around 110 and the number of citations was around 1900, very high when compared to previous years [4].

For the Mediterranean region, this perennial crop shows high potential for energy production due to the high biomass yields that do not require high agronomic inputs (management techniques) [4]. The biological cycle of this crop comprises a vegetative growth in Autumn-Winter, which corresponds to the higher period of rainfall in this region. This fact allows to achieve high biomass yields [2]. Typically, the vegetative re-growth occurs in Autumn, assisted by the root systems which provide a stock of reserve nutrients [2].

According to the Directive 2009/28/EC, until 2020 the energy consumption should be 20% from renewable sources, partly fulfilled by energy crops cultivated in abandoned or marginal lands [5]. The production of biomass for energy generation and bioproducts has a positive environmental impact, including increase of terrestrial carbon sinks and soil protection against erosion [6].

At the Mediterranean region, the protection of soil from erosion is crucial because typically they are characterized by a low or very low organic matter content, being more susceptible to deterioration and fertility decrease [7].

Besides the importance for bioenergy production (biodiesel or solid biofuel), *Cynara cardunculus* L. can be cultivated for several other purposes. Their leaves are used as a diuretic compound or hepatoprotector. They also showed some antidiabetic and cholagogue effects [8]. In the capitula there are flowers that can be used to produce aqueous extracts (aspartic proteases - cardosins A, B

and cyprosins) applied as milk coagulant in the manufacture of traditional sheep cheese, providing that unique texture and flavor [4, 8]. In addition, Cardoon shows high content of phenolic compounds and flavonoids (with bioactive properties) [8]. Several studies referred the bioactivity of cardoon, for example the antioxidant potential of artichoke or antibacterial activity [8]. In Portugal, Cardoon is only cultivated with the objective to obtain the flowers and extract the vegetable coagulant used for traditional cheese manufacture. Those cheeses benefit from the special status of “Protected Designation of Origin - PDO” (EC Nº 1107/96) being mandatory to use the endogenous aqueous extracts of *Cynara cardunculus* L. as coagulant.

Alongside their economic potential, it is also relevant to mention another particular market segments, for instance, the cheese production with natural enzymes that serve for lacto-vegetarian consumers or even to overcome religious restrictions, the ecological markets [9-11].

Besides the flowers, the remaining portions of the plant are not usually recovered/collected and are disposed of on the field (including the capitula with seeds).

Despite that, the other parts of the crop show some potential, for example, the green leaves have considerable nutritive value for green forage and the high digestibility coefficients making cardoon an alternative crop for forage (see section 5.4). The other parts of crop (dry leaves and crop residues) need supplementary conditioning to achieve good digestibility [4]. Press cake (obtained after mechanical oil extraction) can be used as organic fertilizer, improving the content of organic matter in the soil due to the high content in carbon (50.08 wt.%) [12]. As a result of the high seeds’ oil content (24 wt.%), cardoon arises as alternative crop to the conventional edible crops used for biodiesel production [4, 13]. For energy purposes, under rainfall conditions, aerial biomass can be explored simultaneously (as solid biofuel) with the production of oil from the Cardoon seeds (ranges 603 – 1300 kg.ha⁻¹) [12, 14]. Seeds’ yields and oil content are variable, depending on the genotype and edaphoclimatic conditions [2, 15]. Figure 4.1 depicts capitula (head or capitula) morphology, where the seeds are present. The highlighted yellow area represents the “receptacle” (portion of the stem that bears the flower). The white area corresponds to the seeds and pappus structures.

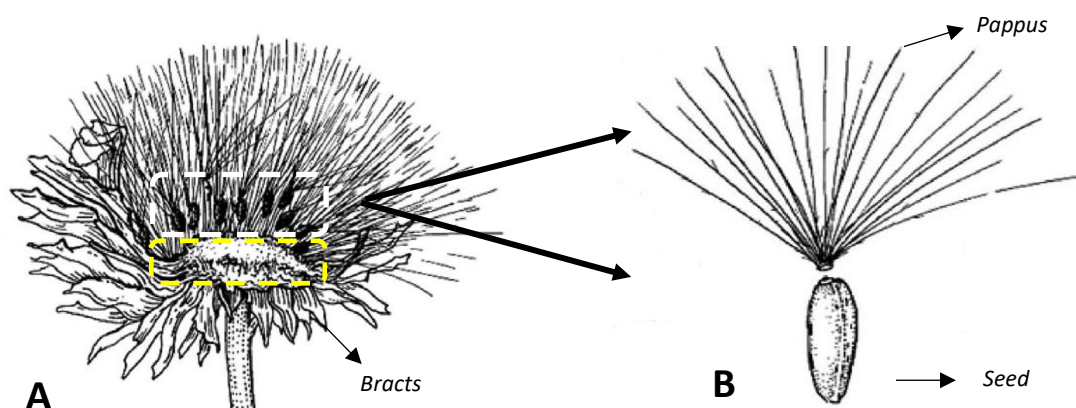


Figure 4.1. Morphology of *Cynara cardunculus* L. capitula (A) constituted by bracts (external lawyer); and pappus and seeds (B) — adapted from [4].

In this study the characteristics of the cardoon oil from different genotypes, both from cultivation and from wild genotypes found in Portugal were evaluated. The study follows all the process steps, from oil extraction until biodiesel production through alkaline and enzymatic transesterification. The final quality of the product was assessed considering some parameters of the European biodiesel quality standard (EN 14214). The study of this crop as alternative source of oil was mainly justified by the fact that Cardoon is nowadays cultivated in Portugal only with the purpose to obtain vegetable coagulant (from the flower). For that reason, more studies should be developed to increase the economic return obtained from Cardoon cultivation, with additional environmental benefits. The studies should also include the characterization of biodiesel obtained using different seeds' genotypes, as alternative feedstock to the conventional edible oils used in Europe.

4.2. SPECIFIC OBJECTIVES

The main focus of the work was to follow the *Cynara cardunculus* L. cultivation in the North of Portugal, to assess the seed yields obtained as well as the potential for biodiesel production from the extracted oil. Accordingly, the study aims to evaluate the following:

- evaluate the viability of *Cynara cardunculus* L. cultivation in the bioclimatic conditions of the North of Portugal;
- evaluate the agronomic conditions required for the establishment of the crop;
- quantify the seed yields in two consecutive cycles of grow;
- determine the seeds oil content from different seeds' ecotypes;
- analyze the effect of flower cutting in the seed oil content;
- establish the relationship between the weight of the capitula (capitula) and the amount of

seeds collected;

- determine the influence of intraspecific variability of *Cynara cardunculus* L. in the characteristics of the oils;
- assess the viability of producing biodiesel from cardoon oil.

4.3. MATERIALS AND METHODS

4.3.1 Plant Materials

The seeds of *Cynara cardunculus* L. used in the agronomic study were provided by “Sementes de Portugal” and belong to a wild specie native from “Serra do Sicó”. The cultivation was performed in a field at Northern Portugal (41°02'05.3" N; 8°33'03.1" W) with agronomic control conducted between October of 2016 and September of 2019.

4.3.2 Agronomic conditions

The biological cycle of this crop comprises a vegetative growth in Autumn-Winter at the period where more rainfall occurs in Mediterranean regions, allowing to achieve high biomass yields.

With the objective of obtaining cardoon plants with high success, the seeds were sown in a nursery beds (10 cm of depth), shown in Figure 4.4, and the plants were transplanted to the field after 40 days of growth (October of 2016). The space adopted between lines and plants was 0.50 m with a density of 8600 plants.ha⁻¹, as suggested by the seeds' supplier.

The chemical composition of the soil in Table 4.1, showed high levels of fertility for potassium and phosphorus macronutrients, and for calcium and magnesium, the level of fertility was considered low.

Table 4.1. Chemical composition of the soil used for cardoon cultivation

pH	P (mg/kg)	Organic matter (wt.%)	Ca(mg/kg)	K (mg/kg)	Mg (mg/kg)
5.8	>200	1.97	790	>200	47

Due to the low content of calcium and magnesium in the soil, dolomitic limestone was added during ten days before the sowing. Before the transplanting step, 50 m² of soil were prepared with a plough at 25 cm of depth.

The weather conditions were assessed using a local weather station (Froggit® WH1080) and the results (monthly basis) are presented in Figure 4.2.

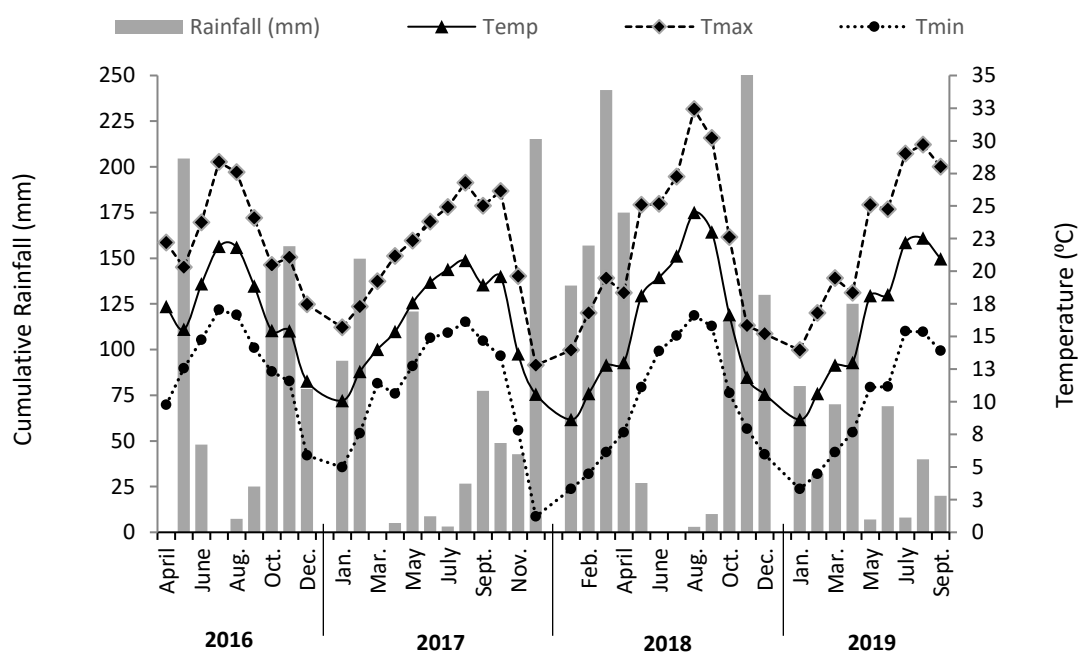


Figure 4.2. Maximum, minimum and mean temperatures and cumulative rainfall (monthly) verified in 2016 – 2019 (1st, 2nd and 3th years of crop installation).

Table 4.2 presents the average temperature and rainfall in the years of cardoon cultivation.

Table 4.2. Average temperature and accumulated rainfall verified during Cardoon growth

	2016	2017	2018	2019*
Rainfall (mm)	1209	793	1260	455
Temperature (°C)	16.5	16.0	15.4	15.6

* average values obtained until September.

The year of 2017 was the second most hot since 1931, being also the third with the lowest value of precipitation since then. For 2018, the weather conditions were considered “normal” regarding the average temperature and rainfall. During this year some atypical events occurred, namely Leslie’s tropical storm and adverse weather during March (the second rainiest March since 1931). In the first year, the capitula’s harvest was not performed. Only at the end of second and third year (2018 and 2019) the harvest of the biomass was carried out.

4.3.3 Extraction and analysis of *Cynara cardunculus* L. seeds

The *Cynara cardunculus* L. seeds, of around 4 mm, are inside the capitula. For this reason and to remove impurities such as plumose pappus hair and portions of capitula, the biomass were shredded to make the collection of the seeds easier. After that step, the obtained biomass was sieved with sieves of 6.7 mm, 4.75 mm and 3.35 mm. Finally, the retained mass, mostly constituted by seeds, was submitted to an air flow to remove the light impurities. The capitula residual biomass was finally weighted. Figure 4.3 shows the apparatus used for seeds separation.

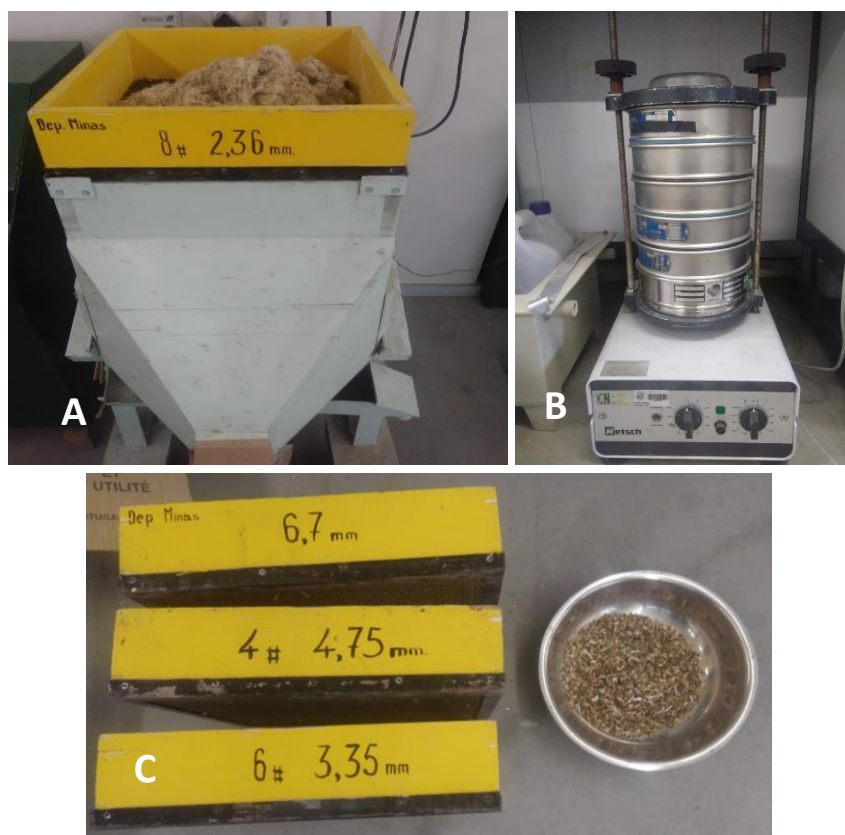


Figure 4.3. Equipment used to separate seeds: A – pilot scale sieving; B – laboratorial sieving;

Several characteristics of the seeds were determined, namely: (i) moisture content, (ii) one thousand seeds' weight; (iii) seeds' oil content; and, (iv) percentage of viable seeds.

Seeds' moisture content was determined after drying the seeds in an oven at 105°C, overnight. The weight of one thousand seeds (TSW) was determined after manual collection and counting. The amount of seeds per square meter of cultivated soil was also calculated considering the amount of

seeds collected (seeds' yields) and the density of plants in the field. Seeds' oil content was determined by chemical extraction according to the standard NP EN ISO 659 2002.

The concept of viable seeds is related with the seeds' germination ability after being sown and it was associated to the color and density of seeds obtained from different plants. The procedure adopted to evaluate this characteristic was first drying the seeds and following placing them into a recipient with water. The seeds that settled at the bottom of the recipient were considered the most viable seeds and thus potentially showing high germination capacity and viability. Its percentage relatively to the total number of seeds is taken as the index of viability of these seeds.

Ten plants were selected with the objective of understanding whether or not the cutting off the flowers might lead to an incomplete development of the seeds; and, consequently, if it affects the seeds' oil content. In these plants the flower was cut in June, when it showed a purple color. These ten plants selected were chosen according to their location in the field (allowing an easier access/control).

The morphological characteristics of the plants were also evaluated. Capitula collected from the agronomic field showed different sizes and weight, and, for that reason, they were separated by size. The objective was to understand if seeds' content and seeds' oil content varied according to the capitulas' size (weight). Those were divided into 6 different sizes.

4.3.4 Preparation of seeds and extraction of *Cynara cardunculus* L. seeds oil

The oil extraction was performed according to referred in section 3.2.3.2.

4.3.5 Oil properties

Crude oil was characterized in terms of acid value, oxidation stability, mono-, di- and triglycerides and fatty acid profile. The acid value was determined according to EN 14104 (titrimetric method). The oxidation stability was measured in agreement with the EN 14112 by accelerated oxidation using a Rancimat equipment (Metrohm). The fatty acids profile was assessed through the fatty acid methyl esters profile of *Cynara cardunculus* biodiesel using gas chromatography and following the standard EN 14103.

In 2017 it was not possible to collect any seeds, and for that reason six different cultivated ecotypes of *Cynara cardunculus* (A, B, C, D, E, F) provided by "Escola Superior Agrária de Viseu" were used in

preliminary studies of cardoon oil quality. The characteristics of these seeds and the oil obtained from them were compared with the results from the seeds harvested in 2018 (sample G – wild ecotype).

For sample F (cultivated variety) and sample G (wild variety) the total glycerol, monoglycerides, diglycerides and triglycerides were evaluated in the oil according EN 14105. GC analyses were conducted using a Dani Master GC with a DN-1 HT capillary column of 15 m, 0.32 mm internal diameter and 0.1 μm of film thickness. The temperature program was as follows: 50 °C hold for 1 min, rate of 15 °C.min⁻¹ up to 180 °C, followed by 7 °C.min⁻¹ up to 230 °C and 10 °C.min⁻¹ up to 370 °C, holding 5 min at this temperature.

For the oil obtained from these two varieties other parameters were determined, namely, (i) the refractive index at 20 °C according the EN ISO 6320/2017 (*Abbe refractometer of Optic Ivyman System*®); (ii) density at 20 °C, according the Portuguese standard - (NP) 938/1988; (iii) iodine value by titration method using Wijs reagent (NP EN 14111/2009); and, (iv) peroxide value by titration method (EN ISO 3960/2017) .

4.3.6 Biodiesel Production

Biodiesel was produced from *Cynara cardunculus* L. oil (10 g) using the experimental conditions referred before [16], namely a temperature of 65 °C, in a batch reactor, using a methanol:oil molar ratio of 6:1 and a catalyst concentration of 1 wt.% of NaOH (relative to mass of oil), during 1 hour. After that, the products were left to settle under gravity in a separatory funnel and the upper layer (biodiesel) was recovered. The excess of methanol was recovered in a rotary evaporator and the biodiesel washed with 50 vol.% of hydrochloric acid (0.5 wt.%) and with 50 vol.% of distilled water until the washing water was close to neutral pH.

Due to constraints of seeds availability, biodiesel was produced using only seeds of the variety F (cultivated genotype) and variety G (wild genotype).

For the oil obtained from variety G, due to the high content of free fatty acids (> 2 mg KOH.g⁻¹), enzymatic transesterification was performed, using a methanol:oil molar ratio of 6:1 and an enzyme concentration of 5 wt.% ($w_{\text{enzym}}/w_{\text{oil}}$) [17]. The oil (10 g) was added to a batch reactor in an orbital shaking incubator (Agitorb 200IC), with constant stirring of 200 rpm, during 24 h at 35 °C according to the literature [18]. In fact, by the enzymatic route the yields of the transesterification reaction is

not affected by the presence of FFA, because simultaneously occurs the production of methyl esters from FFA and from mono-, di- and triglycerides.

Methanol 99.9% (analytical grade, Fischer Chemical) and NaOH 97% powder (analytical grade, Aldrich) were used in the transesterification reaction (biodiesel production). The enzyme used was lipase *Thermomyces lanuginosus* (Lipolase 100L, activity $\geq 100,000$ U/g), purchased from Sigma-Aldrich. The laboratorial apparatus used in the homogenous transesterification reaction was similar to the presented in section 3.3.3.5.

The fatty acid profile was assessed through the fatty acid methyl esters profile of *Cynara cardunculus* L. biodiesel using gas chromatography and following the standard EN 14103. GC analyses were conducted as referred in section 3.3.3.3.

4.3.6 Statistical Analysis

To evaluate the significance of the results, analysis of variance (ANOVA) were performed with the support of the Excel® software (Analysis ToolPak). For the parameters/properties with significant difference ($p < 0.05$), Tukey test was performed with objective to identify, for the same property, the groups of results (growing seasons) among which differences were relevant.

4.4 RESULTS AND DISCUSSION

4.4.1 Agronomic Conditions

Considering the temperature and rainfall data shown in Figure 4.2, the climatic conditions were those typical of a temperate region with a rainy winter and dry and not too hot summer (Csb climate according to the Köppen classification). According to the records, the mean long-term annual climatic characteristics between 1967 and 2018 for the region of Porto were: 1136 mm of rainfall and 14.8 °C of air temperature. Comparing the rainfall values recorded in the agronomic field with the long-term values it is possible to conclude that the year of 2017 (793 mm) was very dry. For 2016 (16.5 °C) and 2017 (16.0 °C), the mean temperature recorded was higher than the long-term average suggesting a hot year for the region where the agronomic study was performed. The year of 2018 is described as a normal year in terms of rainfall and temperature.

To start the development of the cardoon's plants protected from the climatic conditions, the seeds were sown in nursery beds, shown in Figure 4.4, in a place far from the rainfall and frost. Although 112 seeds were sown, only 91 germinated (germination rate of 81%). After approximately forty days, the plants were transplanted to the field respecting the space between plants defined previously.



Figure 4.4. Development of cardoon in nursery beds after 7 days (A) and 15 days (B).

Figures 4.5, 4.6 and 4.7 the different stages of cardoon's transplant, including soil preparation, might be observed.



Figure 4.5. Preparation of the field: ploughing (A) and correction of soil's pH (B).



Figure 4.6. Transplant of cardoon's plants.



Figure 4.7. Cardoons growth 10 days after the transplant.

The transplant of plants (60 plants) to the field was performed in October of 2016. The first capitula of cardoon were expected available only after 1 year. During the year of 2017, weeds' control was performed. The irrigation was done in the months of April, June and July (one time per week), when the rainfall was very low.

The amount of water added to each plant was around 1 liter per irrigation. Because the goal of the study was to stablish cardoon crop in rainfed condition after 2017, irrigation was not performed. During the first year of the cardoons' growth no diseases were observed. Figure 4.8 presents the aspect of the crop in July of 2018. The first harvest of cardoons' capitula was forecasted on August of 2018.



Figure 4.8. Experimental field experiment of cardoon in June of 2018.

Typically, the development of cardoon's flowers occurs during the month of June as shown in Figure 4.9.



Figure 4.9. Flowering of cardoon specie (June of 2017).

The harvest of the majority of the capitula was carried out after August, when the flower started to show a brown color, as seen in Figure 4.10.



Figure 4.10. Appearance of cardoon crop in September of 2018 (capitula with brown color).

- **First Capitula Harvest (Summer of 2018):**

Only in September of 2018 capitula seen in Figure 4.11 were collected for the first time, because capitula's moisture was high, which made harder to perform the shredder step.



Figure 4.11. Capitula collected from the field: before (A) and after shredding (B).

In Chapter 5, the results of a nutritional assessment will be presented for the biomass depicted in Figure 4.13-B after the extraction of the seeds. Regarding the capitula biomass, it was divided into two different fractions, a light fraction (plumose pappus hair) and a heavy fraction (bracts and receptacle).

Figure 4.12 and 4.13 show some of the 10 selected plants.



Figure 4.12. Selected plants: 2, 3, 5 and 6.

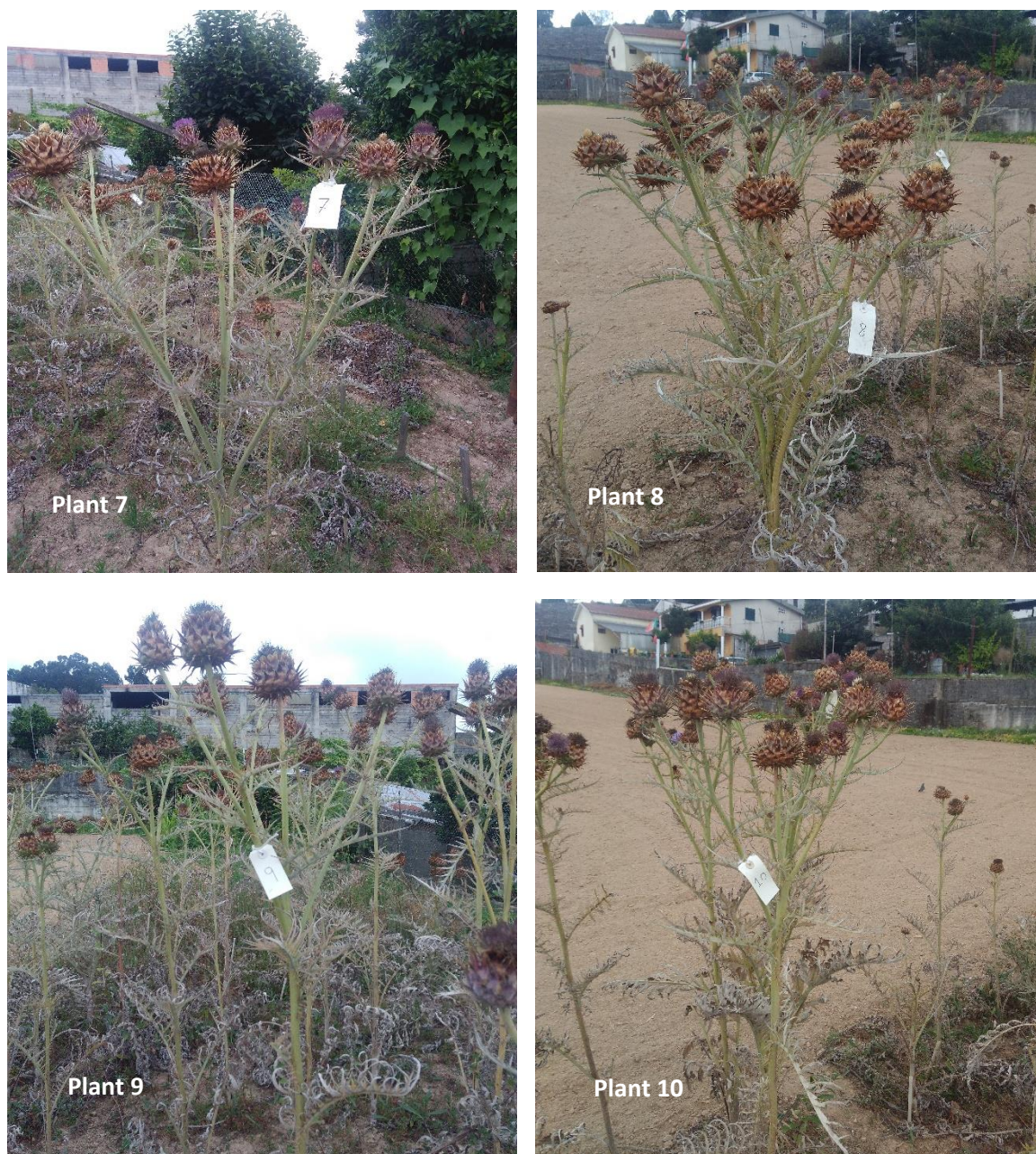


Figure 4.13. Selected plants: 7, 8, 9 and 10 (continued).

Figure 4.14 shows the seeds of the 10 plants selected those colors vary, being some samples lighter than others. The color of the seeds was related with the seeds' germination capacity. The darkest seeds exhibit generally higher germination capacity, being more viable to originate new cardoons' plants.

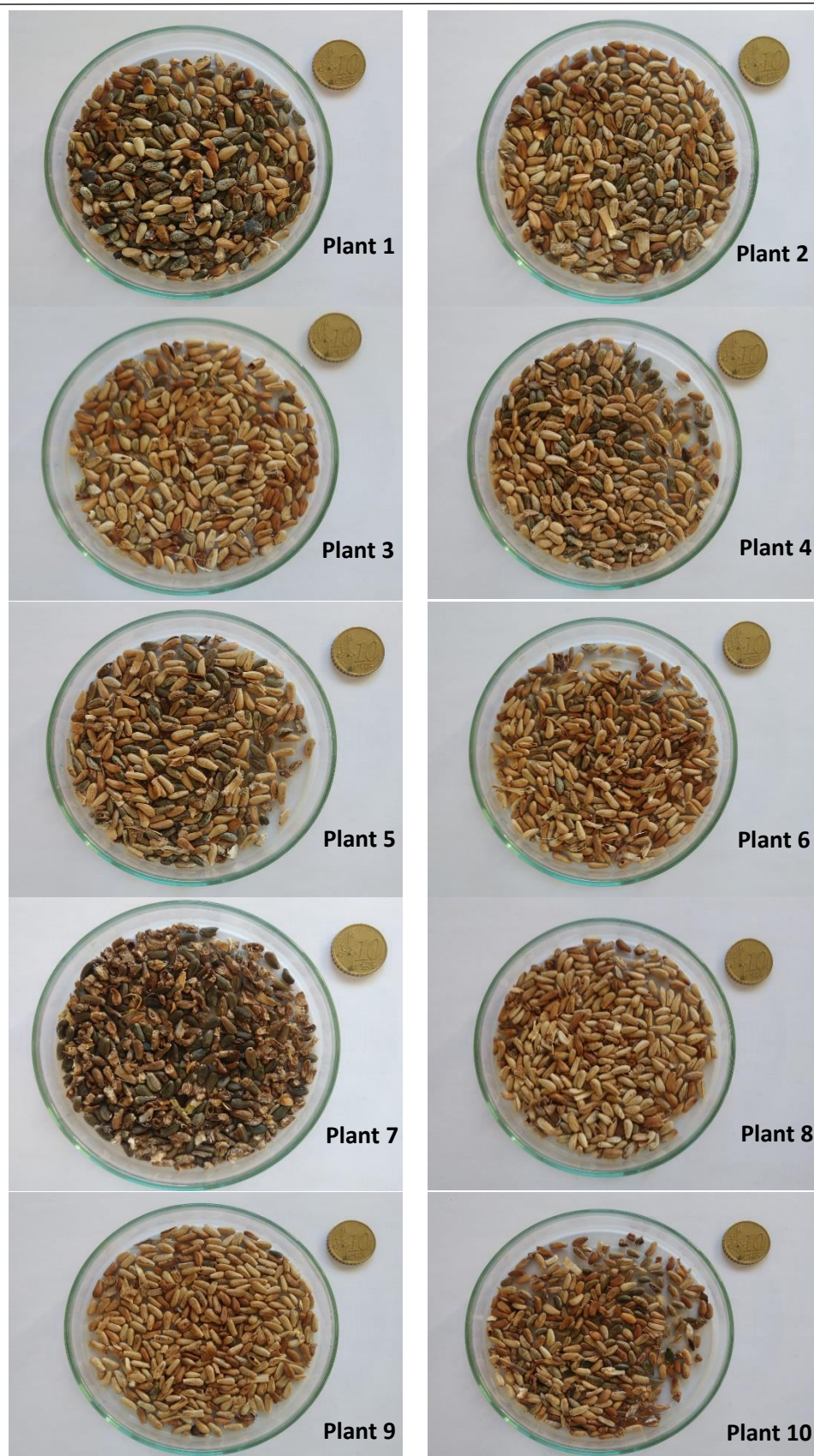


Figure 4.14. Seeds collected from the 10 plants previously selected.

For this group of ten plants their morphologic characteristics were described and the main results are present in Table 4.3. The height of each plant, diameter of the main stalk, number of capitula, height of the first ramification and the number of primary and secondary ramifications were determined.

Table 4.3. Morphologic characteristics of the ten selected plants (first harvest)

Plant	Height (m)	Diameter (cm)	Capitula	Height of first ramification (m)	N.º Primary ramifications	N.º Secondary ramifications
1	1.80	9.0	12	0.94	4	9
2	1.70	9.5	12	0.98	4	7
3	2.05	11	20	1.25	5	8
4	2.09	7.0	15	-	-	-
5	1.70	13	15	0.62	5	11
6	1.48	6.5	9.0	0.72	3	7
7	1.74	7.1	9.0	0.92	4	9
8	2.04	10	16	1.02	4	8
9	1.85	6.2	7.0	1.27	3	6
10	2.02	6.1	10	1.43	3	6
Average	1.85	8,5	13	0.92	4	7

Taking into consideration the results presented in Table 4.3, it was possible to verify that in 2018 all the plants were higher than 1.50 m. This fact makes the harvest of the capitula and flower more difficult. The morphological results are in agreement with the average of 1.70 m reported in other study using a cultivated variety of cardoon [19]. For wild varieties the height of Cardoon plants are usually lower [19, 20].

The number of capitula per plant, according to the literature, (average of 13 in this study), depends on the variety of cardoon studied. Capitula number can vary between 6 and 25.0 per plant, 17.1 on average [15]. According to Salvatore *et al.* [15], the year of the cultivation influences the number of capitula per plant more than the genotype. In the referred study, for wild cardoon variety, the number of capitula obtained was 15.2 on average [15].

Regarding the primary ramification, the studies reveal a value usually between 1.5 and 3 [15, 19]. In this study the average number of 4 for primary ramification was slightly higher than that reported.

For each plant, capitula harvest was performed aiming to obtain cardoon seeds yields' and several other parameters as the TSW, the seeds' oil content, the percentage of viable seeds and the weight

of capitula. Table 4.4 presents some of these results and highlights those (in bold) corresponding to the highest values found for each parameter.

Table 4.4. Capitula's assessment (biomass and seed's characteristics) and seeds characterization (first harvest)

Biomass data				Seeds characteristics		
Plant	Seeds (g)	Seeds/biomass processed (%)	Seeds/capitula (g.capitula ⁻¹)	1000 Seeds weight (g)	Seeds oil content (wt.%)	Viable seeds (%)
1	68.9	14	5.75	38.9	27 ± 2	71.3
2	61.4	5.7	5.12	32.9	25 ± 3	60.2
3	60.9	8.5	3.04	18.8	22.1 ± 0.3	29.8
4	27.0	7.0	1.80	21.9	30 ± 1	62.0
5	44.9	8.5	2.99	32.1	25 ± 2	63.5
6	14.6	5.4	1.63	15.9	26 ± 4	43.8
7	27.4	3.8	3.05	34.6	17 ± 2	40.1
8	33.4	5.3	2.08	21.3	20 ± 2	45.7
9	29.4	9.6	4.20	22.6	24.9 ± 0.5	30.6
10	17.4	7.1	1.74	29.9	26 ± 4	38.2

Although belonging to the same cardoon genotype, the results suggest a high intraspecific variability.

The weight of each capitula is in the range of 21.3 – 90.2 g and the weight of the seeds obtained per plant varied between 14.6 and 68.9 g. The results obtained did not support the idea that increasing the capitula's weight guarantees the increase of the amount of seeds collected. Overall, 385 g of seeds and 5350 g of biomass (from sieved capitula) were obtained corresponding to a seeds/biomass percentage of 7.1%. from these ten plants.

Figure 4.13 shows a linear relationship ($r^2 = 0.58$) between capitula's weight and seeds oil content with the results in the range 20 – 80 g of capitula's weight (inversely proportional). In this case, the increase of the capitula's weight corresponded to a decrease of seeds oil content.

The seeds oil content parameter and the percentage of viable seeds seems to have a positive linear relationship with $r^2 = 0.49$, which means that seeds oil content increases with the amount of viable seeds.

ANOVA analysis for the seeds oil content parameter, makes possible to conclude that these plants are significantly different (p value < 0.001). With the Tukey test it was possible to identify the groups of results (plants) where the differences were relevant (p value < 0.05): plant 7 vs plant 1, 2, 4, 5, 6,

9, 10; plant 4 vs plant 3, 8; and plant 2 vs plant 10. In fact, Plant 7 is significantly different from the remaining plants due to its low seed oil content (the lowest value obtained). Plant 4 stand outs for having the highest seed oil content determined.

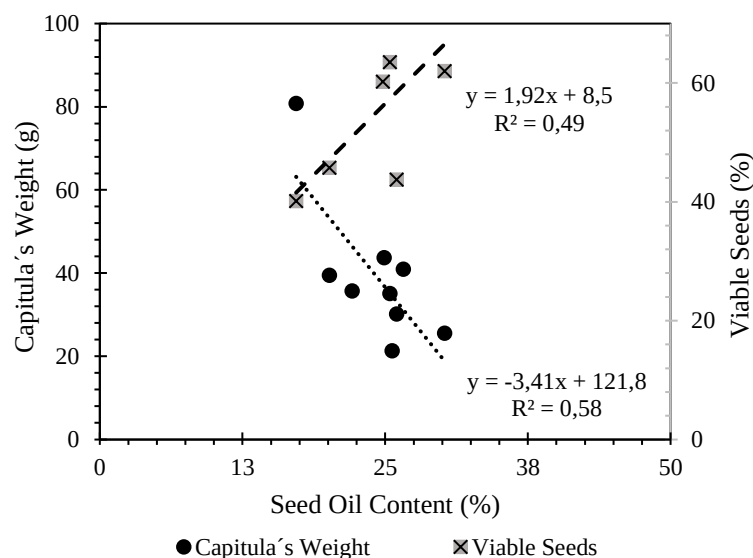


Figure 4.15. Correlation between seed oil content and capitula's weight.

A linear positive relationship was also observed between the amount of seeds collected from each plant and either the weight of 1000 seeds or the viable seeds parameter, as shown in Figure 4.16.

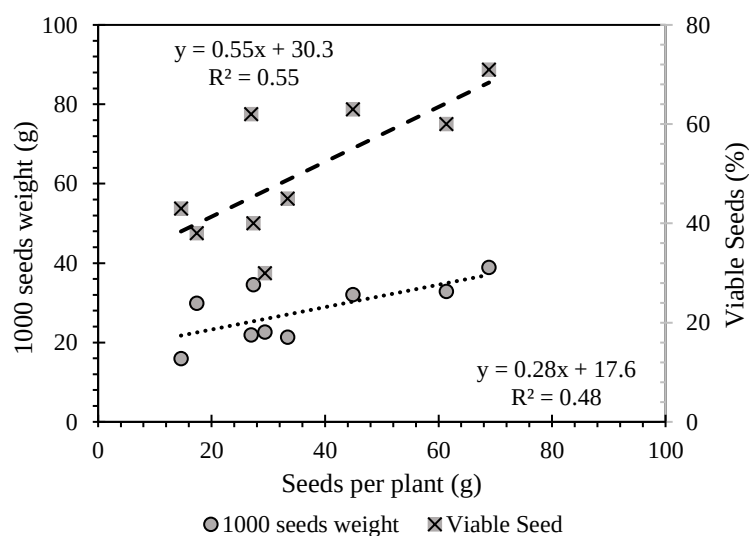


Figure 4.16. Correlation between the amounts of seeds obtained for each plant and the parameter 1000 seeds weight and viable seeds.

According to the Figure 4.16, the increase of seeds' weight per plant gives origin to an increase of 1000 seeds' weight, as expected. Also, between the parameter "seeds per plant" and the value of "viable seeds" a directly proportionality seems to exist. From the two linear relationship referred before, it was also expected that the increase of the 1000 seeds weight value was followed by the increase of the percentage of viable seeds as obtained in equation 4.1 with $r^2 = 0.63$ (no shown in the Figure).

The established equation was:

$$y = 1.53x + 6.8 \quad (4.1)$$

It was also verified that the increase of the number of capitula collected by plant led to increase the number of seeds obtained, as seen in Figure 4.17. It was not possible to find results in the literature to compare with those obtained in the present study.

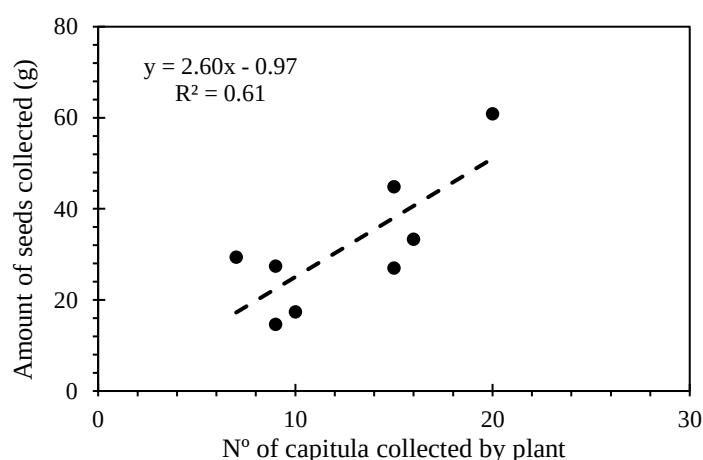


Figure 4.17. Evolution of amount of seeds collected with the number of capitula harvested.

Capitula's harvest was also carried out to all the plants present in the field beyond the ten plants previously referred. Table 4.5 presents the morphologic characteristics (on average) of these 50 plants.

Table 4.5. Morphologic characteristics of the remain population of cardoon (first harvest)

Nº of capitula per plant:	1 to 4	5 to 8	> 9
Height (m)	1.6 ± 0.1	1.7 ± 0.1	1.9 ± 0.1
Diameter (cm)	4.1 ± 0.8	6 ± 1	9 ± 2
Nº of capitula	2.9 ± 0.9	6 ± 1	13 ± 3
% of each class in the population	40	24	36

According to these results, the increase of the number of capitula per plant relates directly to the growth of the plant's structure, namely the stalk's diameter and its height. Overall, the population of wild cardoon cultivated is mainly constituted by plants with a number of capitula above 5.

Comparing the results from the population of 10 plants (previously presented) with these 50 plants, the average number of 13 capitula per plant in this first group is above that for remaining cardoon population. Only 36% of the plant population had a number of capitula around 13. The same tendency is verified for diameter and height of the plants which means that the 10 plants selected correspond to a population sample with a bigger size. After the harvest, as seen in Figure 4.18, the capitula were divided into six groups according to their size.

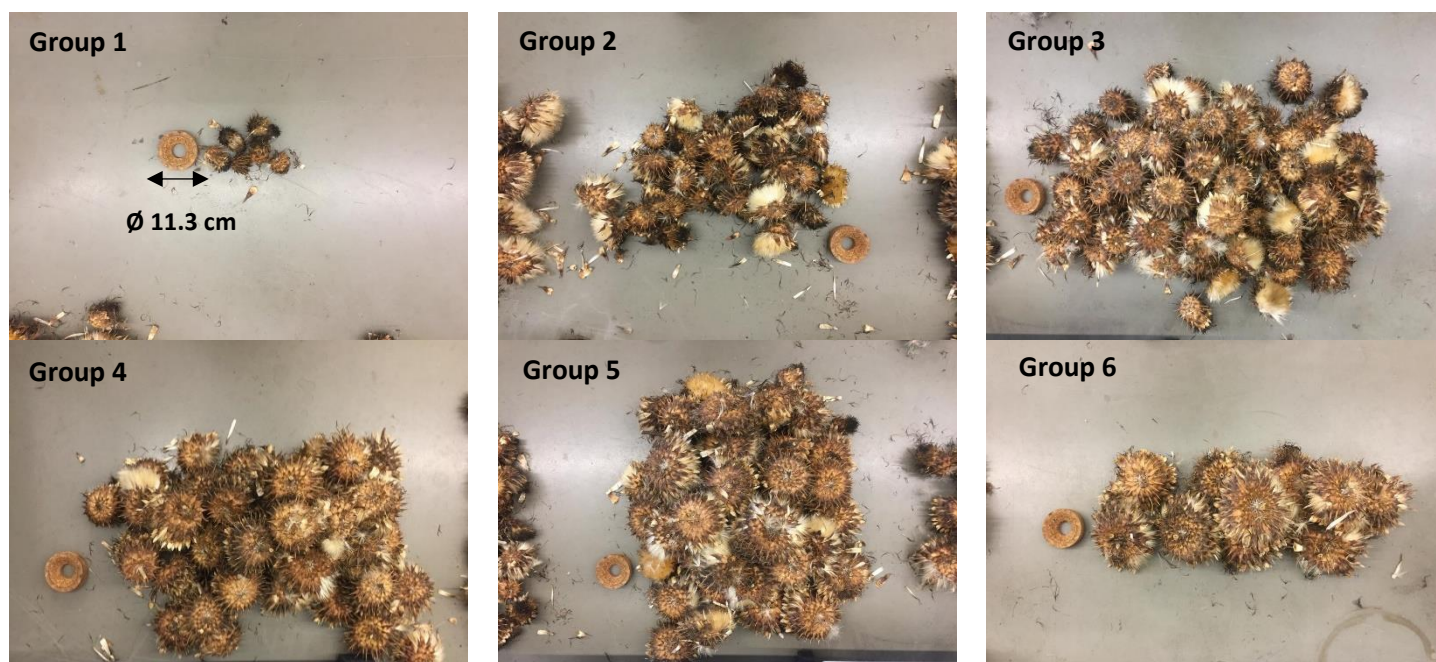


Figure 4.18. Size comparison between the six groups of capitula.

To verify if the increase of capitula's weight is related to the increase of seeds' weight, the capitula were weighted in each group and the heaviest capitula separated. Following, for each group, seeds from capitula with similar weight were separated as well as the seeds from the heaviest capitula.

Table 4.6 presents these results. The size range of the capitula was between around 6 cm (group 1) and 17 cm (group 6).

Table 4.6. Capitula's weight verified for the different groups

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Capitula with regular weight	6 ± 1	12 ± 3	20 ± 5	25 ± 7	29 ± 6	39 ± 6
Heaviest capitula	10 ± 1	21 ± 4	33 ± 2	42 ± 2	47 ± 8	48 ± 1
% of each group	6.0	17	32	21	19	5.0

According to them, the increase of the capitula size corresponds, as expected to a higher capitula's weight. For some groups, the heaviest capitula had almost more 10 g than the capitula with "normal" weight (approximately 45% more heavier). Figure 4.19 shows the aspect of the seeds obtained at the end of the sieving process and the groups are identified with the symbol * which correspond to the seeds obtained from the heaviest capitula.

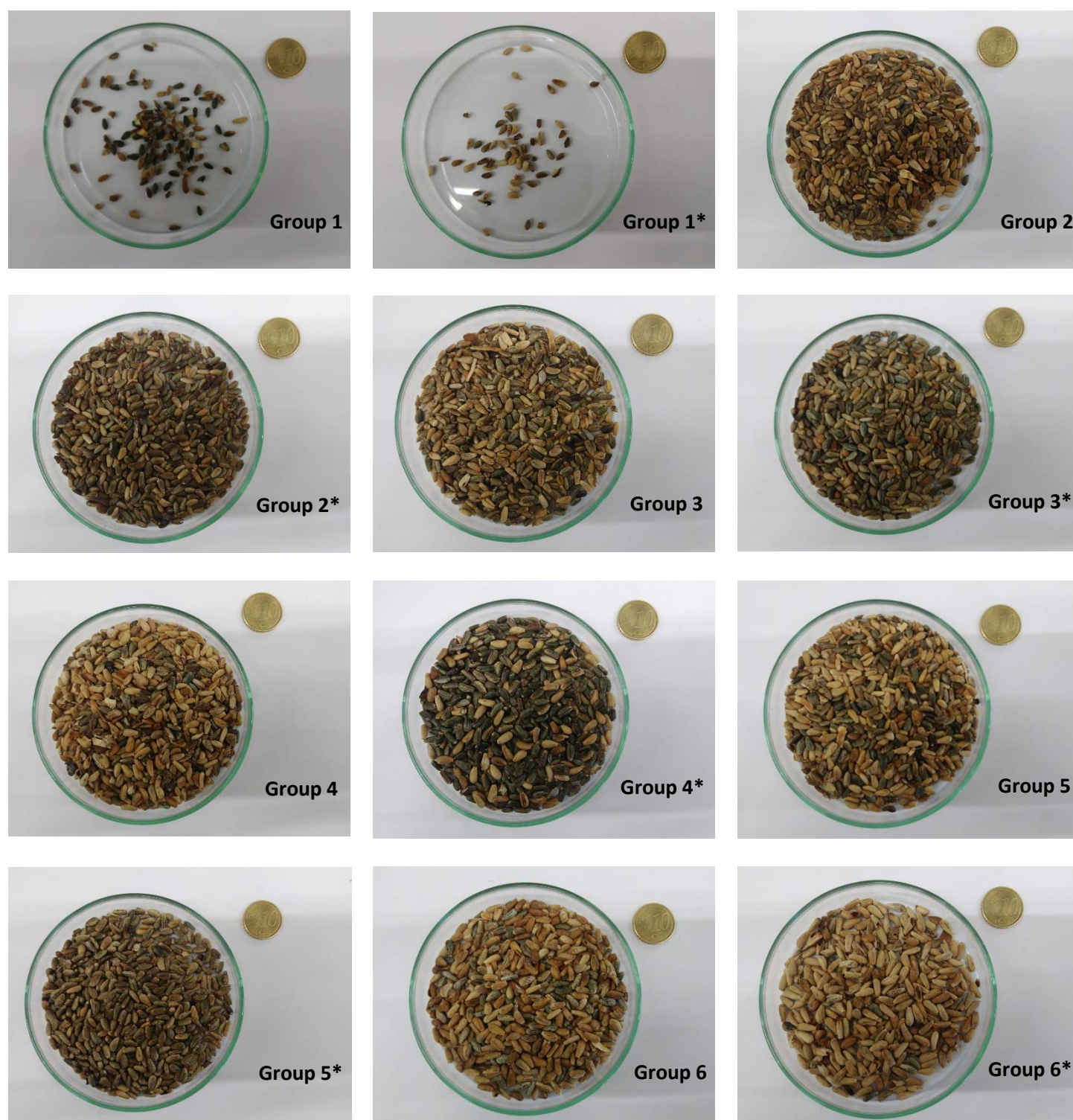


Figure 4.19. Morphologic aspect of seeds obtained from the different groups of capitula. Groups identified with * correspond to seeds obtained from the heaviest capitula.

For the same capitula size group, it was expected that the seeds had similar aspect, although for most of the groups (except group 6) the seeds had no color uniformity. The seeds belonging to the heaviest capitula had a dark color (related to the viable seeds concept), meanwhile the seeds from capitula with regular size showed the trend of having brown color (inviable seeds). Table 4.7 presents the main results of this analysis.

Table 4.7. Capitula's assessment (biomass and seed's characteristics) per group and seeds characterization (first harvest)

Group	Biomass data				Seeds characteristics		
	Number of Capitula	Seeds (g)	Seeds/biomass processed (%)	Seeds/capitula (g.capitula ⁻¹)	1000 seeds weight (g)	Seeds oil content (%)	Viable seeds (%)
1	10	1.10	2.0%	0.11	10.4	-	-
1*	3.0	0.60	1.9%	0.19	11.2	-	-
2	31	18.4	5.1%	0.59	-	17.5 ± 0.7	-
2*	5.0	13.7	13.2%	2.73	25.8	18.2 ± 0.2	58.7
3	61	110	9.3%	1.81	26.4	26.0 ± 1.6	76.8
3*	5.0	30.5	15.4%	5.09	30.3	26.2 ± 2.8	85.3
4	42	116	11.0%	2.78	25.1	18.1 ± 0.9	51.2
4*	3.0	28.6	22.8%	9.54	38.2	24.8 ± 2.1	83.4
5	37	137	12.7%	3.71	27.3	19.9 ± 0.3	70.8
5*	3.0	43.6	30.8%	14.5	35.6	27.2 ± 0.6	95.2
6	8	45.0	14.6%	5.62	33.2	18.5 ± 0.9	51.9
6*	2.0	11.9	12.5%	5.97	-	22.1 ± 0.8	-

These preliminary results make possible to conclude that the weight of seeds obtained from each group (1, 2, 3, 4, 5 and 6) increases with the capitula's size. As an example, for group 1 (which correspond to the smallest size), the seeds/biomass percentage was 2.0, although for group 6 (biggest capitula's size), seeds/biomass percentage was almost 15 %.

For the same amount of biomass processed a higher amount of seeds is obtained at the heaviest capitula (higher seeds/biomass processed ratio), regardless the size of the capitula (groups).

Overall, with this ecotype of cardoon 558 g of seeds were obtained and were sieved 5520 g of biomass with 211, capitula corresponding to a 10.1% seeds/biomass processed ratio.

ANOVA analysis on the seeds oil content parameter from the different groups makes possible to conclude that only for "group 5", the *p* value was below 0.05 (meaning a significant variation). For the other groups, the difference on the seeds oil content from capitula with regular weight and the heaviest ones was not statistically different (*p* value > 0.05).

The results of seeds oil content in the first group of 10 plants and the remaining cardoon population ranged very similar to both groups (group of 10 plants: 17.2 ± 1.6 – $30.2.2 \pm 1.4$; remaining plants: 18.1 ± 0.9 – 27.2 ± 0.6). So, it is possible to conclude that cutting off the flower did not seem to affect the seeds oil content. ANOVA analysis brought the same conclusion through a $p > 0.05$ comparing the results from the 10 plants (which suffered a flower cut) with those from the remaining cardoon plants. Also, for the 1000 seeds weight parameter, ANOVA analysis gave a $p > 0.05$ (no significantly different TSW on the 10 plants and the remaining cardoon population).

On the other hand, cutting off the flower seemed to affect the percentage of viable seeds. For the group of 10 plants, the average of viable seeds lower, being 49 ± 15 wt.% and for the remaining cardoon plants it was 72 ± 16 wt.%. ANOVA analysis confirmed this conclusion of a significantly different percentage of viable seeds for the population of 10 plants from the remaining plants ($p < 0.05$).

Figure 4.20 presents the seed/biomass processed ratio vs the size of the capitula roughly equivalent to the amount of seeds (weight) per amount of biomass sieved. The highest values were found to the “Size 5”, having the highest amount of seeds per gram of capitula sieved. The heaviest capitula also gave the highest seeds per biomass processed ratios (except for groups 1 and 6). Accordingly, when the objective is to have a cardoon crop for bioenergy production, the seeds of the 5th group should be cultivated aiming to obtain plants which allow high seed and oil yields.

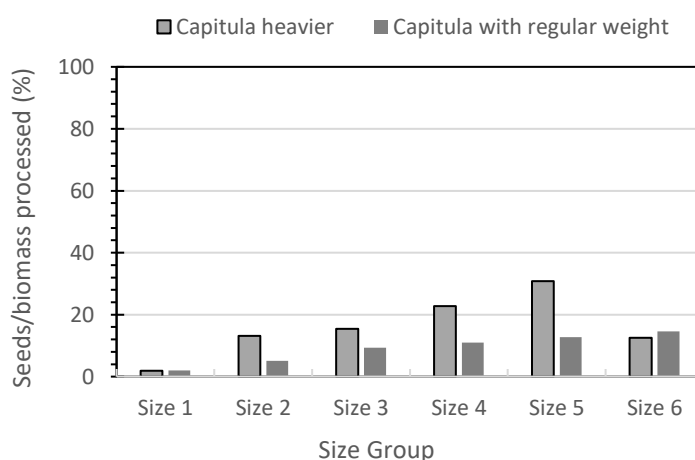


Figure 4.20. Amount of seeds (g) per capitula among the different capitula's size.

Figure 4.21 presents the amount of seeds obtained per each capitula (total amount of seeds divided by the number of capitula obtained in each group).

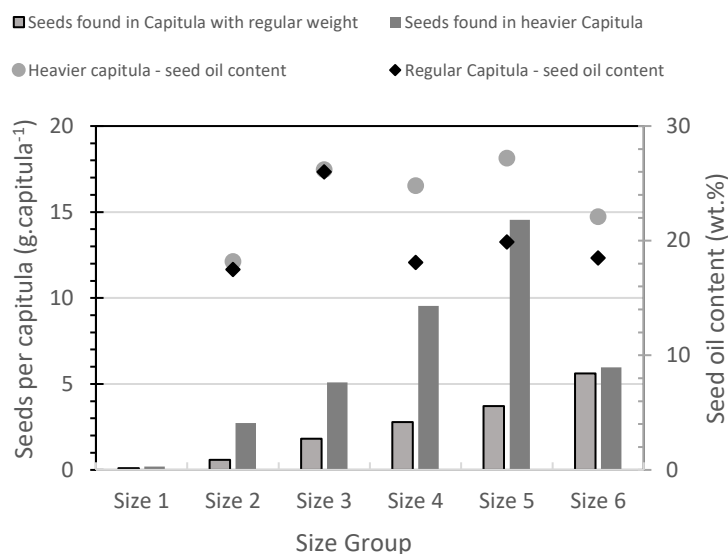


Figure 4.21. Evolution of seeds/biomass ratio and seed oil content according to the capitula's size.

Capitula from "Size 5" had the highest weight of seeds per capitula. Comparing the capitula with regular weight with the heaviest ones, the amount of seeds obtained per amount of biomass processed is higher in the last ones. So, one may conclude that as heavier the capitula is, more weight of seeds it contains. On the other hand, the biggest size - "Size 6" was the group with the highest amount of seeds per capitula in case of regular weight, as expected.

In what concerns to the seed oil content parameter (Figure 4.21), the biggest differences for the same size of capitula, mainly happened on the highest sizes, i.e., in groups 4, 5 and 6.

The increase of the capitula's size is not followed by increase of seeds oil content, according to Figure 4.21. The highest seeds oil content among the capitula with regular weight was found on size 3, while for the heaviest capitula the oiliest seeds were found in size 5. On the other hand, one can say that heaviest capitula gave the oiliest seeds in all the groups studied.

Taking into account the results present in Table 4.7, a linear relationship between the % of viable seeds and the seeds oil content was established with $r^2 = 0.88$, as shown in Figure 4.22. Thus, selecting seeds to sow with a typically dark color generally means obtaining oiliest seeds what may be explored in future agronomic studies regarding bioenergy production. In the literature it was not possible to find any information about the percentage of viable seeds typically present in the capitula of cardoon to compare with the results of the present study.

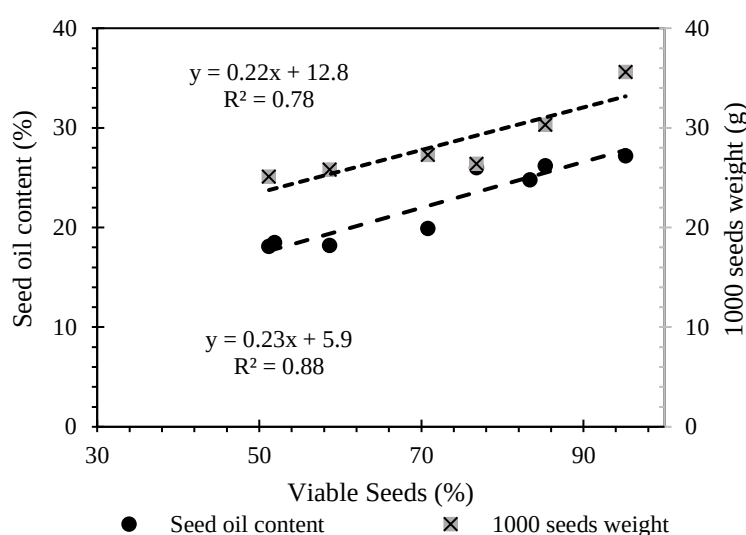


Figure 4.22. Linear relations between viable seeds and seed oil content as well 1000 seeds weight.

Figure 4.22 also shows a linear relationship between the percentage of viable seeds and the 1000 seeds weight. It justifies the hydrogravitic test used to select the viable seeds and it also agrees with the increase in their weight due to the higher content of oil on the viable seeds comparatively to the others.

One another relationship that was assessed was between the number of capitula harvested and the total amount of seeds that was possible to obtain in the cardoon plant. If this type of regression works, it may be very useful in the field to estimate the amount of seeds very quickly by counting the capitula in the plant. Figure 4.22 shows the result of this linear regression.

A high determination coefficient of $r^2 = 0.90$ was obtained through a linear regression between the biomass processed (sieved) and the amount of seeds collected. In large cultivation areas of cardoon, a sequential harvest of seeds and lignocellulosic biomass may be done, so the amount of biomass processed is very often unknown, but estimable by a regression equation such as that presented in Figure 4.23 [21].

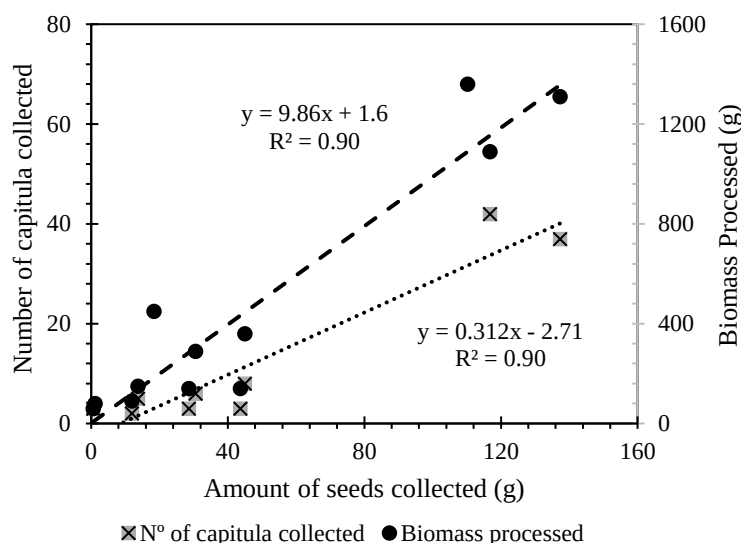


Figure 4.23. Linear relationship between the amount of seeds collected and the number of capitula collected and the amount of processed.

In the 50 m² of cardoon cultivated area studied, the total amount of seeds obtained was 943 g (in 2018), corresponding to a seeds' yield of 188.6 kg.ha⁻¹. This value is lower than that usually found in the literature for this crop (600 – 4300 kg.ha⁻¹) [4]. This low seeds' yield may be justified by several factors, namely the low agronomic inputs (irrigation and fertilization) applied, the wild variety of cardoon used and the low plant density of 8 600 plants.ha⁻¹. In the literature plant densities of 28 444 plants.ha⁻¹ are found, much higher than that, leading to highest seeds' yields of 603 kg.ha⁻¹ [21]. For a plant density of 80 000 plants.ha⁻¹, seeds' yields can achieve 2 800 kg.ha⁻¹, with a cultivated genotype, recognized well above the wild genotypes of cardoon with lower seeds' yields of 1 210 – 1 790 kg.ha⁻¹ [2].

In the literature, the studies present cardoon crop cultivated using chemical fertilization, certified seeds and with some irrigation. In our study, the cultivation of cardoon was carried out without fertilization of the soil and irrigation was only performed to guarantee the plants' survival. Cardoon seeds used belong to a wild variety, perhaps not the most suitable to maximize the seeds' yields although with other favorable characteristics, for example a superior adaption capability and resistance.

Comparing the seeds' yields between cardoon and the conventional crops cultivated for bioenergy purpose, for example, the rapeseed crops without irrigation (1 724 kg.ha⁻¹ – 2 144 kg.ha⁻¹) and the soybean crops (1 630 kg.ha⁻¹ in rainfall conditions), the values are very similar to those reported in

the literature for cardoon crops, and although not obtained in the present study for the reasons explained, it shows a reasonable potential of this specie as source of feedstock for bioenergy production [22, 23].

- **Second Capitula Harvest (Summer of 2019):**

In September of 2019 cardoon's capitula were harvested for the second time. The procedures to treat the biomass and obtain the seeds were similar to those carried out in the year 2018, referred before. Because cardoon is a perennial crop, the morphologic characteristics of the plants were expected to be similar to those obtained in 2018. For this reason, the morphologic characterization of the plants from 2019 was only evaluated for the group of ten plants already studied in 2018. The main results are in Table 4.8. It should be referred that in 2019, plant 4 and 6 (not shown in the table) did not develop any above ground biomass and in the place of plant 2 and plant 3 two new plants grew (second growth cycle).

Table 4.8. Morphologic characteristics of the group of 10 plants previously selected (second harvest)

Plant	Height (m)	Diameter (cm)	Nº capitula	Height of first ramification (m)	N.º Primary ramifications	Nº Secondary ramifications
1	1.95	3.5	11	1.38	3	4
2	1.88	6.5	15	0.98	4	8
2	1.86	4.0	10	1.12	3	3
3	1.65	1.6	5.0	1.49	2	1
3	1.60	1.9	5.0	1.50	2	3
5	1.80	1.5	3.0	1.60	2	1
7	1.30	2.0	4.0	0.71	3	3
8	1.55	1.0	3.0	1.42	2	1
9	1.65	1.6	-	1.30	2	1
10	1.80	1.5	4.0	1.64	3	1
Average	1.65	1.9	4.8	1.35	2.4	1.8

The height of these 10 plants in 2019 was lower than that verified in 2018, respectively 1.65 m to 1.85 m on average. Overall, the plants were smaller in height, diameter of the stalk and the number of capitula, differences which are mostly justified by the less favorable climatic conditions during this last year. ANOVA analysis aiming to compare the years of 2018 and 2019 in all the parameters measured, i.e., height, diameter, nº capitula, height of first ramification, number of primary ramifications, number of secondary ramifications gave results significantly different (p value < 0.01). These results were not expected since the harvested plants showed a more developed roots system in 2019 than in 2018, thus the weather conditions may be the main explanation. As already noticed,

rainfall between December of 2017 and September of 2018 was 961 mm and in an identical period between 2018 and 2019 was 590 mm. These decrease of water availability mostly during the Winter-Spring season may justify the less developed plants.

Table 4.9 presents the main results for the 10 plants of cardoon previously selected. The highest values for each parameter are highlighted in bold.

Table 4.9. Capitula's assessment (biomass and seed's characteristics) and seeds characterization (second harvest)

Biomass data				Seeds characteristics		
Plant	Seeds (g)	Seeds/biomass processed (%)	Seeds/capitula (g.capitula ⁻¹)	1000 seeds weight (g)	Seed's oil content (wt.%)	Viable seeds (%)
1	39.7	12.5	3.61	36.9	23.4 ± 0.0	65.9
2	33.1	8.6	1.32	26.0	26 ± 2	38.3
3	16.5	10.2	1.65	16.2	11.3 ± 0.5	4.50
5	10.7	6.3	3.57	24.6	27 ± 3	44.7
7	6.6	9.7	1.65	17.6	30.1 ± 0.2	13.1
8	6.1	8.2	2.02	15.1	14 ± 4	5.30
10	6.31	11.5	1.58	18.0	29 ± 2	36.1

The results suggest seeds' yields below those verified in 2018 for most of the plants. In case of the plants 4, 6 and 9 any capitula was harvested either because plants did not have it or simply because plants did not growth.

ANOVA analysis of the results gave seeds' oil content significantly different (p value <0.003) among the plants. A Tukey test identified groups of results (plants) with relevant differences (p value <0.05): plant 3 vs plant 2, 5, 7, 10; and plant 8 vs plant 5, 7, 10. Plant 3 and 8 are significantly different from the remaining plants due to their low seed oil content. Plant 8 stand outs for having the highest seed oil content determined.

The results obtained in the two consecutive years for the same 10 plants showed that the year of 2018 was more favorable for the collection of seeds. Generally, the amount of seeds obtained by capitula in 2018 was higher than the verified in 2019. Overall, in 2018, 385 g of seeds were collected in 5350 g of biomass of cardoon against 119 g collected from 1230 g (biomass) in 2019.

The characteristics of the seeds in these two years are presented and compared as follows. The "1000 seeds weight" in 2018 and 2019 was 26.9 g and 22.1 g, respectively, on average. The seed oil content was similar in 2019 and 2018, i.e., 23 ± 2 and 25 ± 4, respectively. Overall, the results found were not significantly different (p value > 0.05) through ANOVA analysis.

Viable seeds parameters were significantly different in 2018 and 2019 (p value <0.05), in the first year being 48.5%, on average, and in the second, only 29.7%. However, the values for “weight of 1000 seeds” in the years of 2018 and 2019 were not significantly different (p value >0.05).

Considering that for the 50 m² of area cultivated with cardoon and that the total amount of seeds obtained in 2019 was 480 g, a very low seeds’ yield was obtained, of 96 kg.ha⁻¹.

Despite this low value, the seeds oil content was similar in both years. Figure 4.24 shows the linear relationship between the amount of viable seeds and seeds oil content valid for a range of seeds’ oil content between 10 wt.% and 30 wt.% with a coefficient of determination near 0.60.

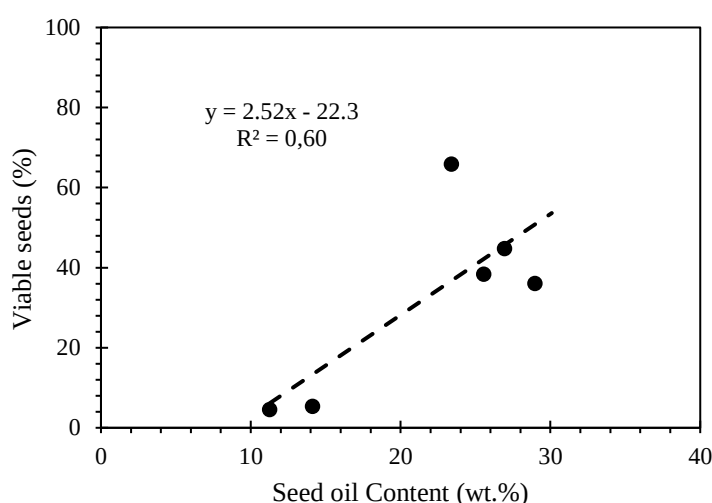


Figure 4.24. Linear correlation between viable seeds and seeds’ oil content obtained for seed collected in the second harvest.

The seeds’ oil content was on average similar in both years for the 10 cardoon plants. Despite that, there were differences in the plants in those years. For example, the oiliest plant in 2019 was plant 7 (30.1 ± 0.2 %), contrary to 2018 with 17 ± 2 % of oil, the lowest value obtained. The decrease on the viable seeds, in 2019, is a clear evidence how the rainfall can affect the plants’ development and their reproductive ability.

From the remaining population of cardoon plants, 360 g of seeds were obtained after processing 3740 g of biomass, corresponding to 231 capitula. The ratio of 9.6% for seeds/biomass is very close to the value registered in 2018 which was 10.1% for 5520 g of biomass collected. In agreement, 10 wt.% of capitula’s weight is a good estimate for seeds amount using this *Cynara cardunculus* L. ecotype. Table 4.10 shows the main results obtained for the biomass characteristics of the different sizes of capitula.

Table 4.10. Characterization of the biomass and seeds' obtained from capitula of different sizes and weights (second harvest)

Biomass data					Seeds' characteristics		
Group	Number of capitula	Seeds (g)	Seeds/biomass processed (%)	Seeds/capitula (g.capitula ⁻¹)	1000 seeds weight (g)	Seed oil content (%)	Viable seeds (%)
1	31	5.9	3.10	0.19	13.5	n.a	17.5
1*	7.0	2.8	3.40	0.40	10.8	n.a	35.0
2	37	16.2	4.80	0.44	17.5	20.1 ± 0.2	25.6
2*	8.0	16.7	11.7	2.09	17.0	25.0 ± 0.9	17.8
3	35	34.7	8.40	0.99	26.6	21.1 ± 0.1	30.6
3*	11	29.6	13.1	2.69	16.5	16.7 ± 0.1	72.9
4	65	49.1	3.90	0.76	25.3	18.8 ± 0.1	33.7
4*	10	154	46.1	15.4	25.6	19 ± 1	27.0
5	10	4.5	2.00	0.45	22.4	21 ± 3	26.3
5*	5.0	10.2	6.20	2.04	21.9	24 ± 6	14.3
6	9.0	16.4	6.70	1.82	23.7	15.5 ± 0.3	13.7
6*	3.0	21.0	16.7	7.01	23.6	19 ± 2	4.0

n.a. – not available (the amount of seeds collected did not allow the performing of Soxhlet extraction);

According to the results, for the heavier capitula (*) the amount of seeds obtained per capitula was higher than that obtained for the capitula with regular weight, regardless the size of the capitula (groups). This fact shows that increasing capitula's weight (size) increases seeds content, as previously observed (Table 4.7). Figure 4.25 depicts seed yields in 2018 and 2019.

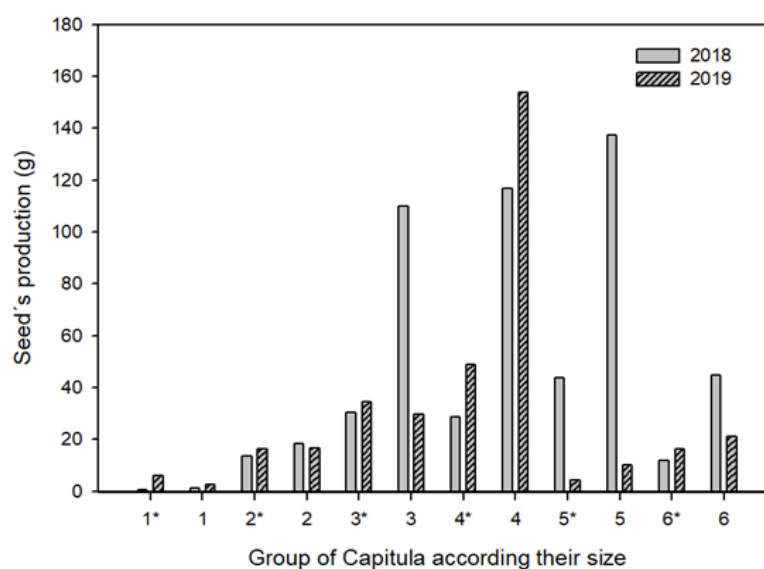


Figure 4.25. Seeds yields per capitula type, obtained in 2018 and 2019 (* heavier capitula).

As referred before, in 2019 there was less capitula harvested and consequently the amount of seeds decreased. In the two seasons, the largest differences were verified in groups 3, 5 and 5*.

In both years (2018 and 2019), the increase in capitulas' size corresponded to the increase in the weight. Concerning the seeds/biomass ratio in Figure 4.26, the results were very similar in both years for groups 1, 2*, 2, 3*, 3, 4 and 6*. The biggest differences were verified in group 5*, because the seeds collected in 2019 corresponded only to 10 % of the total collected in 2018. For group 4*, the decrease in the seeds/biomass ratio was mostly due to the higher amount of biomass processed which was not followed by the increase in the seeds' collected. Overall, these results indicate that in 2019, the growth of cardoon was affected, which was reflected in low yields.

Viable seeds, on average, suffered an abrupt reduction from 72% in 2018 to 27% in 2019, difference considered significant by ANOVA analysis (p value < 0.05). The 1000 seeds weight parameter also decreased from 30.2 g in 2018 until 20.4 g in 2019.

Seed oil content seems similar in average in both years, respectively 22 ± 4 for 2018 and 20 ± 3 for 2019, conclusion confirmed by ANOVA analysis and also for the 1000 seeds weight (p value > 0.05).

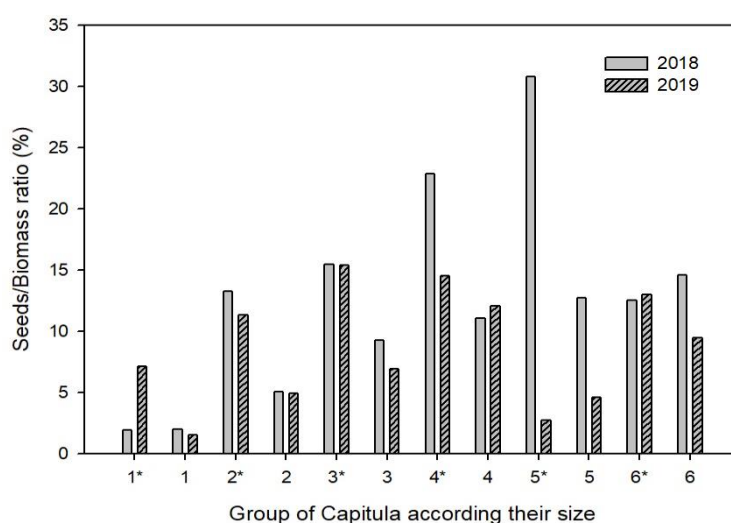


Figure 4.26. Seeds' yields obtained in 2018 and 2019.

Seeds' oil content was however significantly different from ANOVA analysis in capitula's group 2 and 3, as indicated by $p < 0.05$, contrary to the other groups having capitula with regular and highest weight (p value > 0.05).

In 2019, the seeds' oil content of 23 ± 2 for the group with the 10 selected plants and the remaining cardoon population with 20 ± 3 do not differ significantly, therefore it is possible to conclude that cutting the flower did not seem to affect the oil content in the seeds. This was true in 2019 as well as in the year before. ANOVA analysis also confirmed that seeds' oil in the population from the

sample of 10 plants with flowers cutting it was not significantly different from the remaining cardoon plants population (p value > 0.05). The same conclusion was obtained for the percentage of viable seeds as well as for the 1000 seeds weight (p value > 0.05).

According to all these results it is possible to conclude that cutting off the flowers didn't affect the characteristics of the seeds, i.e., seeds oil content, % of viable seeds, 1000 seeds weight. In the two years studied the seeds' oil content and the 1000 seeds weight did not vary significantly. The changes in the climatic conditions in those years were mostly reflected in terms of biomass and seeds yields (crop development).

4.4.2 *Cynara cardunculus* L. oil properties

The *Cynara cardunculus* oil properties were studied in six different ecotypes, one of them the seeds collected from our field study (sample G – first harvest – 2018). Figure 4.27 shows the aspect of some of the seeds' samples used.

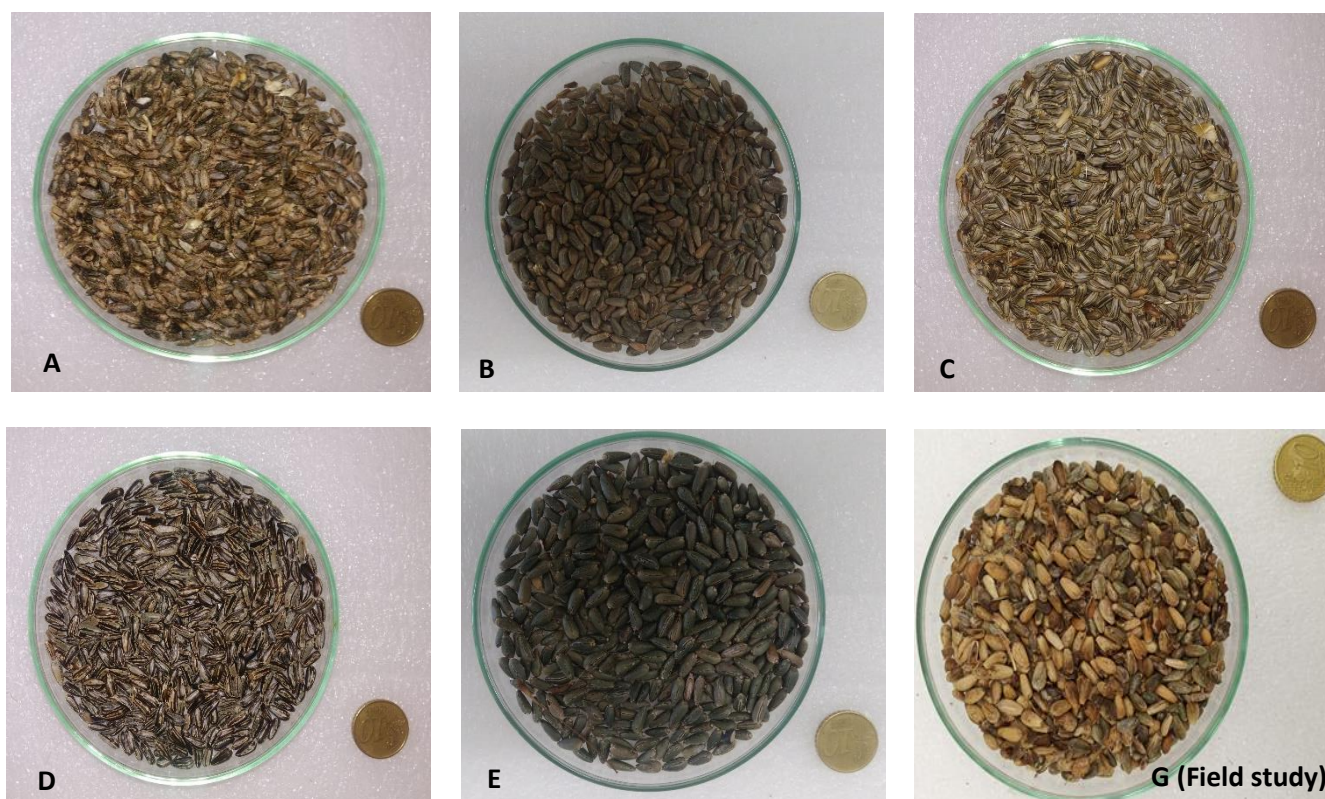


Figure 4.27. Morphologic aspect of seeds obtained from different ecotypes studied (A,B,C,D,E,G).

It might be observed that the main difference by naked-eye is the different color from the six ecotypes shown, whose shape and size does not appear to be of much difference.

However, Table 4.11 summarizes the seeds' properties and oil characteristics obtained from each sample.

Table 4.11. Seeds' characteristics and oil's properties obtained for the different ecotype

Seeds' Properties				Oil's Properties	
Variety	1000 seeds weight (g)	Seeds moisture content (%)	Seed oil content (wt.%)	Acid value (mgKOH.g ⁻¹)	Oxidation stability (h)
A	29.97 ± 0.02	6.1 ± 0.2	24.1 ± 0.1	5.02 ± 0.01	-
B	40.37 ± 0.04	6.4 ± 0.2	24.9 ± 0.1	3.8 ± 0.5	2.0 ± 0.2
C	41.18 ± 0.04	5.9 ± 0.1	23.8 ± 0.1	5.20 ± 0.01	3.6 ± 0.2
D	47.46 ± 0.03	5.7 ± 0.0	25.3 ± 0.6	4.10 ± 0.02	3.2 ± 0.2
E	53.07 ± 0.01	5.9 ± 0.0	25.2 ± 0.2	4.3 ± 0.4	3.6 ± 0.5
F	-	6.8 ± 0.0	23 ± 1	2.6 ± 0.3	2.3 ± 0.2
G	27.5 ± 0.8	-	23 ± 4	21.2 ± 0.1	1.6 ± 0.3
Sample Factor	**	**	ns	**	**

Results between all samples significantly different ** $p \leq 0.01$ (ANOVA). ns – Not significant.

The 1000 seeds weight parameter varied from 27.5 g up to 53.1 g, a range slightly higher than the 20 – 39 g referred in literature [21, 24]. ANOVA analysis confirmed that significant differences ($p < 0.01$) existed and the Tukey test identified as groups significantly different the following: A vs E, D vs G and E vs G.

The seeds moisture varied between 5.7% and 6.8%, characteristic that in the literature can oscillate between 5.6% and 11% [2, 4]. The moisture is related with the cardoon ecotype but also with weather conditions in the harvesting year [25]. ANOVA analysis confirmed significant differences in moisture in the results from the same period ($p \text{ value} \leq 0.01$) and Tukey test gave $p \text{ value} < 0.05$ comparing A vs F, B vs D, C vs F, D vs F and E vs F.

Seeds oil content varied between 23.2 wt.% and 24.9 wt.% which is in agreement with the values reported in several studies for cardoon crop (domestic and wild type) [14, 15, 26]. ANOVA analysis showed that the type of seeds didn't influence the amount of oil found in them ($p \text{ value} > 0.05$).

Compared to the conventional feedstocks used in Portugal for biodiesel production, i.e., soybean and rapeseed, it is possible to conclude that cardoon oil content is similar to soybean seeds (18 – 22 wt.%) and less than rapeseeds (37 – 50 wt.%) [27]. Besides the amount produced it does matter the quality of the oil that must be evaluated to guarantee the viability of biodiesel production.

Conventionally, biodiesel production is carried out in presence of an alkaline homogenous catalyst, but for that the feedstocks should have an acidity lower than 1 wt.% [28, 29]. Due to this reason,

the acid value was determined in the oils from the different ecotypes. The oil from seeds collected in our agronomic field had a high acid value, of 21.2 mg KOH.g⁻¹. ANOVA analysis gave significant differences in the results (*p value* < 0.01), mostly verified between sample G (wild variety) and the others (A, B, C, D, E, F), as expected. Also, significant differences were verified between oils from samples A vs F, C vs F and E vs F.

The oxidation stability of the oils is related with oil's fatty acid composition and with the presence of natural antioxidants such as tocopherols and carotenes. This parameter is essential to predict the stability of the biodiesel produced. Factors such as soil composition, climatic conditions, irrigation and degree of seeds' maturation might influence the stability of the oils [30]. According to the results in Table 4.11, the oxidation stability of the cardoon crude oil varied between 1.6 h and 3.6 h. ANOVA analysis showed results significantly different (*p value* < 0.01). The differences were mostly verified between sample G and the others (A, B, C, D, E, F). In the literature no information was found related with the cardoon oil's oxidation stability. When compared with the oxidation stability for soybean crude oil (3.6 h) or rapeseed crude oil (4.5 h) it is possible to verify that cardoon oil had lower oxidation stability [31, 32]. According to EN 14214 a commercial biodiesel should have at least an oxidation stability of 6 h, for this reason, it is expected that biodiesel produced from this type of cardoon oil will require the addition of antioxidants, improving oxidation stability until acceptable levels.

The total glycerol, monoglyceride, diglyceride and triglyceride were determined for sample F (cultivated variety) and sample G (wild variety). In Table 4.12 are the results obtained.

Table 4.12. Monoglyceride, diglyceride and triglyceride in samples F and G

Variety	Mono- (wt.%)	Di- (wt.%)	Tri- (wt.%)	Total (wt.%)
F	1.19 ± 0.02	2.255 ± 0.006	79.8 ± 0.03	83.3
G	1.181 ± 0.008	2.43 ± 0.04	65.12 ± 0.004	68.7

For both samples, no glycerol was detected (as expected) and the amount of monoglycerides and diglycerides was similar. According to the results, the major difference between oils' composition was identified for triglycerides concentration, lower in the oil obtained from seeds of ecotype G. Generally, it is possible to identify several different compounds in the oils, like free fatty acids, glycerol, monoglycerides, diglycerides, triglycerides, phospholipids, soaps, water and impurities. Aiming biodiesel production, the oils used as feedstock, should be mostly constituted by mono-, di-

and triglycerides. For this reason, the oil obtained from seeds of ecotype F is the most suitable for biodiesel production due to their high content in glycerides (83 wt.%) and low free fatty acid content (2.6 mg KOH.g⁻¹).

For the *Cynara cardunculus* L. oil, few information was found about its composition. Only in the study developed by Pasqualino [33] monoglyceride and diglyceride concentration were determined in unrefined oil. According this study, unrefined Cynara's oil showed monoglyceride and diglyceride content of 0.08 wt.% and 2.78 wt.%, respectively. According to Wassner *et al.* [34], mono- and diglycerides concentration were affected by the seeds' harvest date. Also, the conditions of storage, the age of the oil, the presence of water and *in-situ* lipases can influence oil's composition [35].

The difference of triglycerides concentration verified between sample G and F should be mostly related with the variety of the seeds (cultivated vs wild). Seeds from ecotype G probably had low germination ability (brown color – Figure 4.27).

Table 4.13 presents a supplementary determination of oil's characteristics in samples F and G (refractive index, density, iodine value and peroxide value).

Table 4.13. Oil's characteristics from samples F and G

Variety	Refractive Index	Density (g.cm ⁻³)	Iodine value (g I ₂ /100g)	Peroxide value (m _{eq} O ₂ /kg)
F	1.4735	0,9159 ± 0,000	120.49 ± 0.03	5.116 ± 0.007
G	1.4726	0,9164 ± 0,002	118.4 ± 0.6	6.1359 ± 0.0006

According to the results it is possible to confirm that the refractive index is different in the two samples. Refractive index measures the velocity that the light goes through some medium, being a measure of the colors of the different samples (dimensionless parameter). Lower values are obtained in lighter and clearer samples. According to the results, light travels faster in a sample of oil from variety G than F (refractive index and speed of the light in the medium are inversely proportional), showing that the first presents a lighter color. Refractive index may also varies according the acid chain length and the degree of unsaturation [36]. For biodiesel production the common edible oils used as feedstock have similar refractive index than that obtained for sample G (sunflower oil – 1.4748; soybean oil – 1.4671 and rapeseed oil – 1.4750 [37-39]).

The results showed densities of cardoon oil's very similar. According to Bassam [40] cardoon's oil showed a density of 0.916 g.cm^3 . The density is also similar to that found for other oils (soybean, sunflower) [41, 42].

The iodine value of cardoon's oil was around $120 \text{ g I}_2/100 \text{ g}$, being lower than that reported for sunflower oil ($127 \text{ g I}_2/100 \text{ g}$) or soybean oil ($129 \text{ g I}_2/100 \text{ g}$) [43]. The iodine value is closely related with oil composition, that measures the presence of unsaturated fatty acids.

For the peroxide value parameter, the results suggested a small difference ($1 \text{ m}_{\text{eq}}\text{O}_2/\text{kg}$) between the two types of oils. This parameter is used as an indicator of the early stages of oil's oxidation, since peroxides are primary oxidation products [44]. Thus, the oil from ecotype F showed a lower oxidation degree, in agreement with the oxidation stability previously determined, although in this case the values were more similar. According to Tan *et al.* [45], sunflower oil, rapeseed oil and soybean oil showed peroxide values of $9.1 \text{ m}_{\text{eq}} \text{O}_2/\text{kg}$, $6.68 \text{ m}_{\text{eq}} \text{O}_2/\text{kg}$ and $2.4 \text{ m}_{\text{eq}} \text{O}_2/\text{kg}$, respectively [45]. In agreement, cardoon oil showed a peroxide value in the range of other oils conventionally used as feedstock for biodiesel production.

Comparing the characteristics of cardoon oil (obtained from wild variety) with crambe oil (other alternative oil cultivated and studied – section 3.3), it is possible to verify that both types of oils had similar density, although crude crambe's oil acidity ($0.61 \text{ mg KOH.g}^{-1}$) and iodine value ($89 \text{ g I}_2/100 \text{ g}$) were lower than the values verified for cardoon's oil [29]. Meaning, crambe oil showed a considerably lower content in unsaturated fatty acids (low iodine value) and in free fatty acids (low acid value) when compared with cardoon's oil from the wild variety. From the cultivation of crambe in Portugal, seeds had oil content of $26 \pm 4 \text{ wt.}\%$, similar to value obtained for cardoon's seeds ($24.2 \pm 0.8 \text{ wt.}\%$) [29]. Figure 4.28 shows the shape, size and color of cardoon and crambe seeds. Cardoon seeds typically have oval shape and a dark color and crambe seeds show spherical shape and a brown color (which suggest that the seeds completed their maturation). Cardoon seeds are bigger (3.2 to 7.5 mm) than crambe's seeds (0.8 to 2.6 mm).

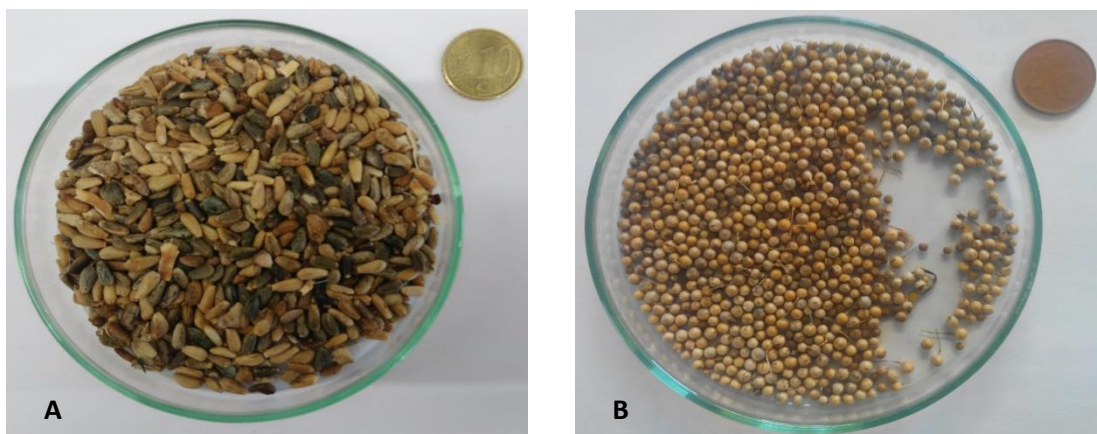


Figure 4.28. Naked-eye observation of *Cynara cardunculus* L. (A) and *Crambe abyssinica* (B) seeds.

4.4.3 Transesterification of *Cynara cardunculus* L. oil

Table 4.14 shows the FAME content obtained at the product resulting from cardoon oil transesterification (cultivated and wild variety).

Table 4.14. FAME content for the final product obtained by transesterification using oil obtained from seeds of variety F and G

Variety	FAME Content (wt.%)
F	88.3 ± 0.4
G	86.6 ± 0.3

By alkaline transesterification (oil from variety F) was possible to produce a biodiesel with high purity, although with the FAME content was lower than the require by the standard EN 14214 (96.5 wt.%). Due to the high content of FFA, oil from seeds with variety G was subjected to enzymatic transesterification for biodiesel production with a purity of 86.6 wt.%. Enzymatic transesterification is a heterogeneous catalyzed process and thus avoids soaps formation and wastewater production. Using crambe oil (see section 3.3) as feedstock for biodiesel production, it was possible to obtain a FAME content of 96 ± 1 wt.% by alkaline transesterification reaction under the same experimental conditions performed with cardoon oil.

Figure 4.29 shows the biodiesel produced from cultivated (F) and wild (G) varieties of cardoon where it is clear the color difference between the biodiesel samples.

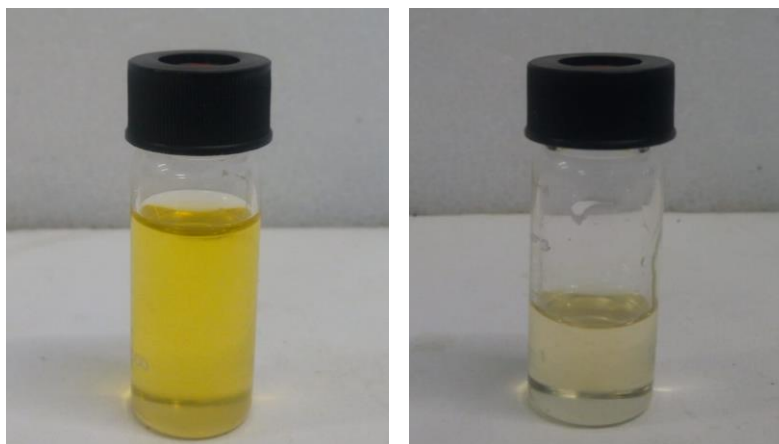


Figure 4.29. Biodiesel obtained by seeds of variety F (left) and G (right).

The fatty acid profile of the oils is usually predicted from the resultant methyl esters profile of biodiesel, which is shown in Table 4.15.

Table 4.15. Fatty acid methyl esters profile in biodiesel obtained from *Cynara cardunculus* L.: cultivated (G) and wild (F) varieties

Methyl ester of the following fatty acids	Palmitic (C16:0)	Stearic (C18:0)	Oleic (C18:1)	Linoleic (C18:2)	Others (<2 wt% each)
Biodiesel from sample oil F	10.5 ^a	2.5 ^a	21.1 ^a	66.0 ^a	-
Biodiesel from sample oil G	12.0 ^b	3.3 ^b	20.1 ^b	61.8 ^b	2.8
[46]	11 - 14	3 - 4	25	56 - 60	-

^{a,b}, Mean values with different letters in the same column vary significantly ($p \leq 0.01$).

According the results in Table 4.15, the linoleic and linolenic acid methyl esters concentration are slightly different of the values reported in the literature [46]. Cardoon's oil is mostly constituted by linoleic acid (> 60 wt.%) being the composition very similar to both the sunflower oil and the soybean oil [47, 48]. Linoleic acid is an omega-6 fatty acid, essential for human diet [14]. By enzymatic transesterification, it was possible to identify other fatty acids, with concentration below 2 wt.% (in terms of FAME), namely eicosanoic (C20:0), docosanoic (C22:0) and docosenoic (C22:1) acids.

Iodine value was determined in the samples of biodiesel produced according the Annex B of EN 14214. For biodiesel produced from the varieties F and G, iodine value calculated was 132 g I₂/100 g and 125 g I₂/100 g, respectively. These values are slightly higher than the determined before by titration methods. The biodiesel obtained from the cultivated variety (F) had the higher iodine value, being this result in agreement with the concluded before for cardoon's crude oil. For biodiesel

produced from crambe seeds collected in Portugal, iodine value calculated was 89 g I₂/100 g. As for the oil, the iodine value reflects the degree of unsaturation of biodiesel. According EN 14214, the maximum iodine value admissible in a commercial biodiesel is 120 g I₂/100 g. The presence of methyl fatty acids with double bonds can lead to polymerization of glycerides by heating (internal combustion engine). For biodiesel produced from cardoon's oil, iodine value was higher than threshold value imposed by EN 14214. In the study developed by Pasqualino [33], biodiesel of cardoon's oil showed an iodine value of 123.7 g I₂/100 g.

The linolenic acid methyl ester content (C18:3) indicates the degree of instability of the biodiesel, because this polyunsaturated fatty acid is very oxidizable when exposed to the light or oxygen. In case of the biodiesel produced from cardoon's oil, linolenic acid methyl ester was not detected. In the literature is reported that biodiesel produced from cardoon's oil does not have linolenic acid methyl ester [33, 46]. The maximum admissible linolenic content in biodiesel according to EN 14214 is 12 wt.%.

With the composition profile of biodiesel is possible to calculate the molecular weight of the cardoon's oil. This value is important for biodiesel production because it allows to calculate, more accurately, the amount of reagent necessary in the transesterification reaction. For cardoon's oil obtained from varieties F and G, the molecular weight (determined according to section 3.3) was 873.4 g.mol⁻¹ and 878.6 g.mol⁻¹, respectively. In the study developed by Pasqualino [33] using a wild *Cynara cardunculus* L., the oil's molecular weight obtained was 873.2 g.mol⁻¹, value in the range of the found for oil from varieties F and G.

4.5 CONCLUSIONS

This study showed that *Cynara cardunculus* might be cultivated in Portugal aiming at seeds production. The seeds showed a relatively high oil content and seeds' yields obtained were close to 189 kg.ha⁻¹. The scale-up of cardoon production also depends on the integrated use of all the cardoon biomass resources. The seeds' yields found was lower than the usually found in the literature for this crop ranging from 600 to 4300 kg.ha⁻¹. The low seeds' yields obtained can be justified by several factors, namely the low agronomic inputs (irrigation and fertilization) used, the wild variety used and low plant density employed (8 600 plants.ha⁻¹).

For seed oil content, no significant differences were found between the results obtained in 2019 (23 ± 2 wt.%) and 2018 (25 ± 4 wt.%). Linear relationship ($r^2 = 0.58$) between capitula's weight and seeds'

oil content (inversely proportional); and, between seeds' oil content and the percentage of viable seeds ($r^2 = 0.88$, directly proportional) were established. Overall, the results suggested that for the wild *Cynara cardunculus* L. ecotype, 10 wt.% of capitula's weight corresponds to seeds.

Comparing the results in the two consecutive years, 2018 was more favorable for the collection of seeds. Generally, in this year, the amount of seeds obtained by capitula was higher than that verified in 2019. The decrease of water availability mostly during the Winter-Spring season might justify the lower yields obtained in 2019.

Viable seeds seem to be the parameter that best reflects the climatic variation verified in the years (2018 - 48.5%; 2019 – 29.7%).

Based on the results in both years, it was possible to conclude, that the cutting off the flowers does not affect seeds oil content parameter and the 1000 seeds weight.

Cardoon's ecotype did not influence seeds' oil content (p value > 0.05), contrary to the other parameters evaluated (1000 seeds weight, moisture content, acid value, oxidation stability) for which cardoon's ecotype influenced significantly the results (p value < 0.05).

Despite the relatively low seeds' yield obtained, this crop may be considered an alternative source of oil for biodiesel production, since the oil content of the seeds is similar to that of soybean seeds (18 – 22 wt.%), a common source of oil for biodiesel production. The plants density and irrigation adopted, variables to be improved in other experimental fields, may be decisive in increasing the yields of this crop under different weather conditions.

Biodiesel produced with *Cynara cardunculus* L. oil showed high purity (FAME content > 86 wt.%), although less than the minimum limit imposed by EN 14214 (96.5 wt.%). Similar results were obtained using a homogeneous and a heterogeneous catalyzed process. Generally, biodiesel obtained from cardoon oil showed high level of unsaturated fatty acids (high iodine value), being expected to have low oxidation stability.

The work carried out, particularly the agronomic assessment, has shown that to cultivate this crop low inputs are required, at least when compared with other oilseed crops, and to maximize seeds' yields a cultivated variety should be used.

The biodiesel produced using cardoon oil is expected to be technically viable and it may constitute a promising complementary alternative to the conventional edible oils generally used for biodiesel production in Portugal.

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CHAPTER 5 *CRAMBE ABYSSINICA* AND *CYNARA CARDUNCULUS* *CROPS – NUTRITIONAL ANALYSIS OF BIOMASS WASTES*

ABSTRACT

Cynara cardunculus and *Crambe abyssinica* are crops referenced in the literature as having high potential for renewable energy production. A comprehensive study regarding the nutritional value of the seeds and biomass wastes (press cake and meal, biomass from capitula and straw) was developed. Cardoon and crambe were cultivated during three consecutive years in Portugal. From the collection of seeds, several biomass wastes are obtained, namely, straw and biomass from capitula. Both from the chemical and mechanical oil extraction results a solid product. Biomass waste presented a protein content between 6.6 wt.% and 7.2 wt.%. Crambe's straw presented a low protein content (3.85 wt.%) and a high content of fibers, being the energetic value of this material determined as 301 kcal/100g dw. The meal and press cake from oil extraction showed a higher protein content (25 wt.%). Typically, the optimum level of protein for animal feed is in the range between 16 wt.% and 22 wt.%. Considering that, it was concluded that crambe meal, crambe press cake and cardoon meal have a high potential to be used for animal feed. The assessment of the nutritional value showed that the different biomass coproducts are rich in protein, oil and fibers, having potential to be used as animal feed. Although, for future studies, it is also very important to assess the presence of antinutrients and toxic compounds.

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5.1 SCIENTIFIC RELEVANCE

As for any economic activity, the agricultural sector generates by-products and wastes. From agricultural activities, organic wastes are predominant, including manure, plant waste (crop straw) and wastes from further plant processing (skins, husk) [1]. Some of those materials, when not properly managed (e.g. burning practices and uncontrolled biological degradation), cause environmental problems related to the release of CH₄ (manure decomposition), N₂O, SO₂ and smoke (e.g. agricultural waste burning) [1, 2]. In the last 50 years, the agricultural production increased three times [3]. Such increase of productivity led to an expansion of the soil's area for agricultural use, creating environmental pressure and thus causing relevant impacts [3, 4]. The agricultural sector is responsible for 21% of GHG emissions and for this reason it is essential to create a new management model, more sustainable and environmentally friendly [5]. As an example, in China (2013) the production of agricultural wastes was 1.75×10⁹ ton and the total waste production is expected to increase at a rate of 5 – 10% a year [2].

Beside the environmental problems, economic losses associated to the traditional disposal should also be considered, mostly in the developing countries. The exposure to air pollution is estimated to represent welfare losses of 5.11 trillion dollars (in the world) [1]. For example, in India, the welfare losses are estimated as 7.69% of the gross domestic product (GDP) [1]. The improper management of straw, which is conventionally burned in China (open air), is estimated to cause an economic loss corresponding to 0.14% of the GDP [6].

Most of the agricultural wastes correspond to biomass, with an estimated world annual production of 100 billion metric tons [2, 7]. Biomass waste comprises different types of materials like forestry residues, crop straw or fruit processing waste [7]. According to the Directive 2009/28/CE, biomass refers to the biodegradable fraction of products, waste or residues of biological origin from forestry activity, agriculture activity and others (excluding organic materials such as fossil fuels). In Europe, 250 Mt.year⁻¹ of agricultural waste are produced [8].

Such materials are considered of low-value and thus not widely used; however, alternatives should be developed to make economically feasible their recovery [9]. In fact, biomass biorefineries can produce more compounds than the refineries based on petroleum [9]. The recovery of the agricultural waste should thus be further developed to contribute to the economic development, also reducing the related environmental impacts.

The sustainable production of chemicals and products is an industrial key issue; thus, biomass waste might be introduced as a resource for the production of value-added products. Figure 5.1 shows the different value-added products (of high economic value) obtained from biomass waste (biomass biorefineries).

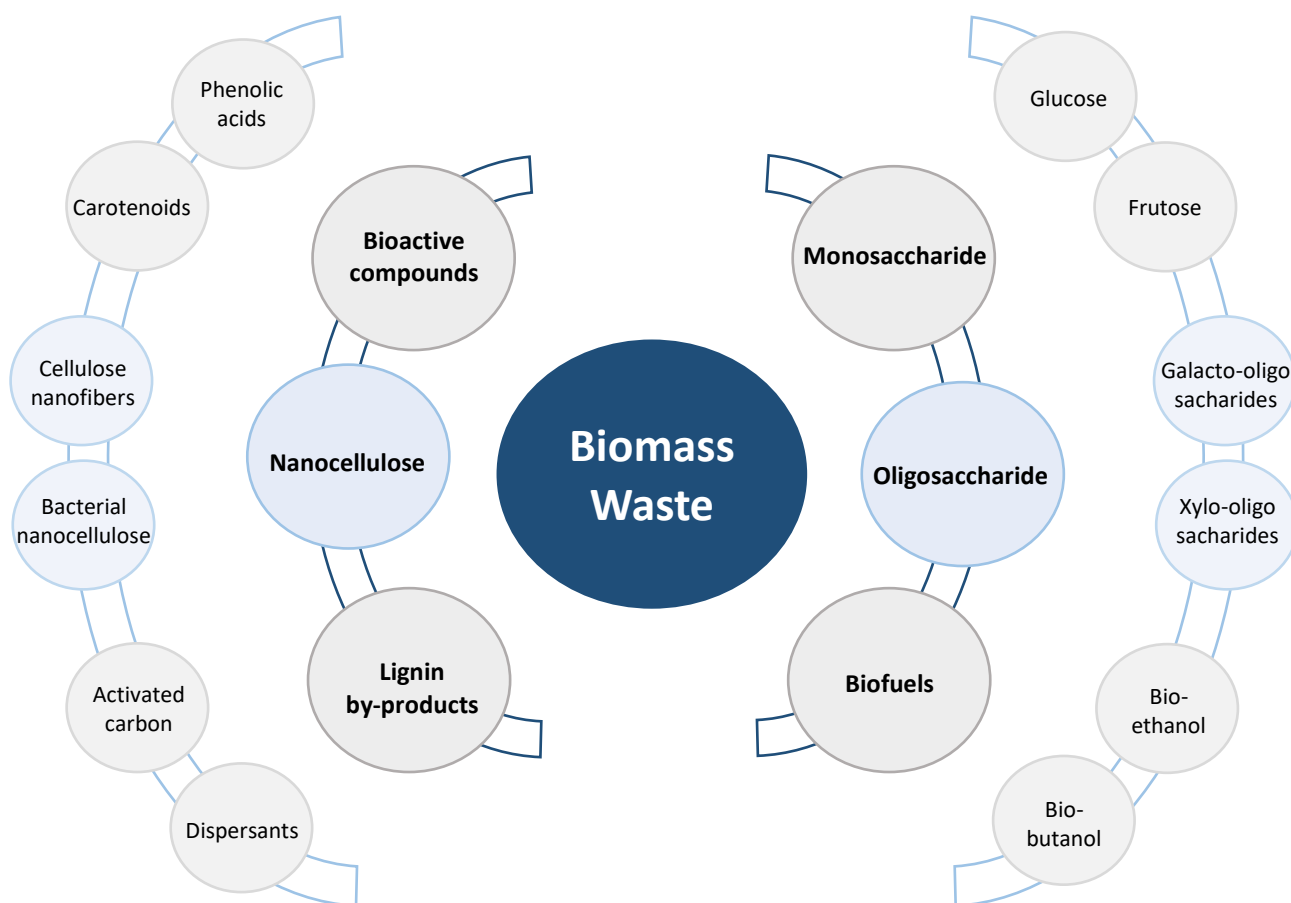


Figure 5.1. Products with economic interest obtained from biomass waste recovery – adapted from [7].

Nowadays, most of the biomass wastes are used as feedstock for bioenergy production [8, 10]. Generally, the most used biomass wastes are the ones that result from woodworking, forest activities and farms which are burned in power plants [8]. Biomass wastes can also be used as raw-materials for building products, like ceramic bricks (incorporation until 5 wt.% of waste) [8]. From the agricultural sector, the most abundant residues, estimated for 2013, were rice straw (1119 Mt), corn straw (1016 Mt), wheat straw (1069 Mt), sugarcane bagasse (563 Mt) and soybean residues (276 Mt) [8, 11]. Forestry and food wastes (citrus waste, grape pomace, apple pomace, olive

pomace) and spent coffee grounds are types of biomass waste produced in considerable amounts [7].

Forestry wastes have high contents of cellulose and hemicellulose, suitable to produce ethanol or butanol from the fermentable sugars. Food waste resulting from the fruits and vegetables processing evidence also high cellulose/hemicellulose content. In spent coffee grounds it is possible to find a high fraction of hemicellulose (30.1%) being mannan (polysaccharide, 19.3 wt.%) an excellent source for the production of valuable mannose (food supplement) [12]. In sugarcane bagasse, rice straw, wheat straw or other wastes it is possible to identify xylo-oligosaccharides, polymers used for several purposes, namely, as food ingredient, for pharmaceutical application or yield enhancer in the agriculture [13]. Summing up, several compounds can be recovered from biomass wastes, like flavonoids, phenolic acids or carotenoids, which are bioactive compounds that exhibits antioxidant, anti-inflammatory, antimicrobial and antiallergenic activities with application in several industries (pharmaceutical or cosmetic industry) [14].

Typically, wood wastes and agricultural residues are constituted by lignin, which is the second most abundant natural polymer [7]. Economic studies showed that lignin recovered from these wastes leads to an economic profit 10 times higher than that obtained using the wood/agricultural residues for burning (energy or steam production) [15]. In fact, from lignin, it is possible to produce value-added chemicals like vanillin, vanillic acid, catechol or muconic acid (chemical compounds) [15].

Some biomass wastes can be considered as nonconventional feedstocks for animal feed, used directly or indirectly (as an ingredient) [16]. The use of these substances for animal feed requires studies on their nutritional value. The studies should analyze the digestibility and feed value (nutritional) [16]. One relevant aspect is that a small improvement in animal feeding can represent a considerable economic improve at a farming level, because animal feed is expensive, representing almost 70% of the total costs with animal production [16].

Nonconventional animal food corresponds to the materials that are not used for animal feeding or incorporated in commercially prepared meals [16]. These alternatives are mostly constituted by wastes from perennial crops and the food's processing (for human consumption) [17]. For example, from seed's processing (milling industry) several wastes/by-products like bran, waste flour or seed's hulls are obtained [16]. From the oil industry, cakes from solvent extraction (meal) and from mechanical extraction (press cake), gums and other products produced during oil's refining step (lecithin and fatty acids) have high potential to be used for the production of animal feed [16].

With the crops growth, the stems and leaves can be used for animal feed (cattle feed) due to their high contents of digestible proteins [18]. However, the use of these wastes for animal feed depends on several factors, such as protein concentration, digestibility, fats, carbohydrate content, fiber content and presence of toxic substances (antinutritional factor) [16]. Usually, the ruminants can tolerate higher concentrations of toxic compounds when compared with nonruminants animals. There are several methodologies (detoxification processes) that allow to minimize the effect of toxins, consequently increasing the nutritional value of the residue [16].

Aiming to evaluate the feasibility of using biomass wastes for animal feed, a nutritional characterization was performed to quantify the different macronutrients (water, fats, carbohydrates, proteins) and micronutrients (minerals, vitamins and fibers). These compounds are essential for the metabolism and survival of the organisms. Figure 5.2 presents the parameters analyzed (green boxes).

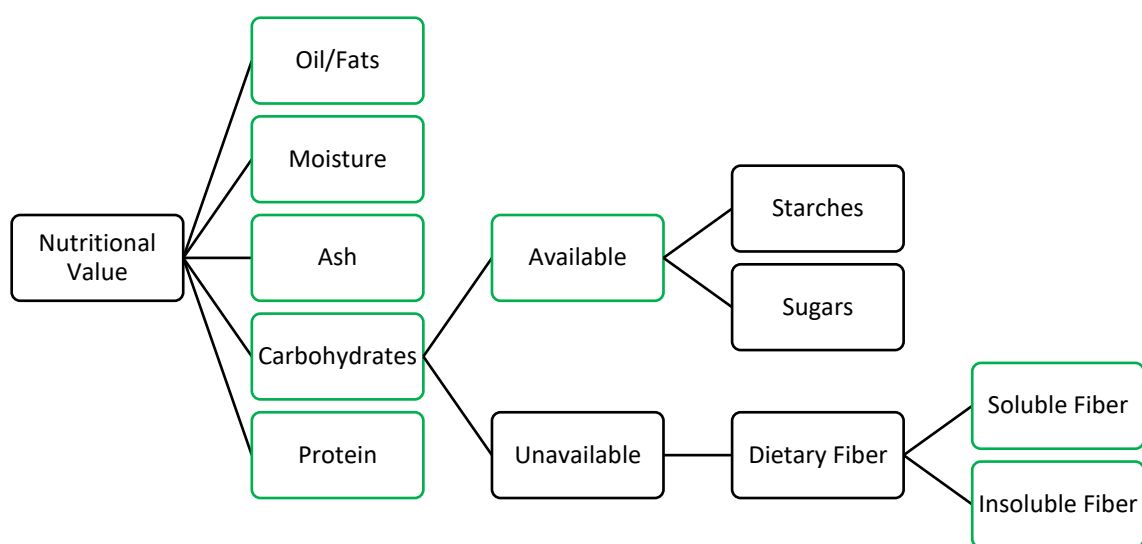


Figure 5.2. Classification of the different macronutrients and micronutrients evaluated in the present study (nutritional value assessment).

Proteins are essential for the organism activity, being composed by amino acids which contain carbon, hydrogen, oxygen and nitrogen, being known as the main source of the last [19]. The synthesis of proteins is possible due to the natural plant fixation of nitrates and ammonium from the soil (natural nitrogen cycle) [19]. Proteins are the main constituent of the muscles, corresponding to 17% of the human body, being essential for tissue building and an important source of energy (1 g of food protein = 4 calories) [19].

By photosynthesis, the plants can produce carbohydrates using water (from the soil) and carbon dioxide (from the air) [19]. Carbohydrates are constituted by oxygen, carbon and hydrogen. The main function of carbohydrates is to supply energy to the body. The simple carbohydrates (small molecules) consist in mono- (glucose, fructose, galactose) and disaccharides (maltose, lactose, sucrose), easily absorbed by the body (digestible) [19]. On the other hand, fibers (other type of carbohydrates) are considered large and complex molecules which contain several hundred of small sugar units which are slowly absorbed [19]. Polysaccharides are considered complex carbohydrates, being some of them classified as indigestible (cellulose, hemicelluloses, lignin) [19]. When ingested in excess, carbohydrates are converted into fats or storage as glycogen, being mobilized when the body needs energy [19]. Pectin, cellulose and hemicellulose are substances present in the skins of fruits, in the coverings of the seeds and in the structural parts of edible plants, being usually called “fiber”. Some parts of the plants which are indigestible (not digested by the enzymes of the intestinal tract) are entitled of “dietary fibers” [19]. For the human body, the enzymes present in the intestine are not able to break the dietary fibers, although these indigestible substances are useful to eliminate intestinal wastes and stimulate gastrointestinal movements [19].

The dietary fibers are usually divided in two types: insoluble and soluble fibers. Insoluble fibers require an unusual and consequently improved food chewing. These fibers absorb water, increasing their size and consequently the surface area for enzymes action, improving the digestion, and also create a feeling of fullness [19]. Soluble fiber binds bile acids and cholesterol and helps to excrete these substances [19].

Usually, the carbohydrates can be classified in two groups: “available” and “unavailable”. The available carbohydrates correspond to those which are digested and absorbed by the intestine (sugars for example). The unavailable carbohydrates are digestion-resistant nutrients [20, 21]. As consequence, dietary fibers correspond to the unavailable fraction of carbohydrates.

Fats belong to an important chemical group called lipids. They are a very important component of the body (human or animal), protecting the vital organs (brain, heart, liver) of injuries [19]. Lipids composition is similar to the carbohydrates (carbon, hydrogen and oxygen). They also can contain phosphorus and nitrogen. Lipids are a very relevant form of stored energy and the energy released per g of lipid is higher than that released by carbohydrates (around 2.25 times) [19].

Water is the last macronutrient described, although it can be considered the most important (a few days without water can lead to death). The presence of water is essential for cells/body and water

content vary among the different tissues. Brain, liver or muscles have an higher water content than bones or fat tissue [19]. Water allows the transport (universal solvent), inside of the body, of several substances, participating in chemical reactions and allowing body's temperature control [19]. This nutrient is also important to excrete soluble metabolic wastes [19].

Considering the aspects previously referred, a nutritional value assessment of biomass wastes was performed. The biomass wastes studied result from the cultivation and oil's extraction of *Cynara cardunculus* L. and *Crambe abyssinica* crops. For each crop, different structural parts of the plant were studied. The main objective of this work was to perform a preliminary assessment of the potential of the biomass wastes, from these two crops, for animal feed.

5.2 SPECIFIC OBJECTIVES

The following specific objectives were established:

- To evaluate the macronutrients content (proteins, carbohydrates, water and fats) present in different structural parts of by-products and wastes resulting from *Cynara cardunculus* L. and *Crambe abyssinica* crops.
- To evaluate the ash content, insoluble fiber and fiber content present in selected samples.
- To compare the results with the literature and conclude about the potential of such products for animal feed.

5.3 MATERIALS AND METHODS

5.3.1 MATERIALS

Cynara cardunculus L. and *Crambe abyssinica* crops were cultivated in Portugal to obtain oil for bioenergy production. For this reason, it was necessary to cultivate the crops, to harvest the seeds and to extract the oils from the seeds. It should be highlighted that different biomass wastes and by-products were produced from the different phases of the thesis.

For *Cynara cardunculus* L. crop the nutritional value was assessed at the: seeds (sample A); cardoon meal (sample B); light biomass from capitula/head (sample C); and heavy biomass from capitula/head (sample D). The sample C ("light biomass from head") mostly corresponds to plumose

pappus hair (see section 4.1), while sample D (“heavy biomass from head”) is composed by bracts and receptacle parts (section 4.1) [22]. Figure 5.3 shows the different samples analyzed.

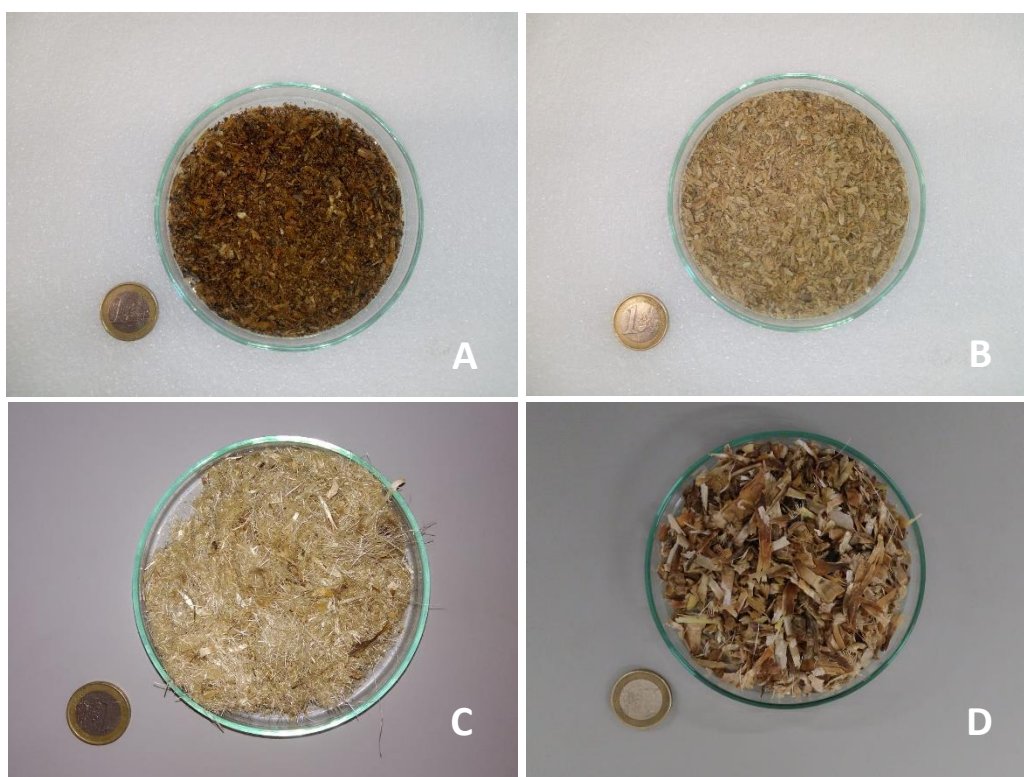


Figure 5.3. Samples used for the assessment of nutritional value of different wastes and by-products obtained from *Cynara cardunculus* L. cultivation and oil extraction: A- seeds; B- cardoon meal; C- light biomass from head; D- heavy biomass from head.

For *Crambe abyssinica* crop, the nutritional value was assessed at the: seeds (sample I); crambe meal or de-oiled meal (sample II); crambe straw (sample III); and, press cake (sample IV). Crambe straw corresponds to the biomass (stem) after seeds harvesting. Usually, this biomass stays in the land, being integrated in the soil. The press cake (sample IV) results from the mechanical extraction of crambe oil (section 3.3.4.1). Figure 5.4 shows the different analyzed samples.



Figure 5.4. Assessment of nutritional value of different samples belong to *Crambe abyssinica* crop: I- seeds; II- crambe meal; III- crambe straw; IV- press cake.

All the types of biomass were previously grinded at the laboratory (“Bimby® TM6”) with objective to obtain homogenous samples.

The assessment of the nutritional value of the different samples requires the use of several reagents. For the oil content determination, chemical extraction was performed using n-Hexane (>95% of purity, VWR). For the protein content determination, samples were digested with sulphuric acid (96%, Merck) and this reagent was also required for titrimetric analysis. The determination of fiber content required the use of sodium phosphate dibasic (Na_2HPO_4) and sodium phosphate monobasic monohydrate (NaH_2PO_4). This determination also required the use of α -amilase enzyme (Kit Sigma – A 3306), amiloglucosidade enzyme (Kit Sigma – A 9913), protease, hydrochloric acid (37%, VWR) and ethanol (96%, VWR). All the reagents required for analytical procedures were of analytical grade.

5.3.2 ANALYTICAL METHODS

The nutritional value of the selected samples was determined according to several standards and for each parameter (water content, protein content, total fiber, ash content, oil content) the procedure was performed in triplicate.

The moisture content was determined using an automatic infrared balance (Figure 5.5), at 105 °C.



Figure 5.5. Infrared balance for automatic determination of moisture content determination.

The oil content determination was performed by solvent extraction using a 100 mL Soxhlet extractor and n-hexane, according to NP EN ISO 659 (2002). Figure 5.6 shows the apparatus used for this determination.

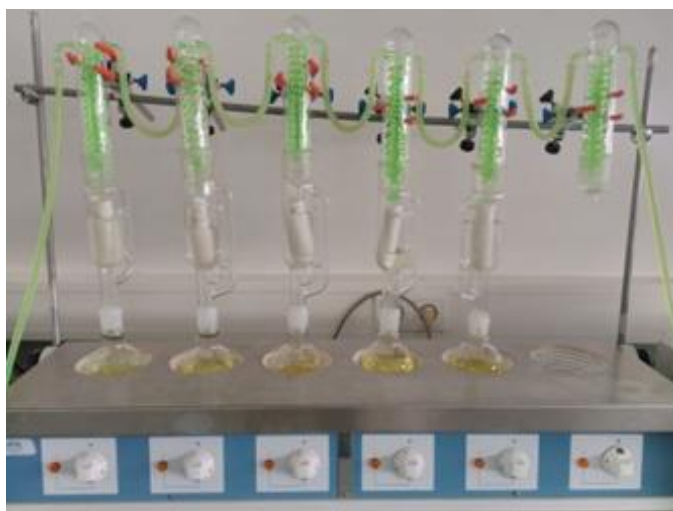


Figure 5.6. Soxhlet apparatus used for oil content determination.

The ash content was determined following the procedure specified in the Official Methods of Analysis of AOAC International (AOAC 920.153) [23]. This methodology suggests the calcination of the samples at 450 – 500 °C during 24 h. Figure 5.7 shows the muffle used for the calcination.

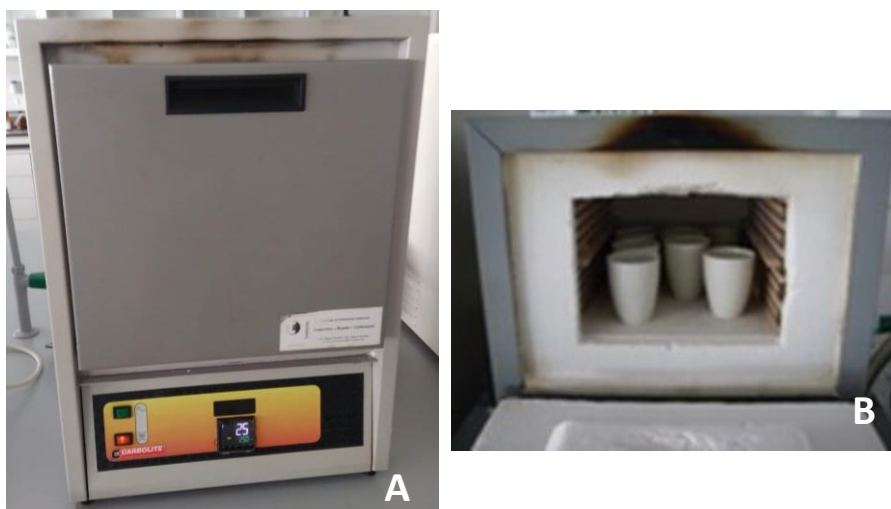


Figure 5.7. Furnace (A) used in ash content determination with the porcelain crucible inside (B).

The total dietary fiber content was determined through an enzymatic-gravimetric method (AOAC 985.29). This procedure requires an enzymatic digestion with three different enzymes (α -amilase enzyme, amiloglucosidade, protease); at the end ethanol 96 % V/V was added to precipitate the soluble fiber. After that, the solution is filtrated and the residue analyzed (protein and ash content determination). The total dietary fiber corresponds to the weight of the residue, discounted by the protein and ash content. As described before, beside the total fiber it is very important to know the amount of soluble fiber and insoluble fiber. The determination of insoluble fiber was also performed without the addition of ethanol 96 % v/v (AOAC 985.29). At the end it is possible to calculate the amount of soluble fiber by difference. For *Crambe abyssinica*, it was considered that the fiber content of the sample I (seeds), sample II (solvent extracted cake) and sample IV (press cake) were similar (the major difference between these three samples corresponds to the oil content parameter), and due to economic constraints (enzyme costs), the fiber content was only determined for sample I (seeds) and sample III (straw).

For *Cynara cardunculus* L. crop the determination of soluble fiber was performed only for samples A and D, since it was considered that the sample B should have a similar content of sample A, and sample C similar to D. Figure 5.8 shows the equipment used for the determination of fiber.



Figure 5.9. Vacuum system used for sample filtration aiming fiber determination.

Protein content was determined according AOAC 928.08 (Kjeldahl method) [23]. The first step of this methodology consists in the digestion (Figure 5.9 - A) of the sample with sulphuric acid (96%) for two hours followed by a titration step with sulphuric acid (0.2M). The amount of nitrogen (N) present in the samples is consequently determined. The scrubber digester (Figure 5.9) used in this determination allowed the digestion of the organic matter in the presence of sulphuric acid (acid digestion). The distiller equipment allows the distillation of ammonia and its collection. Ammonia titration with the acid allows the determination of the nitrogen present in the sample. To obtain the amount of protein a conversion factor of 6.25 was considered. This value is in agreement with the literature for *Cynara cardunculus* L. and *Crambe abyssinica* crop [24, 25].

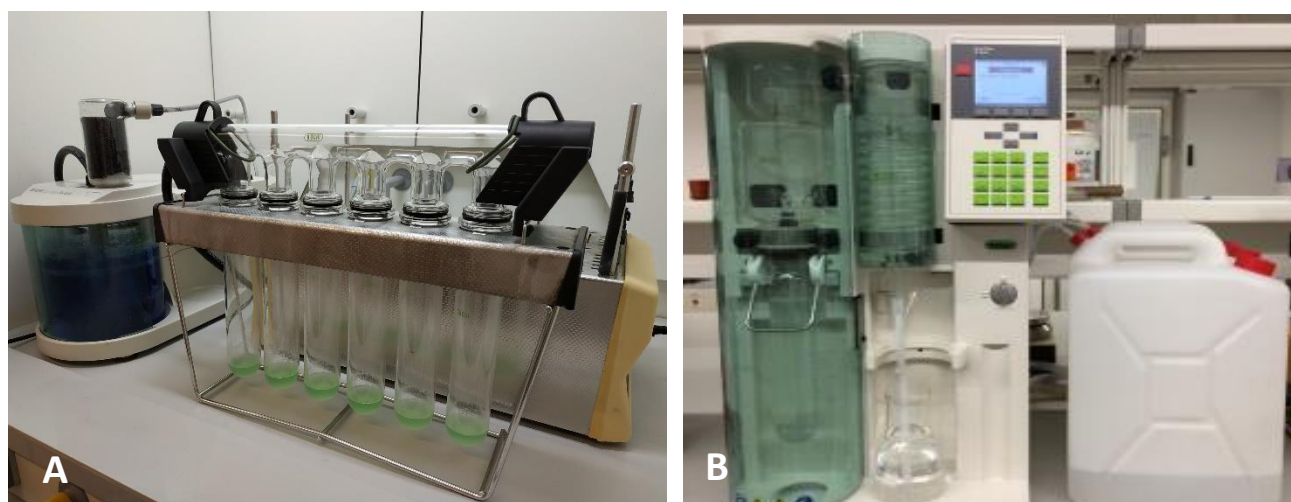


Figure 5.8. System used for nitrogen determination - Kjeldahl method: A- Scrubber digester; B- Distiller.

After all the determinations it was possible to calculate the amount of available carbohydrates (CH) present in each sample. This parameter is determined by difference, according to equation 5.1:

$$CH (\%) = 100\% - Moisture (\%) - Ash (\%) - Protein (\%) - Oil (\%) - Fiber (\%) \quad (5.1)$$

The energetic value is estimated applying the equation 5.2 [24, 26]:

$$Energy \left(\frac{kcal}{100g} \right) = 4 \times [Protein (\%) + CH(\%)] + 9 \times Oil (\%) + 2 \times Fiber(\%) \quad (5.2)$$

All methodologies were performed in triplicate and the variation of the results is expressed as the relative percentage difference to the mean (RPD).

5.4 RESULTS AND DISCUSSION

5.4.1 MOISTURE CONTENT

The moisture content of the different samples is presented in Table 5.1.

Table 5.1. Moisture content determined for the different samples

	Sample	Moisture Content (wt. %)
Cardoon	A- Seeds	5.25 ± 0.00
	B- Meal	3.5 ± 0.3
	C- Light fraction of Head	10.0 ± 0.1
	D- Heavy fraction of Head	10.7 ± 0.1
Crambe	I – Seeds	6.89 ± 0.02
	II – Meal	6.0 ± 0.1
	III- Straw	12.1 ± 0.2
	IV- Press Cake	8.32 ± 0.03

For the biomass samples from cardoon crop (*Cynara cardunculus* L.), the moisture content was similar between sample A and B (seeds and cardoon meal). The same was verified between samples C and D, obtained from the head. In the study developed by Petropoulos *et al.* [24], the seed moisture content varied between 5.1 wt.% and 8.0 wt.%. According to the literature, the meal moisture (4.17 – 4.38 wt.%) is usually lower than that verified for the raw seeds (5.38 – 5.98 wt.%) [27]. The same was verified between the sample A (raw seeds) and sample B (meal). For samples C and D it was not possible to compare directly the results with the literature because the studies usually use fresh

biomass (head) [28], and in the present study the samples are from dry heads (brown color). In the study developed by Claus *et al.* [22], an head's drying was performed in laboratory at 45 °C until constant weight, allowing a moisture decrease from 88 wt.% to 4.63 wt.%. Samples C and D were collected during the summer, being the maximum temperature achieved in this period around 30 °C. Consequently, samples C and D showed higher levels of moisture content (around 10 wt.%).

For the biomass samples from *Crambe abyssinica* crop, samples I, II and IV showed similar moisture. The moisture of raw seeds of *Crambe abyssinica* was according to the reported in the literature (between 6.0 wt.% and 8.1 wt.%) [29, 30]. The moisture content of sample II was near to the described in the study of Togtema *et al.* [31]. Finally, for press cake, the moisture was also similar to the values reported in the literature (10.3 – 11.3 wt.%) [31, 32]. For sample III, which belong to a different typology of biomass (straw) the moisture determined was in the range described in the literature (6.7 – 15.9 wt.%) [31, 33].

Comparing samples A and I (seeds), moisture content was slightly higher for crambe seeds. This fact might be related with the time of harvesting, which for crambe occurred in the beginning of the summer. For cardoon, the heads (and consequently the seeds) were harvested during the summer (higher temperatures). For meal samples (obtained from crambe and cardoon), moisture content was lower than that verified for the respective seeds. This fact could be related with the type and conditions of extraction process employed.

5.4.2 OIL CONTENT

The results concerning oil content determination are presented in Table 5.2.

Table 5.2. Oil content determined for the different samples

	Sample	Oil Content (wt.%)
Cardoon	A- Seeds	20.7 ± 0.3
	B- Meal	2.3 ± 0.2
	C- Light fraction of Head	0.1154 ± 0.0005
	D- Heavy fraction of Head	2.4 ± 0.1
Crambe	I – Seeds	31.0 ± 0.3
	II – Meal	10.3 ± 0.2
	III- Straw	0.0625 ± 0.0001
	IV- Press Cake	14.7 ± 0.1

For cardoon crop the samples that belong to the plant's head (C and D) showed lower oil contents, as expected. Taking into account available literature it was not possible to find studies which used the type of biomass of sample C (plumose pappus hair). For sample D, the oil content was in agreement with that reported in the literature for cardoon receptacle and bracts (1.34 – 2.04 wt.%) [22]. In the study developed by Petropoulos *et al.* [34], the cardoon head (not sieved) showed an oil content of 3.1 wt.%.

The oil content obtained for sample A was in the range reported in the literature (14.5 – 32.4 wt.%) [35]. The ecotype of the seed can influence the oil content. Francaviglia *et al.* [36] cultivated two different wild genotypes of cardoon, and obtained a seed oil content lower than 20 wt.% (in two consecutive years). Finally, for sample B, the amount of oil found in the meal was 2.30 ± 0.17 wt.%, although it was possible to find in the literature higher oil contents for cardoon seed cake (5.3 – 5.8 wt.%) [27]. The efficiency of oil extraction might be related to the type of solvent, the extraction time and the quality of the sample mixture. However, in this case the standard procedures were adopted, being generally used in the literature results allowing a more or less straightforward comparison.

Regarding crambe crop, the results obtained for samples I, II and IV showed that the chemical extraction was not so effective since both the cake and the meal contained relevant oil content. Beside that, the seed oil content obtained was similar to the reported in the literature [37]. As expected, chemical extraction allowed to reduce the losses of oil (less 4.4 wt.% than mechanical extraction), although the extraction time was higher than the required for mechanical procedure. Literature show oil contents similar to those obtained for IV [30, 38]. For sample II, the oil content was higher than the reported (4.0 wt.%) [30, 39, 40], which can be related with the size of Soxhlet extractor used (1 liter – less efficient). As expected, crambe straw showed a very low content of oil. Being crambe an oleaginous crop, lipids are mostly present in the seeds. For cardoon crop, it was also possible to find some oil in the biomass from the cardoon head, which could be related with some seeds' fragments in this sample.

Most of the biodiesel produced in Portugal, in 2017, became from waste cooking oil (56%), followed by edible oil (40%), the last including mostly soybean, rapeseed and palm oil. Comparing the results of oil content at cardoon and crambe seeds with the soybean and rapeseed seeds (Table 5.3) it is possible to conclude that cardoon seeds has an oil content very similar to soybean seeds. For

crambe, seed oil content was (9 – 17 wt.%) is lower than that of rapeseed and higher than the soybean (9 – 13 wt.%) oil contents.

Table 5.3. Oil content for the oilseed crops used for biodiesel production [41-43]

Oilseed Crop	Oil Content (%)
Rapeseed	40 – 48
Soybean	18 – 22
Palm Fruit	50

The consequences of the use of edible-oils for biodiesel production should be carefully analyzed, because the price of, for example, soybean seeds is defined by the demand of oilseed meal (the fraction left after oil extraction) and not directly by the oil market. Oilseed meals are an important component of livestock feed, namely as source of protein (as showed in section 5.4.3) and of key amino acids [44].

5.4.3 PROTEIN CONTENT

The amount of protein determined for each sample are presented in Table 5.4.

The samples of the extracted cake (B, II and IV) have the higher protein content, as expected, once after oil extraction, the remaining fractions of macronutrients are concentrated.

Table 5.4. Protein content determined for the different samples

	Sample	Protein Content (%)
Cardoon	A- Seeds	15.9 ± 0.2
	B- Meal	18.9 ± 0.4
	C- Light fraction of Head	6.6 ± 0.3
	D- Heavy fraction of Head	7.2 ± 0.2
Crambe	I – Seeds	20.17 ± 0.04
	II – Meal	25.37 ± 0.07
	III- Straw	3.9 ± 0.2
	IV- Press Cake	25.4 ± 0.2

For cardoon crop, sample A and B revealed as the main source of protein (15.9 – 18.9 wt.%). According the literature, the protein content of cardoon press cake is around 21 wt.% [45]. No information was found specifically for cardoon meal. For sample A, the value determined was very

near to the reported in other studies. Usually, cardoon seeds showed a protein content between 17 wt.% and 29 wt.% [24, 45-47].

The protein content of samples C and D were very similar. According to the study developed by Claus *et al.* [22], for the bracts and receptacle structures of cardoon heads the protein content was around 10 wt.% and 24 wt.%. This result could be directly compared with sample D (7.22 wt.%), which is mostly constituted by bracts.

According to the literature, the protein content associated to cardoon head ranging 10.25 wt.% and 19.5 wt.% [48, 49]. In the present study sample C and D showed similar protein content.

Regarding crambe crop, the samples generally showed high protein contents, except the sample III (straw) with only 4 wt.% of protein content, value in agreement with the described in the literature [31]. The protein content for crambe press cake was 25.4 wt.%, value very similar (24.67 wt.%) to the reported in the study developed by Canova *et al.* [50].

The value of protein content obtained for sample II (meal) was slightly lower than the usually described for this type of biomass. After de-oiled, crambe cake showed a protein content between 26 wt.% and 39 wt.% [30, 39, 40]. The whole seed showed a protein content of 20.2 wt.%, value very near to the reported in the literature (19.7 – 20.1 wt.%) [40, 51].

The oil extraction for bioenergy production leads to the production of high amounts of press cake. The most common oilseed crops used for bioenergy production are rapeseed, sunflower, soybean or palm. Table 5.5 presents the results related to the protein content for the seeds/fruits of these different crops.

Table 5.5. Protein content for the oilseeds' crops used as source of oil for biodiesel production

Oilseed Crop	Raw Seed's Protein Content (%)	Straw's Protein Content (%)
Rapeseed	18 – 25	4.20 [52]
Sunflower	21 – 29	5.64 [53]
Soybean	36 – 42	4.81 [53]
Palm Fruit	10	—

According to Table 5.5, it is possible to conclude that cardoon and crambe seeds have a protein content similar to the found in other oilseeds crops [42, 43, 54, 55].

In fact, the production of bioenergy is closely linked to the production of animal feed. After the extraction of the oil, most of the press cake or meal is forwarded for animal feed production [44]. For such reason, a high seed oil content allied to a high protein content became bioenergy production more sustainable, being the by-products recovered for animal feed.

Analyzing the amount of protein in the other structural parts of the crops it is possible to verify that the amount of protein found in sample III (crambe straw) is low, although similar to that verified for other biomass (straw) used to feed dairy cattle, like barley (4.4 wt.%), oat (4.8 wt.%), maize (2.74 wt.%) or wheat (4.6 wt.%) [56, 57]. Comparing the protein contents of the different materials, it is possible to conclude that sample III, C and D have very potential to be used as raw material for animal feed.

Typically, the optimum level of protein for animal feed is in the range between 16 wt.% and 22 wt.%. Although, cereal grains (from maize, sorghum, wheat, barley, etc.) have protein levels between 8 – 13 wt.%, while for oilseed meals protein content vary between 33 wt.% and 48 wt.% [44]. Regarding to decrease the protein content, oilseed meals are blended with cereals to reach the optimum protein content [44].

5.4.4 ASH CONTENT

The determination of ash content is a very important, once it measures the amount of minerals present in the biomass (inorganic fraction).

Table 5.6 presents the results obtained for ash content of different samples.

For sample A, the amount of ash determined was similar to the values reported in the literature (2.0 – 4.3 wt.%) [24, 45]. The result obtained for sample B was not directly comparable with the literature (lack of information), but the study of Genovese *et al.* [45] reported for cardoon press cake (similar type of biomass) an ash content of 5.5 wt.%.

Table 5.6. Ash content for the different biomass samples

	Sample	Ash Content (%)
Cardoon	A- Seeds	4.10 ± 0.01
	B- Meal	5.02 ± 0.03
	C- Light fraction of Head	6.6 ± 0.2
	D- Heavy fraction of Head	9.7 ± 0.5
Crambe	I - Seeds	6.6 ± 0.2
	II - Meal	14.53 ± 0.01
	III- Straw	9.5 ± 0.3
	IV- Press Cake	16.24 ± 0.01

For sample D, the ash content obtained was 9.7 ± 0.5 wt.%. In the study developed by Claus *et al.* [22], for a receptacle and bracts structure an ash content of 12.3 wt.% and 5.4 wt.%, respectively, was found. Since sample D was constituted by a mixture of receptacle and bracts it was expected that the ash content was within 5.4 wt.% and 12.3 wt.%.

For the crambe crop, typically the seed's ash content are in the range between 4.8 wt.% and 5.0 wt.% [30, 39], although for sample I the ash content was slightly higher (6.6 ± 0.2 wt.%). For crambe meal, the ash content obtained was 14.53 ± 0.01 wt.%, value higher than the referred in the literature (7 – 9 wt.%) [39, 58]. Also for sample IV, the obtained result (16.2 ± 0.01) was higher than the reported in the literature (5 – 7 wt.%) [31, 59]. Finally, for sample III the ash content obtained was also slightly higher than the expected (7.35 wt.%) [31].

Overall, all the biomass samples which belong to crambe crop showed a mineral content above the expected, according the information provided by the literature. It should be highlighted that this can be related with soil's composition. According to Vieira *et al.* [60], the ash content is related to the amount of nitrogen, phosphorus or potassium present in the soil. Consequently, the high fertility of the soil (see section 3.1.4) where crambe was sown might be one relevant reason for the higher ash content found in the structural parts of the crop.

5.4.5 DIETARY FIBERS

As referred in section 5.1.2, the dietary fibers were only assessed for sample A, D, I and III.

Table 5.7 presents the results obtained for each sample.

Table 5.7. Dietary fibers content obtained for the different biomass samples

	Sample	Total Dietary Fiber Content (%)	Insoluble Fiber Content (%)	Soluble Fiber Content (%)
Cardoon	A- Seeds	55 ± 1	47.2 ± 0.4	7.8 ± 0.9
	D- Heavy fraction of Head	80.0 ± 0.3	77.1 ± 0.3	3.0 ± 0.6
Crambe	I - Seeds	40 ± 1	33.3 ± 0.3	6.9 ± 1.5
	III- Straw	84.7 ± 0.1	80.12 ± 0.03	4.62 ± 0.08

For sample A (seeds of cardoon), the total dietary fiber obtained was 55 wt.%, corresponding almost 86% to insoluble fibers. In the study developed by Foti *et al.* [47], the total dietary fiber content reported for cardoon seeds varied between 18.2 wt.% and 19.1 wt.%. Comparing total dietary fiber present in whole seed of cardoon with the results obtained by the exactly same methodology (AOAC 985.29) for other types of seeds, it was possible to confirm the higher amounts of total dietary fiber obtained at sample A (Chia seed – 37.7 wt.%; Flax – 33.7 wt.%; Sesame – 31.8 wt.%) [61]. The high amounts of total dietary fiber found for sample A also translate the low amount of protein.

For sample D, the value achieved for total dietary fiber was 80 wt.%, corresponding 96 % to insoluble fiber. According to Claus *et al.* [22], bracts plant structure showed a fiber content of 44.2 wt.% and receptacle had 13.1 wt.%. For dietary fibers all the determinations were repeated aiming to dissipate any doubt related with the results obtained.

In Portugal, the demand of pulp fiber for papermaking is increasing, although most of the cellulose fibers came from *Eucalyptus globulus* and *Pinus pinaster*. Considering the dietary fibers' results referred before, cardoon head presents clearly high level of fibers. According to Antunes *et al.* [62], above ground biomass of *Cynara cardunculus* L. is a suitable source of cellulose fibers.

For samples I and III the total dietary fiber found was higher than that reported in the literature (14 – 22 wt.% and 57 wt.%, respectively) [31, 39, 58]. Based on the obtained results, 82% – 95% of total dietary fibers correspond to insoluble fiber.

5.4.6 CARBOHYDRATES AND ENERGY VALUE

As referred before, carbohydrates (Table 5.8) were calculated by difference (equation 5.1). For energy value, the calculation considered Regulation EU Nº 1169/2011 (Food information to the consumers).

For sample A, energy value determined was similar to that found in the literature (479 – 510 kcal/100g dw) [24]. As referred previously, sample D was mostly constituted by bracts and receptacle. According to El Bassam *et al.* [63], the receptacle and bracts structure showed an energy value of 360.5 kcal.100g⁻¹ and 418.1 kcal.100g⁻¹. Sample D showed an energy value in the range referred before.

Table 5.8. Carbohydrates and energy value associated which sample

	Sample	Carbohydrates (%)	Energy Value (kcal/100 g dw)
Cardoon	A- Seeds	4 ± 1	488 ± 1
	D- Heavy fraction of Head	0.7 ± 0.6	372 ± 2
Crambe	I – Seeds	2.0 ± 0.8	529 ± 2
	III – Straw	6.63 ± 0.09	300.9 ± 0.2

For crambe seeds (sample I), the energy value calculated was around 529 kcal/100g dw. In the literature no information was found concerning the energy value of crambe seeds, although based on the nutritional value reports [30, 58], the energy value should be between 501 and 514 kcal/100g dw, calculated according equation 5.1. For sample III (straw), the energy value found was similar to the reported in other study [31]. Despite having a small energy value, straw is a good alternative option for animal feed if mixed with other feedstocks [64].

Beyond the parameters assessed and referred before, antinutrients characterization should also be performed, being essential for conclude about the suitability of using such biomass for animal feeding.

Antinutrients are substances which interfere with the absorption of nutrients, reducing the nutrient digestion and utilization [65]. These substances are naturally produced in the plants for their own defense [65, 66]. For example, crambe crop typically produces glucosinolates which act as a defense

against pests [67]. When ingested by the animal organism, glucosinolates cause a decrease in the production of milk (dairy cattle), leading to problems in the thyroid or affecting the lamb's growth [68]. Different animal species have different levels of tolerance to the glucosinolates [68]. Consequently, the use of crambe's biomass for animal feed requires a detoxification (several processes can be applied) [68]. For crambe oil, the presence of erucic acid (around 55 wt.%) has associated the development of myocardium problems and the increase of fats accumulation in the liver and heart [68].

For *Cynara cardunculus* L., there are few studies in the literature concerning nutritional value of this specie. However, the presence of antinutrients was already reported, namely oxalic acid, which increases the risk of the development of kidney stones. Oxalic acid binds with nutrients, making them inaccessible to the body; in agreement, food with oxalic acid consumed regularly leads to nutritional deficits. For ruminants, oxalic acid is not an important issue because ruminal microflora metabolizes this compound [66].

For cardoon crop, the amounts detected of oxalic acid were within safe limits and lower than found for other edible species [24]. *Cynara cardunculus* L. crop is mostly associated to positive effects in human health (since ancient times), due to their choleretic, diuretic and hepatoprotective effects [35].

5.5 CONCLUSIONS

The results obtained for the nutritional value of biomass of cardoon and crambe crops reveal that such materials have good nutritional characteristics. The low agronomic input required by cardoon and crambe crops makes their exploitation even more relevant. Cardoon revealed to be a crop very suitable to the Mediterranean edaphoclimatic conditions. As expected, the seeds of cardoon and crambe showed a high content of protein (16 — 20 wt.%) and oil (21 — 31 wt.%). After the extraction of the oils, meal and press cake generally showed a higher protein content (18 — 25 wt.%). Typically, the optimum level of protein for animal feed is in the range between 16 wt.% and 22 wt.%. However, cereal grains (from maize, sorghum, wheat, barley and others) have protein levels between 8 — 13 wt.% and oilseed meals protein content varies between 33 wt.% and 48 wt.%. Regarding to decrease protein content, oilseed meals are blended with cereals to reach the optimum values. According to that, crambe meal, crambe press cake and cardoon meal appear to have high potential to be used for animal feed.

The biomass from cardoon heads showed a protein content of 6.6 wt.% and 7.2 wt.%. Although lower when compared with oilseed meal, cardoon heads showed to be a good source of protein, in levels close to those found in the cereal grains. Most of this biomass is constituted by fibers.

The biomass from the crambe stem (the straw) showed a low content of protein (3.85 wt.%) and a high content of fibers, being the energy value 211 kcal/100g dw. When compared with rapeseed straw, barley straw, wheat straw or oat straw it is possible to conclude that the protein content (3.6 – 4.2 wt.%) and energy value are similar (296 – 298 kcal/100g dw). The use of the different types of straw for animal feed should take into consideration that this type of biomass does not provide all the necessary energy and protein content to satisfy the animals' nutritional requirements. However, straw is a good alternative option for animal feed if mixed with other feedstocks.

The nutritional value assessment showed that the different biomass wastes are rich in protein, oil and fiber having potential to be used as animal feed.

To summarize, according the nutritional value results, crambe and cardoon are two crops with high nutritional value and might be considered a potential source for animal feed. However, the digestibility rates and the presence of antinutrients should be taken into consideration for a complete/better assessment.

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CHAPTER 6 ***OVERALL ASSESSMENT OF WASTES AND BY-PRODUCTS AS ALTERNATIVE RAW MATERIALS FOR ENERGY PRODUCTION***

ABSTRACT

In Portugal, several wastes and by-products are produced at domestic and industrial sectors, some of them showing high potential to be used as a source of oils/fats for biodiesel production, simultaneously contributing towards effective waste/material management practices. Examples include waste cooking oil, waste coffee grounds, acid soapstock oil, olive pomace, rice bran, winery waste, among others. In this study, several wastes/by-products with high potential for bioenergy production were identified and explored for the mentioned purpose. The characteristics and amounts of the wastes/by-products available are thus described in each case. Based on the estimated annual amount of each type of wastes/by-product produced in Portugal, the following rank of the seven kinds of wastes/by-products was obtained: Olive Pomace (3.4×10^5 ton) > Winery waste (2.0×10^5 ton) > Spent coffee grounds (1.0×10^5 ton) > Waste cooking oils (6.7×10^4 ton) > Waste animal fat (6.1×10^4 ton) > Rice bran (8.1×10^3 ton) > Soapstock (6.7×10^3 ton).

6.1 SCIENTIFIC RELEVANCE

Waste-to-energy technologies offer promising ways to produce energy, while promoting the reduction of landfilled waste [2]. However, to promote an efficient use of alternative raw materials, for energy purposes, it is necessary to establish synergies between industries and governments or responsible entities for waste management at national level. Wastewater sludge from several activities (slaughterhouses, dairy industry, bakery industry and fish canning industry), waste cooking oil and other industrial wastes present a high potential for bioenergy production due to their characteristics. The establishment of waste recovery policies should consider the economic and environmental viability. Therefore, it is crucial to understand the spatial and temporal variability of waste generation [2]. This includes understanding the waste characteristics, amounts produced, transport requirements and overall energy conversion efficiency, which are essential to develop an accurate strategy [2].

Directive 2008/98/CE, amended by Directive 2018/851, establishes for all member-states of the European Union, measures to protect the environment and human health through the prevention and reduction of the negative impacts related to waste generation and management. According to this, there is waste hierarchy (Figure 6.1) that should be followed, which prioritizes technologies and the first measure to be applied is the prevention of its production. Prevention constitutes the most sustainable measure (e.g. through the adoption of an eco-design or new technologies) allowing the reduction of waste production in some activities or industrial processes.

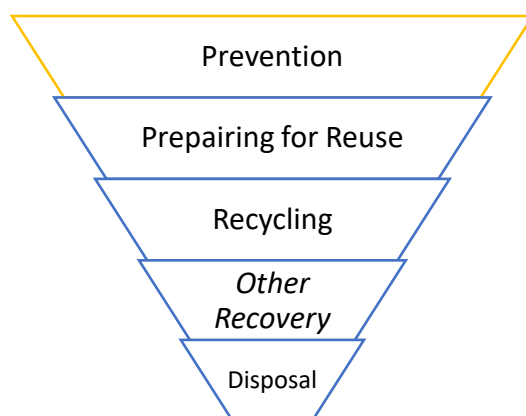


Figure 6.1. Schematic representation of the European Waste hierarchy.

The second level of waste hierarchy proposes the reuse of the product for a similar purpose of the original, enabled by its preparation (e.g. repairing) increasing their lifetime and delaying waste generation.

Some wastes streams can be recycled, being the waste used as feedstock to produce new products, avoiding the extraction and consumption of new resources, while reducing the environmental impacts caused by the manufacturing process. This includes multi-material organic recycling which may pass by composting and anaerobic digestion as well as the production of biodiesel from organic waste materials.

The “other recovery” level is usually related to the energy production with energy recovery nonetheless might include other recovery operations besides recycling such as regeneration, recovery of specific components and some refining processes.

The last level of the waste hierarchy is waste disposal. The wastes are landfilled or incinerated without energy recovery. This solution should be whenever possible avoided since it does not use waste as a resource, being generally the option, which results in higher environmental impacts; thus, it should be implemented when the other management alternatives are not viable.

According to the Waste Framework Directive 2008/98/CE, waste is defined as “any substance or object which the holder discards or intends or is required to discard”. Typically, waste has a negative connotation, even though it can be used as raw material for several processes where its value is recognized [3]. Waste can also be described as a resource, because resources are systematically transformed into waste after human consumption, although it can be redirected back, namely for production processes [4]. Consequently, waste might also be seen as wasted resource.

In Portugal, some products that result from manufacturing processes as a secondary product and which can be directly used, without any treatment, in other productive process are classified as by-products. In this context, by-product and waste are different concepts (Figure 6.2).

Considering the high costs of conventional feedstocks used for biodiesel production (around 80%), the competition with the food market and the soil erosion caused by intensive farming, several researchers have been studying alternative feedstock's for biodiesel production [5, 6].

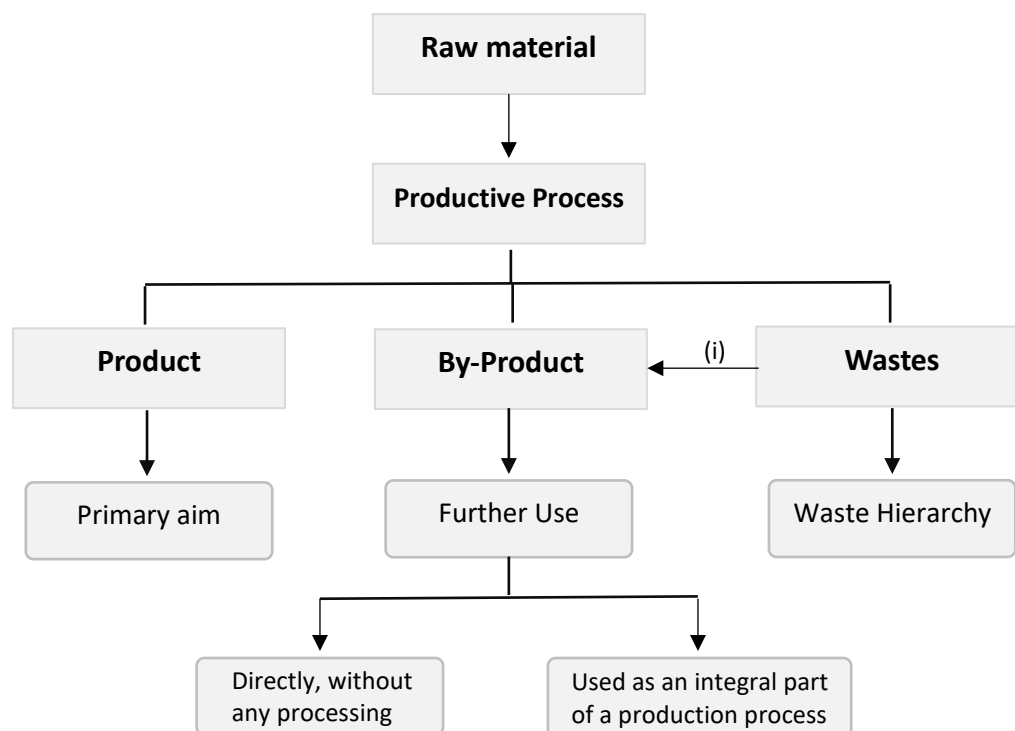


Figure 6.2. Schematic diagram of outputs from a manufacturing process: including products, by-products and wastes (i- end-to-waste criteria).

Biodiesel use can be considered a waste-to-energy technology (WtE), when produced from waste cooking oil, fats, grease and other types of wastes/by-products (WtE resources) [2, 6]. Due to the role of raw-material costs in the biodiesel production process, biodiesel production from wastes might be very relevant regarding the economic viability of the process. Moreover, the production of biodiesel from wastes agrees with what is suggested by the waste hierarchy, reducing waste disposal.

The recovery of oils and fats from wastes or by-products from industrial processes result in a feedstock for biodiesel production with low quality (several contaminants and high free fatty acids content). According to the European List of Wastes (LoW – Commission decision 2014/955/EC) it is possible to identify several categories of wastes with potential for oils/fats recovery for energy/biodiesel production. The wastes from chapter 2 (“wastes from agriculture, horticulture, aquaculture, forestry, hunting and fishing, food preparation and processing”), for example, animal-tissue waste (code 02 01 02), plant-tissue waste (code 02 01 03), sludges from washing and cleaning facilities of industries associated to food processing (meat and fish processing) and from “the preparation and processing of meat, fish and other foods of animal origin” (code 02 02 01), sludges “from on-site effluent treatment” associated to baking and confectionery industry (code 02 06 03),

sludges “from on-site effluent treatment” produce in dairy products industry (code 02 05 02), “wastes from fruit, vegetables, cereals, edible oils, cocoa, coffee, tea and tobacco preparation and processing; conserve production; yeast and yeast extract production, molasses preparation and fermentation “like the coffee grounds (code 02 03 99) and “wastes from the production of alcoholic and non-alcoholic beverages (except coffee, tea and cocoa)” specifically “materials unsuitable for consumption or processing” (code 02 07 04) are some of the categories of wastes with potential for bioenergy production.

Also from the chapter 19 of LoW (“wastes from waste management facilities, off-site waste water treatment plants and the preparation of water intended for human consumption and water for industrial use”), the “sludges from treatment of urban waste water” (code 19 08 05) and grease and oil mixture from oil/water separation containing only edible oil and fats (code 19 08 09) might be pointed out as the type of waste with the higher potential for bioenergy production. From municipal wastes (chapter 20), “edible oil and fat” (20 01 25) can also be used as raw material for biodiesel production (most common waste raw material).

Although having potential for biodiesel production, most of these wastes are not recovered, but landfilled. Among those referred, waste cooking oil is the waste stream most recovered in Portugal for biodiesel production. As previously referred (section 1.4.2.), 56% of the biodiesel was produced from WCO in Portugal [7].

It is also advisable to produce bioenergy from by-products. According to the Directive 2008/98/EC a by-product is defined as “substance or object, resulting from a production process, the primary aim of which is not the production of that item” (article 5). By-products are associated to a wide range of business sectors and their management might cause different environmental impacts. Waste might be classified as a by-product when several conditions are met, namely: i) “further use of the substance”; ii) “the substance or object can be used directly without any further processing other than normal industrial practice”; iii) “the substance or object is produced as an integral part of a production process”; and iv) “further use is lawful, i.e. the substance or object fulfils all relevant product, environmental and health protection requirements for the specific use and will not lead to overall adverse environmental or human health impacts” (End-of-waste criteria).

In Portugal, from the industrial sector several by-products show high potential to be used as raw material for bioenergy production. Soapstock from the vegetable oil refining industry, olive pomace oil from olive oil production, rice bran from the rice industry and brewer’s spent grains from brewery

industry are some examples of by-products that can be used as a source of oil and as a raw material for bioenergy production. The following sections present more detailed information regarding different wastes and by-products with high relevance under the frame of this thesis.

6.1.1 WASTE COOKING OIL

WCO is a type of waste mainly produced in domestic and industrial sectors (hotels and restaurants). In Portugal, the Decree-law nº 267/2009 establishes the legal framework for collection, transport, and the processing of WCO from industrial sources and the domestic sector (waste stream). At the domestic sector, independently of the individual responsibility concerning waste production and management, the management is of the responsibility of the municipalities ($< 1\,100\text{ L}\cdot\text{day}^{-1}$), including the oil's collection. At the industrial sector, the collection of WCO is more efficient and effective as the management responsibility lies directly with the producer.

In EU (specially in Southern Europe) most of WCO comes from the domestic sector, although the collection systems need to be improved [8]. The release of these oils in drainage systems leads to an extra energy consumption to remove oils in the wastewater plants and several technical problems, including the clogging of the sewage system. It is estimated that biodiesel produced only from WCO could replace 1.5% of the EU (27) diesel consumption [8]. In 2017, in Portugal, 56% of biodiesel was produced from WCO, although most of this raw material was imported [7, 9]. Figure 6.3 presents the amounts of food oils sold in Portugal (2015 – 2018) and the amount of WCO collected in the same period according to the “National Entity for the Energy Sector” (*“Entidade Nacional para o Sector Energético”*) [7, 10-15].

The amounts of WCO collected from 2015 to 2018 represented a value between 23% and 48% of the oil sold. The year of 2018 had the higher result for the collection of WCO (48%). For the same year, around $1.79\times 10^5\text{ t}$ of WCO was used for biodiesel production, where 91.4% correspond to imported WCO [7, 10, 11]. For 2018, the amount of food-oils sold in Portugal was around $1.40\times 10^5\text{ ton}$ (for a WCO density of $0.91\text{ g}\cdot\text{cm}^{-3}$), in the same range of the amount of total WCO imported. Considering this, it is possible to conclude that the amounts of food oils sold in Portugal and which lead to the WCO generation could contribute significantly to satisfy the amounts of WCO which were necessary to import, so the WCO collection system should be improved. When compared with the conventional feedstocks (edible oils) used for biodiesel production, WCO can be 2 to 3 times cheaper than the virgin vegetable oils [16].

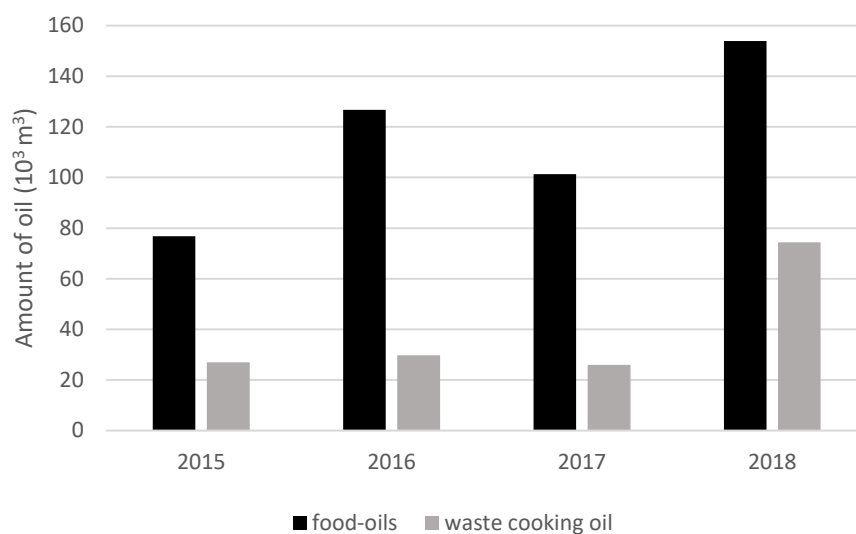


Figure 6.3. Food oils sold in Portugal and WCO collected between 2015 and 2018.

6.1.2 Waste Coffee Grounds

Coffee is one of the most consumed drinks in Europe and America, only surpassed by water [17]. The International Coffee Organization reveals that the global consumption of coffee exceeded 9.8 million kilograms, in 2019, and it is one of the most marketed products in the world, with an annual consumption growth of 1.5% [18, 19]. It is estimated that 1.4 billion coffee cups are prepared everyday [20]. Several studies show the importance of coffee consumption, such as decreased risks of heart disease and some cancers [18, 21]. Related to the coffee consumption, environmental issues have been arising, namely due to waste generation. Coffee drink is obtained from a small number of bean compounds and its consumption produces an huge amount of waste, spent coffee grounds (SCG) consisting in the mass of bean ground remaining after the extraction of desirable compounds, during the brewing process (Figure 6.4).

Similarly to other organic wastes, SCG might have harmful compounds to the environment and for this reason it is mostly forwarded for landfilled instead of being recovered as organic fertilizer [18]. When used as organic amendment, fresh SCG can also cause a negative effect on crop growth due to the competition between roots and microorganisms for nitrogen [22]. Since economic and environmental costs with the landfilling of SCG are considerable, alternative and more sustainable options for dealing with SCG should be considered.

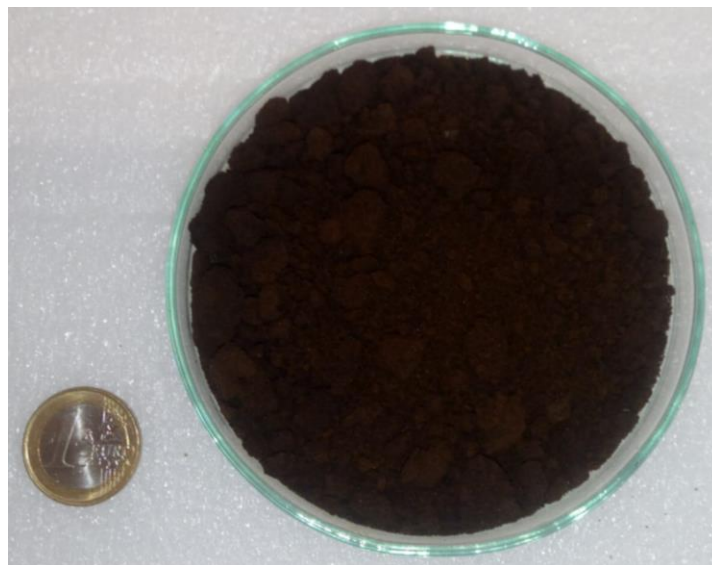


Figure 6.4. Spent coffee grounds, after oven drying.

According to Kamil *et al.* [20], the preparation of 1 kg of soluble coffee leads to the production of 2 kg of SCG (wet basis). Considering that, it becomes crucial to study and develop alternative processes to recover SCG. Additionally, literature shows that considering the number of coffee cups prepared every day, it was possible to estimate the CO₂ emissions caused by SCG landfilling (28.6 million ton of CO_{2eq} per day) which would correspond to the burn of 10.6 million liters of diesel fuel [20, 23].

Coffee beans, depending on its type, present an oil content between 11% and 20% (mass basis), being possible to extract 15% (on average) of oil from SCG [24]. As a consequence, this oil can be studied for biodiesel production. There are some studies where coffee oil is easily converted into biodiesel [24, 25]. After the coffee oil extraction, the grounds keep showing potential as solid biofuel, exploring the natural potential of this waste [19]. In 2008, it was estimated that using SCG oil it is possible to produce almost 11.3% of the total biodiesel produced in the world (1.28 billion L of biodiesel) [20, 26].

Apart from oil, SCG can be a source of other valuable compounds, like, sugars and antioxidants. Due to SCG characteristics, there are several studies regarding the recovery of these compounds, allowing to reduce improper landfilling of this resource. Sugars can also be used for bioenergy production, while antioxidants can be used in dietary supplements. New materials can be created reusing SCG [18]. The SCG has been recycled through composting, mushrooms production or biogas generation [27]. The use of SCG in domestic agriculture has been increasing although without

scientific support regarding the efficiency and the safety for human consumption (bioaccumulation in vegetables and other food products) [27].

The composition of SCG varies according to the brewing method, although typically, the largest components (50 wt.% of dry mass) are polysaccharides (cellulose, hemicellulose) [28]. Lignin and protein correspond to almost 20 wt.% (dry mass) [18, 28]. The remaining fraction of SCG corresponds to oil, ash, phenolic compounds, minerals, caffeine and tannins [18].

Similarly, during the cultivation of coffee crops several wastes are generated, being estimated that only 6 % of the harvested coffee is used for the preparation of coffee drinks. The remain fraction (94%) is considered waste, and is produced during the dehulling of the coffee beans [29]. Fruit pulp and coffee hulls are the most studied coffee wastes due to the high amounts generated and their toxic/pollutant characteristics (leaching of compounds such as caffeine, chlorogenic acid (phenolic acid) and tannins) [30, 31]. The countries that import coffee, like Portugal, are responsible for the management of these wastes, even though only the management of SCG is guaranteed [32].

According to a Portuguese report about food consumption (2012 – 2016), on average, the consumption of coffee was around 11.8 g.inhab⁻¹.day⁻¹ in 2016. Between 2012 and 2016, the consumption of coffee decreased 2.5% [33]. In Portugal, the coffee market is very important and corresponds to 500 million euros.

All the coffee consumed in Portugal is imported. In 2011, the annual amounts imported were around 855 000 bags (1 bag correspond to 60 kg) from five countries: Spain (14.8%), Brazil (12.6%), Vietnam (9.6%), Uganda (8.5%) and Côte d'Ivoire (7.4%) [34]. The internal annual consumption corresponded to 700 000 bags of coffee. Roasted coffee corresponded to 85% of the total consumption and soluble coffee represented 15%. Portuguese coffee consumption corresponds to 0.6% of the total consumption in the world. In Figure 6.5 the amounts of coffee imported to Portugal are represented [35]. More recently (2016-2017), the coffee imported almost achieved the levels verified in 2011 (before the economic Portuguese crisis). In 2017, 833 900 bags of coffee were imported [35].

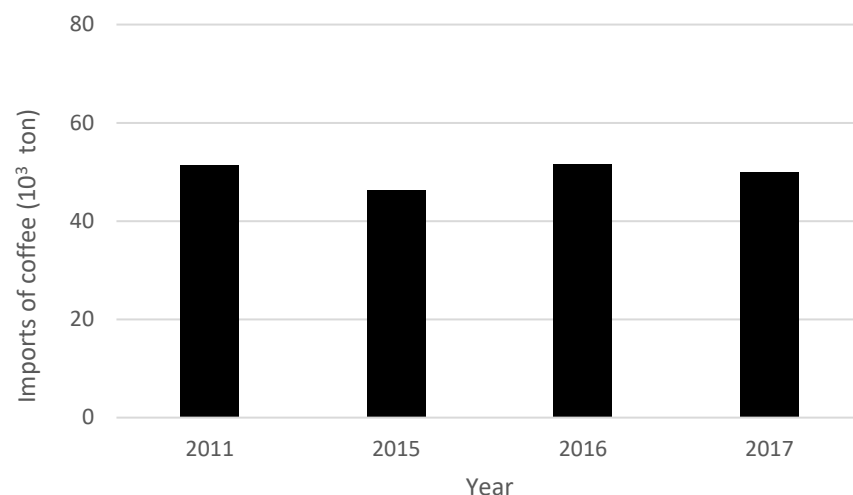


Figure 6.5. Evolution of imports of coffee (green coffee) to Portugal [35].

6.1.3 SOAPSTOCK

The soapstock (SS) is a by-product from the chemical process carried out in the oil refining industry (vegetable oils and fats) [36, 37]. Figure 6.6 is depicted a soapstock sample produced during sunflower oil refining. This by-product, generated in large amounts, results from the neutralization of FFA and corresponds to 5 – 6% (volume basis), or in some extreme cases up to 20% of the crude oil refined [37-39]. Soapstock consists in an alkaline emulsion of lipids, mostly constituted by FFA, phosphoacylglycerols, triacylglycerols, pigments and other nonpolar compounds, varying its composition according to the type of crude oil and the operational conditions of the refining process [38].

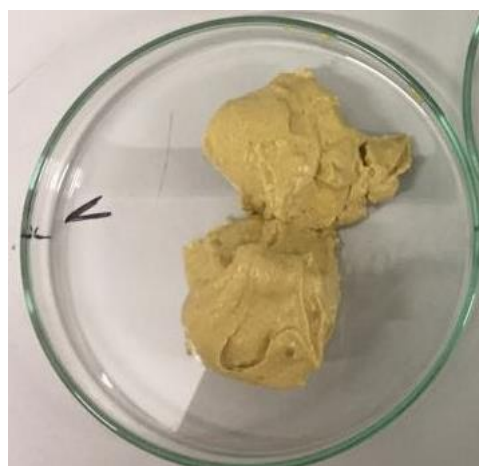


Figure 6.6. Soapstock by-product from crude oil refining of a sunflower.

Almost 50 wt.% of the emulsion is composed by water [38]. The addition of sodium hydroxide to the crude oil aiming to neutralize FFA produces SS. This by-product brings several problems for the vegetable oil refining industry, namely when landfilled due to its high organic content (around 50 wt.%) [37]. In USA, the market value of SS represents one-fifth of crude oil price [37].

Numerous studies have been developed aiming to add value to SS (energy and materials recovery), nonetheless SS is mostly used to obtain acid oil (Figure 6.7) [37]. In fact, from SS it is possible to obtain an acid oil, usually by adding a strong mineral acid (sulfuric or hydrochloric); this process requires the use of water to remove the remaining amount of mineral acid, leading to the production of wastewater.



Figure 6.7. Acid oil from soapstock by-product.

Acid oil can be used as animal feed, for biodiesel production or as boiler fuel [36, 37]. Among these alternatives, the use of acid oil for biodiesel production appears to be the most economically attractive option [16, 36].

In 2016, Portugal produced 8.64×10^5 ton of oils and fats from animal and vegetable sources and sold 1.87×10^6 ton (around 1000×10^6 €) [1]. Almost half of the sells corresponded to imported oils. A substantial fraction of these oils were refined (111 826 ton) [1]. Therefore, to estimate the amount of SS produced in Portugal it was considered that 6% (volume basis) of crude oil refined (1.2×10^5 m³) corresponds to potential SS of 7.3×10^3 m³ (for an edible-oil density of 0.90 g.cm^{-3}) [38].

6.1.4 OLIVE POMACE

Olive pomace is to the solid fraction that remains after olive oil extraction from olives (olive oil industry). It is estimated that 35 – 40 wt.% of the processed olives correspond to a solid waste, the olive pomace (Figure 6.8) [40]. This is a by-product with essentially water, oil, olive skin and olive pulp in its composition [40, 41].

In the Mediterranean region, there is a large production of olive oil, being Portugal a highly relevant producer. Olive pomace has potential for animal feed purposes, organic fertilizer or for energy production, where the use of the oil fraction (5 – 8%, wet basis) arises as an option with economic interest [42, 43]. The moisture content is influenced by the type of the olive oil extraction performed (two-phases or three-phases) [40]. Regarding the recovery of olive pomace oil, due to its high moisture content, it is advisable to dry the by-product before the chemical extractions aiming to increase the oil extraction yield.



Figure 6.8. Olive Pomace after oven drying.

In Portugal, in 2017 the olive production was around 8.58×10^5 ton [1]. For the traditional olive press process 1 ton of olives generates around 400 kg of solid waste (olive pomace), 200 kg of olive oil and 600 kg of wastewater with residual solids and oil [44]. Figure 6.9 represents the evolution of olive oil production between 2015 – 2017.

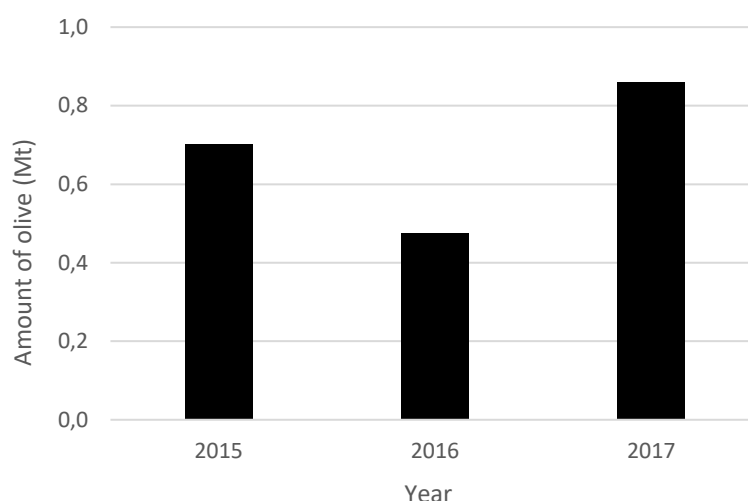


Figure 6.9. Production of olive oil in Portugal (2015, 2016 and 2017) [1].

The increase of olive production verified in 2017 was mainly related with the favorable weather conditions during the flowering stage of the crop and with the good management of irrigation times which allowed the establishment of good conditions for the maturation of the fruits [1]. Pondering the amount of olives collected and the percentage of olive pomace that results from olive oil extraction (40 wt.%), it was possible to estimate that the amount of pomace olive generated was around 3.43×10^5 t. Due to its low cost (between 5 – 25 €·t⁻¹), olive pomace is considered a good alternative to the conventional feedstocks used for biodiesel production [45]. The detection of benzo(a)pyrene in olive pomace oil, a compound classified as carcinogenic to humans, drastically reduced the use of this oil for human consumption [46]. According to the Commission Regulation (EU) 2015/1125, the maximum concentration of benzo(a)pyrene in oils for food consumption is 2 µg·kg⁻¹.

6.1.5 Rice Bran

Rice is the second most consumed cereal in the world, only exceeded by wheat. In Portugal, in 2017, the area of rice sown was around 30 000 ha and an annual production of around 180 kt [1, 33]. It should be noted that the national consumption per capita is 15.3 kg (340 g·day⁻¹), three times bigger than the European average.

By-products such as rice bran (Figure 6.10) might be considered as a non-conventional and low cost feedstock for biodiesel production [47].

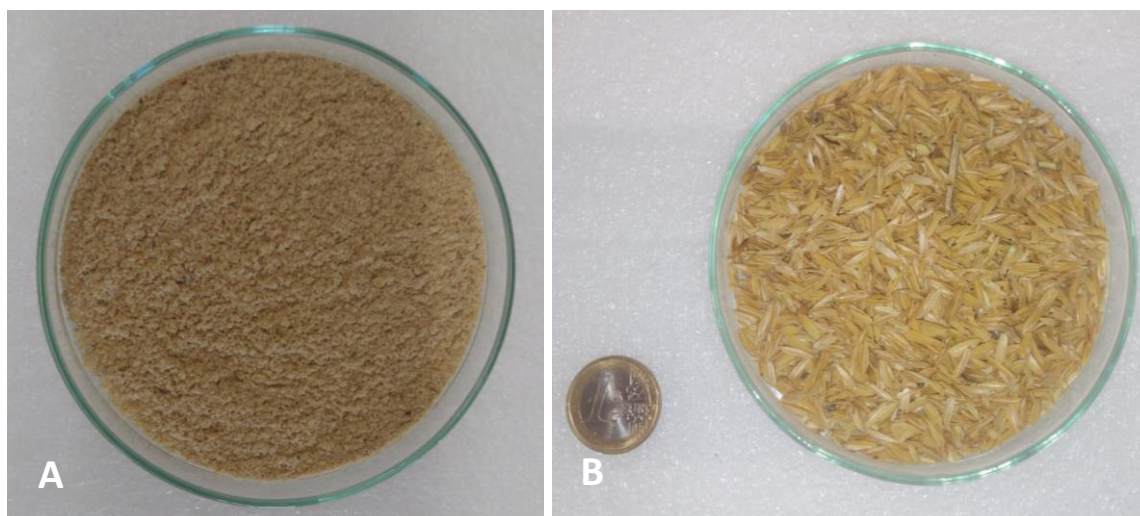


Figure 6.10. Rice bran (A) and rice husk (B) from rice processing.

This by-product results from the milling of the rice grains. The processing involves on the removal of the husk (dehulling) and bran layers, resulting in at white rice product. Dehulling process generates rice husk (Figure 6.10) equivalent to 20 % of the unprocessed paddy rice (rice harvested) [48]. Rice husk is usually used for energy/heat production in industrial practices [48]. Bran residue equals to 10 % of the paddy rice [49]. Numerous studies showed that bran residue has a high oil content (between 15 wt.% and 20 wt.%) and for this reason can be used for biofuels production [47, 50]. After the milling process, the quick deterioration of the bran makes this by-product unsuitable for human consumption [47]. In agreement, rice bran is mostly used for animal feed, namely due to its high protein content [50]. However, it is estimated that only 10 % of rice bran oil is used as edible oil, due to the high content of FFA [47]. In Portugal, rice bran is mostly recovered for animal feed, and in 2017 it was used 8122 ton, an higher value than that verified in 2016 (4592 ton) [51, 52]. Again, in 2017, the use of rice bran for animal feed was approximately 44 % of the total amount of rice bran produced in Portugal. For this reason, it is essential to study alternatives for the recovery of the remaining by-product, which from the gathered information, does not have a clear application.

6.1.6 WASTE ANIMAL FAT

Industry associated to food preparation produces a lot of fatty wastes and effluents that are not suitable to be directly used for biodiesel production [53]. The main animal fat sources are beef tallow, lard, poultry fat and animal fats. Tallow is the beef fat (Figure 6.11), lard is the pig fat and

chicken fat is a type of poultry fat. Animal fats are composed by a mixture of triglycerides, proteins, water and several minerals. These type of wastes can be found in municipal wastewater treatment plants, agri-food industries (slaughterhouses, dairies, canners) or restaurants (grease trap waste) [53]. In the study developed by Costa [54], the oil content determined for a sample from slaughterhouse was 18 wt.%.

The European Regulation (EC) N° 1069/2009 “laying down health rules regarding animal by-products and derived products not intended for human consumption” established that animal by-products are classified in three categories, according to the type of the by-product. In addition, the Portuguese Decree-law nº 152-C/2017 states that when animal fats classified as categories 1 and 2 are used as feedstock for biodiesel production, two biofuel titles are obtained (see section 1.4.1).

From slaughterhouses, the by-products/wastes consist mainly in bones, tendons, skin, blood and animal fats (Figure 6.11). These products correspond to 30 – 40 wt.% of the total weight of the animal and from this fraction, 2 – 3 wt.% correspond to fat with potential to be used as biodiesel feedstock [55].

The biodiesel produced from animal fats has a high calorific value [56], while it is less stable for the oxidation parameter due to the low content of natural antioxidants, compared to the vegetable oils [57].

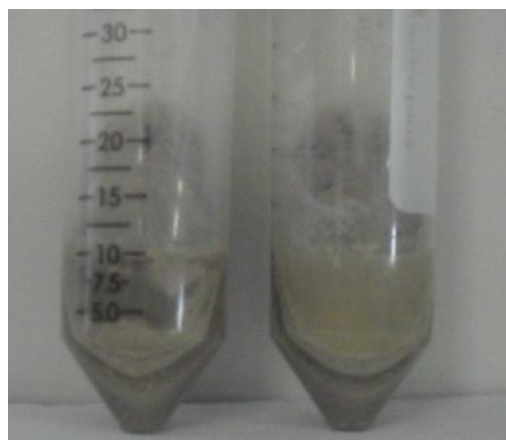


Figure 6.11. Sample from Slaughterhouse after centrifugation (oil content of 18 wt.%).

Another advantage is related to the cold filter plugging point which is higher for the biodiesel produced from animal fats than from vegetable oils [56]. This can be explained by the presence of high levels of saturated fatty acids in animal fats [58]. According to the study developed by United States Environmental Protection Agency [59], the generation of emissions during the combustion of

biodiesel produced from animal fats are lower than the emissions released by the conventional biodiesel [59].

The presence of FFA (5 – 30 wt.%) in this type of raw material makes the direct production of biodiesel unfeasible, being required a pre-treatment to reduce FFA content until it reaches acceptable levels. As an alternative, animal fats can be blended with other raw materials to obtain a mixture with FFA content, ideally lower than 1 wt.%, reducing extensive processing costs [57, 58].

According to the Portuguese Association of Producers of Animal Feed (“Associação Portuguesa de Industriais de Alimentos Compostos para Animais”), in 2017, animal by-products were mainly used for animal feed (6.19×10^4 ton) and animal fats represented 41% (2.5×10^4 ton) of the total animal by-products (2.60×10^4 ton) used [51]. In 2016, 5.2×10^4 ton were used for animal feed and animal fats represented 46% of the total [52]. For 2017, 1.9% of the total animal feed produced came from animal by-products.

In 2017, the total meat production verified in Portugal was around 8.90×10^5 ton, with a decrease of 0.4% when compared with 2016. The total bovine, pig, sheep, goat and horse meat had a decrease of 4.4 %, while poultry meat increased 5.3% (around 3.9×10^5 ton) [1].

Assuming that the total amount of animal fats was used for animal feed (2.5×10^4 ton) and knowing the total meat production (8.90×10^5 ton), it is possible to estimate that more than 2.8% of the animal weight might correspond to fats.

The economic activity related with slaughter of animals, preparation and preservation of meat and meat products was the most valued activity in the food industries with 18.7% of the total sales value (2017) [1].

6.1.7 WINERY WASTE

Winery industry produces huge amounts of wastes (seasonal wastes) which contain some valuable compounds such as polyunsaturated fatty acids (e.g. linoleic acid), tocopherols and polyphenols [60]. The management of this type of waste leads to several issues related with the storage and environmental impacts.

The pomace is constituted by various compounds, which includes seeds, skins and stems (Figure 6.12). From 100 kg of processed grapes, 25 kg are considered wastes (70 wt.% grape skins, 12 wt.%

stalks and 18 wt.% seeds) [61]. Moreover, it is forecasted that 1 kg of grape pomace is produced for each 6 L of wine [62, 63].



Figure 6.12. Winery waste from red grapes, constituted by stem (A) and seeds (B).

The high moisture and nutrients content promotes the development of bacteria and consequently the uncontrolled emission of greenhouse gases due to the natural decomposition of the winery wastes [61]. Landfilling of this wastes represents a high risk for the dissemination of pests/diseases because of the potential infiltration of alcohols and organic acids in the soil and groundwater (environmental impacts) [61].

Among the different wastes from the winery activities, grape seeds show potential to be used for bioenergy purposes, due to their oil content (10 – 20 wt.%) [64]. Grape seed oil shows high content of unsaturated fatty acids, mainly linoleic acid (72 – 76 wt.%) [65]. Besides the potential for bioenergy production, grape seed oil presents tocopherols (antioxidant properties) with a concentration between 63 and 1208 ppm. Studies also refer that grape seeds can be used to prevent cataracts and act as an anti-inflammatory or antihyperglycemic substance, considering their antioxidant properties and radioprotective effects [64, 66]. Other studies suggest that grape pomace when mixed with other products can be used as a soil fertilizer [67].

The annual processing of grapes leads to a production of 30 million ton of press residues and the seeds represent 38 – 52 wt.% of the dry pomace [66].

According to the International Organization of Vine and Wine, in 2016, 7.5×10^6 ha (worldwide) were used for vines leading to a wine production of 267×10^6 hL [61]. In the same year, in Portugal, the vineyard area was around 2.0×10^5 ha, corresponding to a production of 8.0×10^5 ton of fresh grape (6×10^6 hL of wine). Knowing these values and taking into account that from 100 kg of processed grapes, 25 kg are considered wastes (18 wt.% seeds), it is possible to estimate a Portuguese production of 2.0×10^5 ton of grape pomace (wastes) and 3.6×10^4 ton of grape seeds that could be recovered and used for bioenergy production.

In the World, vineyards represent almost 45% of the total agricultural area and only 1.8% of the total used area in the EU [68]. Spain, France and Italy are responsible for three quarters of EU vineyards area in 2015. Nevertheless, Portugal represented 6.1% of the total vineyard area in the EU [68].

According to the International Organization of Vine and Wine, in 2016, Europe produced a total amount of fresh grapes of around 2.8×10^7 ton which corresponds to a production of grape pomace of 7.0×10^6 ton [68]. In agreement, the estimated production of grape pomace in Portugal (2.0×10^5 ton) might represent around 3% of the total amount of grape pomace in Europe. The high amount of wastes produced by the winery industry seems to justify the development of strategies to the recovery of this type of waste, which has Europe as one of the biggest producers.

6.2 CONCLUSIONS

Several organic-rich wastes and by-products are produced from food industry, edible oils production or wastewater treatment as well as at the agricultural sector; however, the majority is disposed of or, in the best scenario, used for the production of animal feed and organic amendment. Such alternatives add little value to the producer or, in most cases, represent significant economic and environmental costs. For this reason, it is highly relevant to develop integrated solutions that enable the implementation of sustainable material management practices (for those materials) and increased value into the economic sector.

Waste cooking oil, waste coffee grounds, soapstock, olive pomace, rice bran, waste animal fat and winery waste are some types of wastes/by-products produced in large amounts in Portugal. Most of these products have a low economic value and consequently, in most cases, are not properly recovered. As a common characteristic, these types of wastes/by-products have some oil content,

which can be used for biodiesel production. Based on the estimated amount of each type of wastes/by-product produced in Portugal, the following rank of the seven types of wastes/by-products was obtained: Olive Pomace (3.4×10^5 ton) > Winery waste (2.0×10^5 ton) > Spent coffee grounds (1.0×10^5 ton) > Waste cooking oils (6.7×10^4 ton) > Waste animal fat (6.1×10^4 ton) > Rice bran (8.1×10^3 ton) > Soapstock (6.7×10^3 ton). Overall, a total amount of 7.8×10^5 ton of wastes/by-products with high potential to be used as source of oils for biodiesel production is generated on an annual basis, in Portugal. For a better understanding of the real potential of each type of waste/by-product as source of oils, further studies are required aiming to estimate the overall recovery of oil, which strongly depend upon the characteristics of the wastes/by-products (moisture content, impurities, among others).

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CHAPTER 7. *SELECTED BY-PRODUCTS AND WASTES AS RAW MATERIALS FOR ENERGY PRODUCTION*

7.1 SOAPSTOCK ACID OIL BY-PRODUCT FOR ENERGY PRODUCTION

ABSTRACT

In this study, the efficiency of esterification vs glycerolysis for the reduction of free fatty acid content of acid soapstock oil ($128.8 \text{ mg KOH g}^{-1}$) was studied. Further, alkaline transesterification of the pre-treated oil mixed with sunflower oil (to achieve FFA content $< 2\%$) was conducted to maximize fatty acid methyl esters (FAME) production. Homogeneous acid esterification was conducted at 65°C , using $2.0 \text{ wt.}\% \text{ H}_2\text{SO}_4$ as catalyst and $9:1$ molar ratio of methanol to oil. Glycerolysis was performed at 200°C using a $6:1$ molar ratio of glycerol:oil. Both reactions lasted 6 h . Final acid value of the product was around 10 mg KOH g^{-1} for both processes, showing negligible differences between them. After transesterification of the mixtures it was possible to obtain biodiesel with around 90% FAME content, thus showing potential for the recovery of such waste material.

7.1.1 SCIENTIFIC RELEVANCE

The soapstock is a waste from the chemical process used in the refining of vegetable oils, resulting from the neutralization of FFA. Usually, it is acidified with a strong mineral acid, such as sulfuric or hydrochloric acid to produce an acid oil that consists essentially of FFA [1]. The use of soapstock for biodiesel production is therefore a very good alternative for cost reduction of biofuel production and to solve environmental problems such as the competition of land use for food production against energy production [2].

Biodiesel is obtained by the transesterification reaction of triglycerides through alkaline catalysis. However, the alkaline catalysis cannot be applied to a material with high FFA (as the acid oil from soapstock) content due to soap formation; the conventional reported limit is 1 wt.% FFA content [3]. When the aim is to lower the FFA content, two processes are highlighted: acid esterification and glycerolysis. Homogeneous acid esterification by which the FFA present in the oil react with an alcohol to produce esters and water, is highly implemented for the reduction of FFA content of highly acidic raw materials, with the main variables being temperature, catalyst concentration and type, and alcohol: oil molar ratio [2]. The drawback of this process is the use of a highly corrosive reagent (mostly sulfuric acid) and the need to refine the products and separate/recover the catalyst. Glycerolysis allows the esterification by reaction of FFA to obtain glycerides (MG, DG and TG) as well as water. This process implies high temperatures, but can be conducted without homogeneous catalyst addition, thus avoiding expensive purification stages.

7.1.2 SPECIFIC OBJECTIVES

The present study, focused on the by-product acid oil from soapstock had as main goal to evaluate its characteristics and viability for biodiesel production. The following specific objectives were established:

- To evaluate homogeneous acid esterification pre-treatment aiming FFA reduction;
- To evaluate glycerolysis pre-treatment as alternative pre-treatment for FFA reduction;
- To maximize FAME production after alkaline transesterification of the pre-treated acid oil in mixture with sunflower oil.

7.1.3 MATERIALS AND METHODS

7.1.3.1 *Materials*

The acid oil from soapstock of vegetable oil refining (mixture of seeds) was provided by the company Nature Light, S. A. Commercial sunflower oil was used. Methanol (Fischer Scientific $\geq 99\%$) and glycerol were used as the acyl acceptor. The sulfuric acid used as catalyst had 98 % of concentration. All reagents were of analytical grade.

7.1.3.2 *Analytical methods*

The physicochemical properties determined in the raw material were acid value and water content. The acid value was determined by volumetric titration, according to NP EN ISO 660 (2009) and it was also determined to evaluate the progression of the esterification reactions. Considering the expected high values of moisture, this parameter was determined by weight loss at $T = 105\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$, until constant weight, according to EN 12880 (2000).

The methyl ester content in the final product (after transesterification) was determined according to referred previously in section 3.3.3.3. All FAME analyses were performed in duplicate and the variation was evaluated in terms of the relative percentage difference (RPD) to the mean. In all cases, RPD was less than 1.5 %.

7.1.3.3 *Pre-treatment of acid oil from soapstock*

All experiments were performed in duplicate and the variation of the results is expressed as the relative percentage difference to the mean (RPD).

- *Homogenous acid esterification*

For the acid esterification reactions, the following reference conditions were selected according to the literature [3]: 65°C , 2.0 wt.% H_2SO_4 catalyst, 6 h and 6:1 molar ratio of methanol to acid oil. In addition, a 9:1 molar ratio of methanol:oil and a 4.0 % of acid concentration were studied, maintaining the other variables.

The setup of the batch reactor and the monitoring of the reaction (0.5 mL of sample from the reaction mixture was collected at different time intervals - 0, 15, 30, 60, 120, 180 and 360 min) was

made according to Dias *et al.* [5]. The amount of sample (acid oil) used for each experiment was 50 g. At the end of the esterification reactions the pre-treated oils were washed to remove excess catalyst and dried for determination of the final real acid value of the product and fatty acid methyl ester (FAME) content.

- *Glycerolysis*

The best conditions and the installation of Costa *et al.* [6] were used ($T = 200\text{ }^{\circ}\text{C}$, 3:1 molar ratio of glycerol:oil, 6 h). The use of a 6:1 glycerol:oil molar ratio was also evaluated, maintaining the same temperature. The glycerolysis was also carried out using 50 g of acid oil and 0.5 mL samples were also collected to evaluate the evolution of the reaction (0, 60, 120, 180, 360 min) [4].

7.1.3.4 Alkaline transesterification of pre-treated oil

Synthesis of biodiesel was made with the best result in terms of acidity reduction obtained from esterification reactions studied. When conducting a transesterification reaction by homogeneous alkaline catalysis, the acidity limit of 1 wt.% was considered. Thus, the reaction products were mixed with sunflower oil to obtain a raw material with such maximum acidity.

The alkaline transesterification was performed in a batch reactor at $65\text{ }^{\circ}\text{C}$ during 1 h [3]. The amount of catalyst was 1 wt. % NaOH and the methanol:oil molar ratio was 6:1. Acid/water washings and drying procedures also agreed with the mentioned study.

7.1.4 RESULTS AND DISCUSSION

7.1.4.1 Raw Materials Properties

The properties of the acid waste oil are presented in Table 7.1.

Table 7.1. Acid waste oil physicochemical properties determined

	Acid value (mg KOH g ⁻¹)	Water content (wt. %)
Result	129.8	2.7
RPD* (%)	0.4	3.5

* Relative Percentage Difference to the mean.

The acid value obtained agrees with those found in the literature, which ranged from 81.9 mg KOH.g⁻¹ to 199.3 mg KOH.g⁻¹ [3, 5, 6]. Similar water content was reported by Pérez-Bonilla *et al.* [7] (2.2 wt.%) for soapstock oil; however, Su & Wei [6] studied an acid oil from soapstock with much less water content, of 0.185 wt.%.

7.1.4.2 Homogeneous Acid Esterification

Figure 7.1. shows the results from the acid esterification of the soapstock oil, under the different reaction conditions studied. It should be highlighted that the acid value was determined in the reaction mixtures and the acid concentration and methanol amount will affect such value; for that reason, initial acid values differ and normalized values to the highest initial acid value (difference between initial acidities corrected for all sampling points) are plotted.

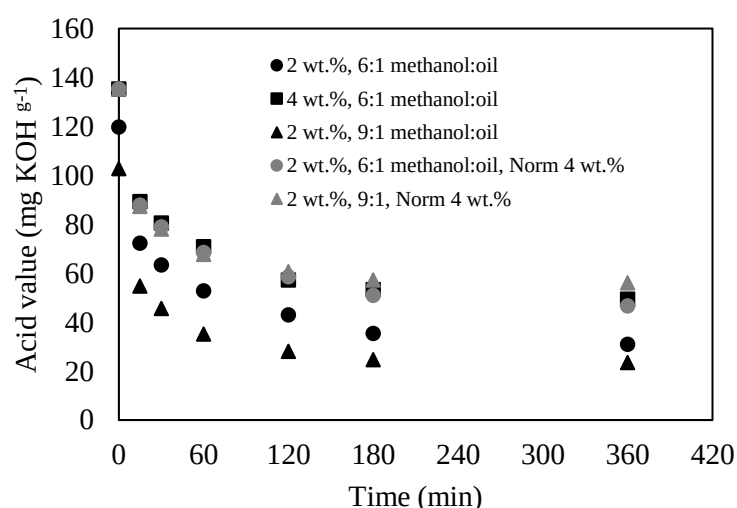


Figure 7.1. Evolution of the acid value during the esterification of the acid oil (65 °C), considering real values and normalized.

The reaction progresses fast until 120 min and then tends to stabilize. The normalized values show negligible differences under the different reaction conditions. Considering the real values, the highest acidity reduction $[(Acidity_{0h} - Acidity_{6h})/Acidity_{0h} \times 100]$, in percentage was achieved when the highest methanol to oil molar ratio was used, being around 77 %, close to that obtained using 6:1 methanol:oil molar ratio and 2 wt.% catalyst (74 %). The lowest reduction, of around 64 wt.% was obtained for the highest concentration studied. Such results differ slightly from what is observed for the normalized values. Considering the results variations (RPD < 7 %), differences are not considered meaningful.

Dias *et al.* [3] selected as the best reaction conditions for acid lard acid esterification (initial acid value of 26 mg KOH.g⁻¹) 65°C, 2.0 wt% H₂SO₄, 6:1 molar ratio of methanol to lard for 5 h. In the present study, despite the use of a raw material with a much higher acidity, the results are in agreement.

The FAME content of the final products was very similar in all cases, being between 68.3 wt.% and 69.3 wt.%. Table 7.2 presents the acid values of the esterification product, after purification. The lowest acid value was in fact obtained when using the highest methanol to oil molar ratio; for this reason, this oil was used for biodiesel production by alkaline transesterification in mixture with sunflower oil.

Table 7.2. Acid value of pre-treated oils after acid esterification at the different reaction conditions (H₂SO₄, 65°C)

Reaction conditions	Acid value (mg KOH g ⁻¹)	RPD (%)
2 wt.%, 6:1 methanol:oil molar ratio	13.86	3
4 wt.%, 6:1 methanol:oil molar ratio	20.71	5
2 wt.%, 9:1 methanol:oil molar ratio	11.46	7

7.1.4.3 Glycerolysis

Figure 7.2. presents the results from the glycerolysis of the acid oil. The glycerol dilution effect affects the acid value of the mixtures but reaction progresses very fast at the beginning leading to high variations for the measurements at time zero; the curves show different behaviours, compared to what was observed under different reaction conditions in section 7.1.4.2, being more accentuated in the case of using the of 3:1 glycerol:oil molar ratio. At the end of the reaction period, differences were minor. Overall, taking into account the initial acid values (zero time of reaction), the FFA reductions were 87% and 83%, using 3:1 and 6:1 glycerol:oil molar ratio.

The lowest acid value of the product was around 10 mg KOH g⁻¹. It should be referred that Costa *et al.* [4] achieved in the first 15 min of glycerolysis reaction a reduction in the acid value of 92% using an oil obtained from an urban wastewater sludge as raw material with 140 mg KOH.g⁻¹ of initial acid value reaching a 8.21 mg KOH g⁻¹ in the final product under the same conditions evaluated in the present work.

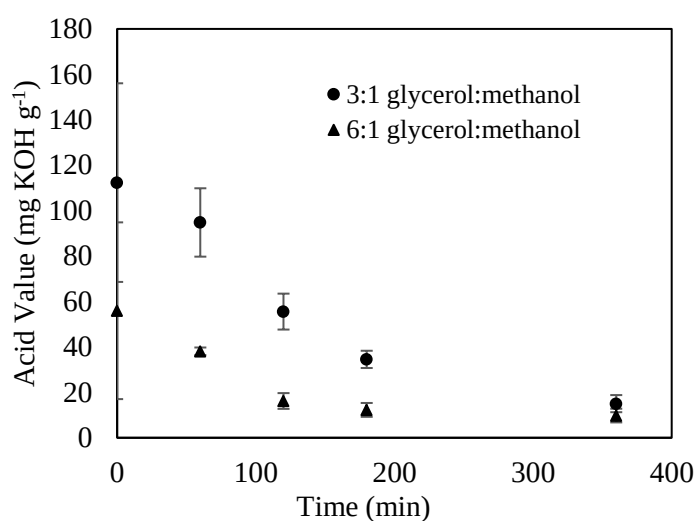


Figure 7. 2. Evolution of the acid value during the glycerolysis of the acid oil (T = 200 °C).

7.1.4.4 Alkaline Transesterification of Oil Mixtures

The biodiesel production was performed using the mixture of sunflower oil with the pre-treated oils from both reactions studied. With the objective to obtain one mixture with an acid value suitable for biodiesel production (max. 2 wt.%), the following equation was used to determine the amount of each component:

$$\text{Acid Value}_{\text{mix}} = (\text{Acid Value}_1 * X) + (\text{Acid Value}_2 * (1 - X)) \quad (7.1)$$

The acid value of commercial sunflower oil was 0.214 mg KOH.g⁻¹ and the value used for the Acid Value_{mix} was 2 mg KOH.g⁻¹ due to reasons explained. FAME content of the final products is presented in Table 7.3.

Table 7.3. Fatty Acid Methyl Ester content of biodiesel produced using different feedstocks

Feedstocks	FAME (wt.%)
Sunflower oil	97.6
Acid Esterification Product + sunflower oil	88.2
Glycerolysis Product+sunflower oil	89.2

According to the values in Table 7.3, the differences between using the two products are negligible, which agrees with the efficiencies previously observed and the final similar acid values of both products. The obtained FAME content is clearly below the requirements for use as automotive fuel (> 96.5 wt. required).

In Table 7.4 is presented the fatty acid profile obtained for soapstock oil and compared with the literature (soybean soapstock acid oil). In the literature was possible found a fatty acid profile for soybean soapstock acid oil.

Table 7.4. Fatty acid methyl esters profile for biodiesel obtained from soapstock raw oil (wt.%.)

Methyl Ester of the following fatty acids	FAME (wt.%)	[5]
Palmitic (C16:0)	10.3	16.5
Stearic (C18:0)	3.9	4.2
Oleic (C18:1)	43.4	33.4
Linoleic (C18:2)	40.3	44.2
Others (< 3 wt.% each)	2.10	1.70

According to the results, soapstock of vegetable oil refining (mixture of seeds) is mostly constituted by oleic and linoleic fatty acid (unsaturated fatty acids). Comparing the results with the referred in the literature is possible verify differences, mostly verified for the concentration of palmitic and oleic acid. The differences can be justified by the fact that the soapstock oils analysed result from different type of oils (mixture of seeds and soybean seeds).

7.1.5 CONCLUSIONS

In the present study, homogeneous acid esterification and glycerolysis of a highly acidic soapstock oil (129.8 mg KOH.g⁻¹) showed potential towards free fatty acid reduction, with a final acid value of the product being close to 10 mg KOH.g⁻¹ using both processes.

After alkaline transesterification of a mixture of the products with sunflower oil, biodiesel with 90 wt.% fatty acid methyl ester content was obtained. Although FAME content is still below the requirements imposed by EN 14214, the pre-treatment procedures show clear potential for the reduction of acidity of this alternative oil.

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7.2 RICE BRAN BY-PRODUCT FOR ENERGY PRODUCTION

ABSTRACT

Rice bran oil might be an alternative low-cost raw material for biodiesel production; however, to enable its use, a pre-treatment process to reduce the free fatty acids content is required. In this study, a preliminary evaluation on the use of this oil was conducted. Two pre-treatment processes, acid and enzymatic esterification, were assessed using an oil with an acid value around 47 mg KOH.g⁻¹. The acid esterification pre-treatment was employed at three reaction temperatures of 45, 55 and 65 °C, with catalyst concentrations of 2 wt.% or 4 wt.% of H₂SO₄ and a fixed methanol:oil molar ratio of 9:1. Enzymatic esterification was conducted at 35 °C using a 6:1 methanol:oil molar ratio and 5 wt.% of lipase *Thermomyces lanuginosus*. Homogenous acid esterification enabled 92 % acidity reduction at the maximum temperature and catalyst concentration after 8 h, with the final product presenting a fatty acid methyl ester content (FAME) of 42 wt.%. After 24 h, the enzymatic process allowed to reach an acidity reduction of 82 % and a FAME content of 87 wt.%, thus clearly revealing the conversion of free fatty acids and glycerides into FAME. The results obtained show the high potential of using rice bran acid oil for biodiesel production and, more specifically, of employing the biocatalysed process for the conversion of free fatty acids and glycerides into FAME, avoiding other pre-treatment steps.

7.2.1 SCIENTIFIC RELEVANCE

Since 1880, the global average surface temperature rise is estimated to be 0.85 °C (in 1880 – 2012) confirming the importance and urgency of world action to minimize climate change [1]. Although having awareness about the impact of greenhouse gases emissions (GHG), the consumption of fossil fuels still shows an increase [2]. The transport sector represents 29 % of the total energy consumed in the world and 65 % of world oil consumption [2], contributing with 24 % of the total CO₂ global emissions. Meanwhile, to face the emissions limits imposed, the use of biofuels has been promoted.

Rice is the second most consumed cereal in the world, only exceeded by wheat. In Portugal, in 2017, the area of rice sown was around 30 000 ha corresponding to an annual production around 180 kt [3]. It should be noted that the national consumption per capita is in average 15.3 kg, three times more than the European average. By-products such as rice bran might be seen as a non-conventional and low cost feedstock for biodiesel production [4]. This by-product results from the milling process of rice grain. The processing consists in the removal of the husk (dehulling) and bran layers, resulting at the end, the white rice product. Dehulling process generates rice husk equivalent to 20 % of the unprocessed paddy rice (rice harvested) [5]. Rice husk is usually used for energy/heat production in the industrial process [5]. Bran residue corresponds to 10 % of the paddy rice [6].

In the Portuguese context, rice bran is mostly used for animal feed, corresponding to an annual consumption of 8122 ton in 2017 [3] which represents only approximately 44 % of the total amount of rice bran produced in Portugal, the remaining by-product does not have an established recovery solution. The literature reports its high oil content (15 – 20 wt.%) which is known to be rich in free fatty acids (FFA), 25 to 50 wt.%, particularly due to presence of endogenous lipases which catalyze the hydrolysis of triglycerides to FFA [7]. At an industrial scale, biodiesel is conventionally produced by a chemical process which includes a homogeneous alkaline transesterification reaction. Alkaline catalysis cannot however be employed when the raw material has a high FFA content. To overcome this drawback, the pre-treatment of the rice bran oil is necessary, namely by acid or enzymatic esterification. Depending on the conditions, both processes might enable both the esterification and the transesterification, although at a slower reaction rates than homogeneous alkali processes [8]. Esterification reaction leads to the production of fatty acid methyl esters (biodiesel) and water. From the transesterification reaction, biodiesel and glycerol are obtained.

In the present work, the potential of acid RBO as a low-cost feedstock for bioenergy production was exploited. For that, the oil was extracted from the by-product and two different catalytic routes were assessed to convert the FFA present into fatty acid methyl esters (FAME), in particular, enzymatic esterification (where no studies exist) and homogeneous acid esterification, in order to contribute for its assimilation by the biodiesel industry.

7.2.2 SPECIFIC OBJECTIVES

The present study focused on rice bran by-product and had as main goal to study the characteristics of the acid oil and its viability as raw material for biodiesel production. Accordingly, the following specific objectives were established:

- To evaluate homogeneous acid esterification pre-treatment for FFA reduction;
- To evaluate heterogeneous enzymatic esterification pre-treatment for FFA reduction;
- To maximize FAME production after alkaline transesterification of a mixture with the pre-treated acid oils and sunflower oil.

7.2.3 MATERIALS AND METHODS

7.2.3.1 *Materials*

The RBO was extracted from rice bran produced by a Portuguese company from the North of Portugal. The alcohol used in the esterification reaction was methanol (Fischer Scientific $\geq 99\%$). The enzyme used as catalyst was lipase *Thermomyces lanuginosus* (Lipolase 100L, activity $\geq 100,000$ U/g), purchased from Sigma-Aldrich. For homogenous acid esterification, concentrated sulphuric acid (H_2SO_4) was employed (Fischer Chemical). For the oil extraction, petroleum ether (LabChem) was used as solvent. All the reagents required for analytical procedures were of analytical grade.

7.2.3.2 *Analytical methods*

The oil extracted from rice bran was characterized in terms of free fatty acids content by volumetric titration (NP EN ISO 660). The oil content was determined in duplicate using a 100 mL Soxhlet extractor, according NP EN ISO 659 (2002). Oxidation stability was determined by accelerated

oxidation using 873 Biodiesel Rancimat equipment from Metrohm. Viscosity at 40 °C, measured according to ISO 3104 using capillary viscometers immersed in a thermostatic bath.

The FAME content in the final product was measured by gas chromatography (GC) according to EN 14103, as referred previously in section 3.3.3.3. All FAME analyses were performed in duplicate and the variation was evaluated in terms of the relative percentage difference (RPD) to the mean. In all cases, RPD was less than 1.5%.

7.2.3.3 Pre-treatment of rice bran oil

Rice bran oil (mostly constituted by C18:1 $\approx$ 46 wt.%; C18:2 $\approx$ 39 wt.% C16:0 $\approx$ 11 wt.%; 883 g.mol⁻¹) was submitted to a heterogeneous and a homogenous catalyzed esterification. Figure 7.3 shows the schematic procedure adopted for RBO FFA conversion into FAME.

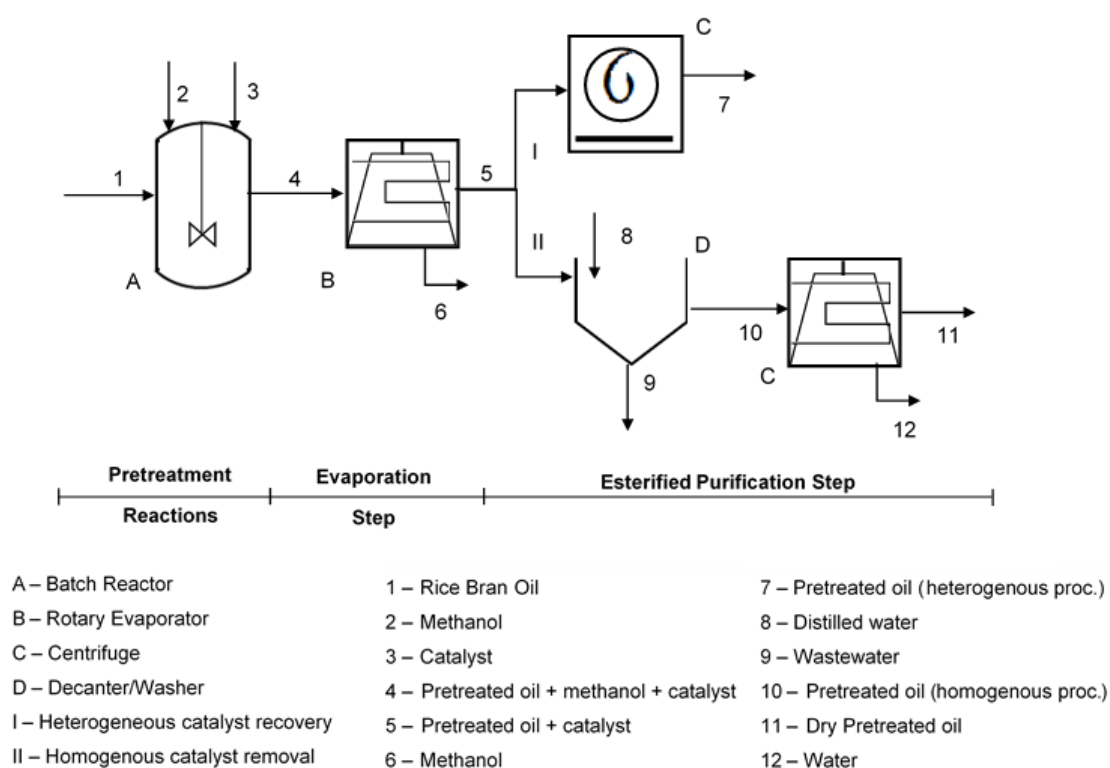


Figure 7.3. Diagram of RBO pre-treatment using both enzymatic esterification and homogeneous acid esterification.

- *Pre-treatment of RBO by homogeneous esterification*

For the homogenous acid esterification reactions, the standard conditions were selected according to previous studies [9, 10]. Each assay required 15 g of RBO. Table 7.5 summarizes the conditions used. Three different temperatures of 45°C, 55°C and 65°C and two catalyst amounts of 2 wt.% and 4 wt.% of the oil were evaluated. The methanol:oil molar ratio used was 9:1, being reported as effective [10].

Table 7.5. Experimental conditions for homogenous acid esterification

Assay	Temperature (°C)	Catalyst (wt.%)	Alcohol:oil molar ratio
1	45	2	9:1
2	45	4	9:1
3	55	2	9:1
4	55	4	9:1
5	65	2	9:1
6	65	4	9:1

To monitor the progression of the reaction, the acid value of the mixture was measured at 15, 30, 60, 120, 240 and 480 min, after the starting of the reaction. At the end of the esterification reactions (480 min), the excess of methanol was removed in a rotary evaporator (T = 65 °C; 200 mbar, 30 min) and the pre-treated oils were washed with 50 vol.% of water to remove the excess of catalyst (Figure 7.3). The acid value of the final product and its FAME content were determined. For the condition with the highest FFA reduction, experiments were performed in duplicate to validate the results.

- *Pre-treatment of RBO by heterogeneous esterification*

In the case of heterogenous enzymatic esterification, the RBO was added to a batch reactor placed in an orbital shaking incubator (Agitorb 200IC) where methanol and the enzyme were introduced. The amount of methanol (6:1 methanol:oil molar ratio) taking into account the oil's molecular weight (883 g.mol⁻¹). The following conditions were used: stirring at 200 rpm, 24 h, and 35 °C, according to previous studies [11] and recommendations of the catalyst supplier. Methanol addition was performed stepwise after being verified that the single addition inhibited the enzyme [8, 11];

this was made as referred in the study of Bonet-Ragel *et al.* [12]. The enzyme concentration selected was 5 wt.% (relative to oil). The enzyme's concentration agree with those mostly referenced in the literature [11, 13]. Samples were also collected from the reactor in different time periods (the zero time was considered when the temperature stabilized after catalyst addition): 15, 30, 60, 120, 240, 480 and 1440 minutes, to measure the FFA content. In the end, the excess of methanol was recovered in a rotary evaporator ($T = 65\text{ }^{\circ}\text{C}$; 200 mbar, 30 min) and the remaining fraction was centrifuged during 5 min at 3500 rpm with the objective to recover the enzyme (Figure 7.3).

7.2.3.4 *Transesterification*

Synthesis of biodiesel was performed, with the best result in terms of acidity reduction, for homogenous acid esterification. The product of homogenous acid esterification (FFA content > 2 wt.%) was previously mixed with sunflower oil (SFO), to obtain a raw material with suitable acidity for alkaline transesterification (< 2 wt.%). For acid esterification reaction, the presence of acid catalyst lead to development of two reactions, namely, transesterification and esterification; however, a low FAME content is expected due to the slow reaction rates [8, 14, 15]. For this reason, transesterification reaction was only performed for the pre-treated oil obtained by acid esterification (lowest FAME content).

Another reason is related to the fact of enzymatic esterification use enzyme as catalyst avoiding the wash of the final product with water. Because that, there was no interest in performed alkaline transesterification, which required the wash of biodiesel with water generating high amounts of wastewater. The alkaline transesterification was performed in a batch reactor at 65°C during 1 h. The amount of catalyst was 1 wt.% (NaOH) and the methanol:oil molar ratio was 6:1 [16]. Acid/water washings and drying procedures were also performed according to previously published procedures [16]. The weight percentage of each oil (pre-treated RBO and sunflower oil) mixed to obtain a blend with acidity close to 2 wt.% was calculated according equation 7.2 (virgin oil presented an acidity of 0.39 %).

$$Acidity_{RBO+SFO} = [Acidity_{RBO} * X] + [Acidity_{SFO} * (1 - X)] \quad (7.2)$$

According equation 7.2, the unknow variable is determined take into consider the acidity RBO+SFO which was defined by the authors according the should value referred in the literature for transesterification reaction (2 mgKOH.g^{-1}) and the acidity of RBO and SFO which was determined.

7.2.4 RESULTS AND DISCUSSION

7.2.4.1 Raw material properties

As referred in section 7.2.3.2, several properties of rice bran oil were assessed before pre-treatment performance. In rice bran was possible found an oil content of 18.9 ± 0.5 wt.%. The high oil content found, open the perspective of use this by-product for energy production. The oil content was in the range found in the literature for rice bran [7, 17]. The properties of the RBO are presented in Table 7.6.

Table 7.6. Physicochemical properties of rice bran oil

	Acid value (mg KOH g⁻¹)	Viscosity at 40 °C (mm².s⁻¹)	Oxidation Stability (h)
Result	46.5	29.0	7.6
RPD* (%)	0.3	5	5

* Relative Percentage Difference to the mean.

RBO had a very high acidity of 46.5 ± 0.2 mg KOH.g⁻¹, close to the maximum values reported in the literature [7]. As previously mentioned, the high acidity is mainly related with the presence of lipases, and, more specifically, due to the rupturing of the cells during the rice milling which leads to a closer contact between the lipase and the oil, causing its hydrolysis. For this reason, during the storage of the rice bran the quality of the oil decreases.

For direct observation, it is possible conclude that rice bran oil is very viscous at room temperature. This aspect was confirmed by cinematic viscosity determination (29.0 mm².s⁻¹). The value found was close to report in the other study (32.2 mm².s⁻¹) [18]. When converted into methyl ester rice bran oil, the viscosity decrease until 6 mm².s⁻¹ [18].

For the oxidation stability property, few information is available in the literature. The value obtained for the RBO oxidation stability (4.77 h) was higher than that found in other study for rice bran oil with 1 month of storage [19]. Due to the lack of information about oxidation stability of RBO, it is suggested that future works should be develop regarding RBO oxidation stability and also the identification of natural antioxidants presents in this oil. It is expected that the biodiesel produced from RBO has an oxidation stability lower than 7.6 h due to the removal of natural antioxidants

during the washing steps of biodiesel purification. According to El Boulifi *et al.* [20] before the storage, biodiesel produce from RBO showed oxidation stability of 1.7 h.

For a commercial biodiesel, the oxidation stability should be higher than 8 h.

7.2.4.1 *Pre-treatment of rice bran oil*

- *Pre-treatment of RBO by homogeneous esterification*

For a better assessment of the homogenous acid esterification process, the acidity of the oil was monitored at different time intervals. Results are presented in Figure 7.4 (A and B).

The use of different amounts of acid catalyst (2 wt.% or 4 wt.%) leads to different initial acid values; thus, direct comparisons should not be performed. In agreement, Figure 7.4 C shows the acidity evolution for a catalyst concentration of 2 wt.% and for normalized values (4% Norm). Normalized values were obtained by discounting the difference between the initial acidity of the products (from 2 to 4%), which results from the excess of mineral acid, to compare the results at the different catalyst concentrations.

According to the Figure 7.4 (A and B), when increasing the temperature from 45°C to 55°C or 65°C, the rate of the esterification reaction increases significantly during the first two hours. After 8 h, similar results are obtained. According the Figure 7.4 (C), the results indicate the relevance of increasing catalyst concentration from 2 to 4 wt.% for the temperatures of 55°C or 65°C whereas at 45°C no benefit is evidenced. Results using 55°C or 65°C were similar and this was corroborated by the acid value determination after purification, which was very similar for products obtained under such conditions (Table 7.4). In fact, several studies suggest that the optimal reaction temperature to pre-treat acid oil by esterification reaction is in the range of 55°C to 65°C [21, 22].

Under the best conditions (65°C and 4 wt.% of catalyst), after 60 min of reaction, the acidity tends to stabilize, showing no relevance in increasing the reaction time.

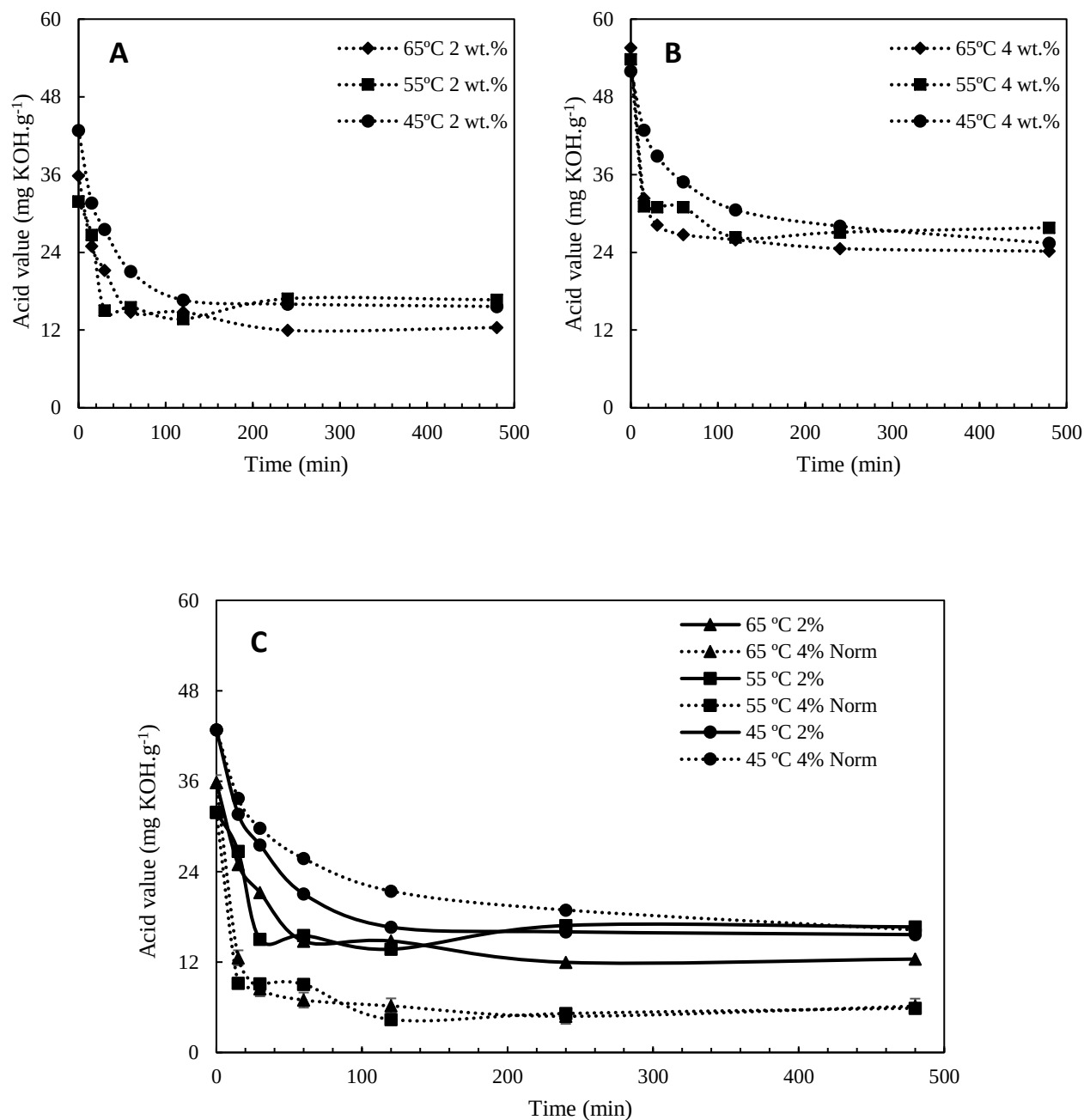


Figure 7.4. Evolution of the acidity during the homogenous acid esterification of the acid oil for a catalyst amount of 2 wt.% (A) and 4 wt.% (B) at different temperatures; results comparing the use of 2 wt.% catalyst and normalized values in the case of 4 wt.% (C).

Table 7.7 shows the acid value and the FAME content of the final product obtained at the different experimental conditions.

Table 7.7. Characteristics of the homogenous acid esterification final product

	65 °C, 4 wt. %	65 °C, 2 wt. %	55 °C, 4 wt. %	55 °C, 2 wt. %	45 °C, 4 wt. %	45 °C, 2 wt. %
Acid value (mg KOH.g ⁻¹)	3.7	5.4	4.5	7.7	6.8	5.8
FAME content (wt.%)	42	30	35	29	28	24

By considering only the esterification reaction, it is expected that lower acid values correspond to higher FAME contents; however, in some cases it is possible to observe that although the acid value is higher, the FAME content is also higher (e.g. comparing the results at 45°C using 2 wt.% and at 55°C using 2 wt.% of catalyst). Besides that, with a lower expression compared to what is verified for the heterogeneous process further described, the results suggest that, apart from the FFA, also mono-, di- and triglycerides were converted into FAME by the homogeneous acid transesterification reaction, thus affecting such trends.

It is reported that sulphuric acid can catalyze the transesterification reaction in the temperature range from 55°C up to 88°C [23]. As an example, according to the study developed by Canacki *et al.* [24], transesterification reaction can be performed at 65°C with 3 wt.% of sulphuric acid, leading to a final FAME content of 88 wt.% after 48 h of reaction. In the study developed by Costa *et al.* [10], which applied homogenous acid esterification (65°C; 2.0 wt% of catalyst; 9:1 methanol:acid oil molar ratio) using soapstock acid oil (with an initial acid value of 128.8 mg KOH g⁻¹), after 7 h a product with less 77 % of acidity was obtained, presenting a FAME value of 69 wt.%.

- *Heterogeneous enzymatic esterification pre-treatment*

The use of heterogenous catalysts in the esterification reaction provides easier purification of the final product, being pointed out as a more eco-friendly alternative process [25].

Based on the literature, it is expected that 5 wt.% of catalyst concentration should be enough to reduce the acidity of the oil until acceptable levels. Although having the possibility to reuse the enzyme, due to economic constraints, the optimization of enzyme's amount is of high relevance

[26]. Figure 7.5 shows the evolution of acidity reduction at 35°C, after addition of methanol in five steps for 5 wt.% of catalyst concentration.

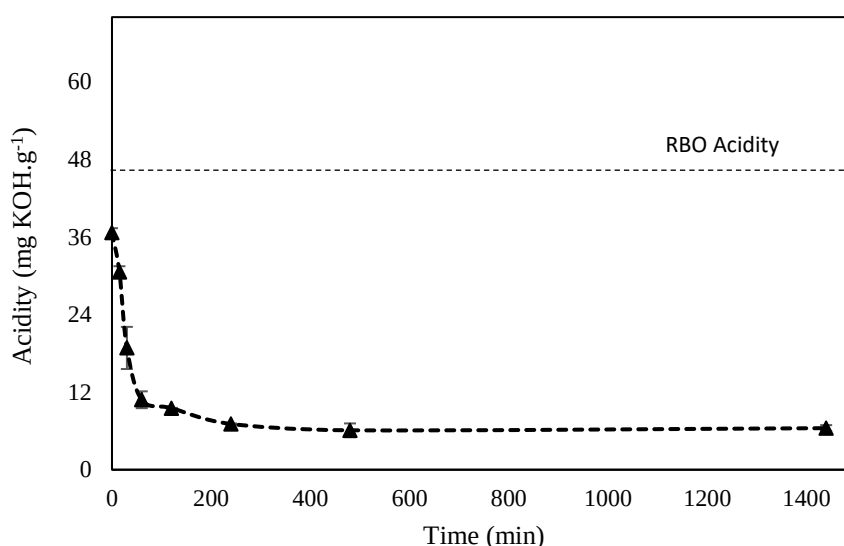


Figure 7.5. Acidity reduction during heterogenous enzymatic esterification reaction at 35 °C, for 6:1 methanol:oil molar ratio with stepwise alcohol addition (5 wt.% of catalyst). Errors bars show maximum and minimum values of the experiments in duplicate.

According to Figure 7.5, in the beginning of the reaction, the acidity of the mixture decreases very fast, for almost 36 mg KOH.g⁻¹ (initial acidity of the oil was 46.5 mg KOH.g⁻¹). Some of this acidity reduction is due to the addition of methanol (dilution effect). After only 60 min, it was possible achieved an acidity reduction of 70 % (10.8 mg KOH.g⁻¹).

After purification (removal of methanol and enzyme), the rice bran pre-treated oil showed an acid value of 7 mg KOH.g⁻¹ (Table 7.8).

Table 7.8. Characteristics of the product obtained after heterogenous esterification of RBO

	35 °C, 5 wt. %
Acid value (mg KOH.g ⁻¹)	7
FAME content (wt. %)	87

As initial FFA content represents only around 23 wt.% of oil's mass (46.5 mg KOH.g⁻¹ of acid value for unrefined RBO) which can be converted into FAME, it is possible to conclude that esterification and transesterification reactions occurred simultaneously; and, in this case, transesterification was

extensively promoted. Similar results were previously found [11, 14, 27]. The first 8 h of reaction corresponded to the period with the highest reduction of acidity (82 %). After that, the acidity reduction is very low, with a final result after 24 h being similar to those obtained after 8 h. In the work of Mulalee *et al.* [13] which studied the esterification reaction of acid palm oil (5 wt.% of enzyme; 45°C; 2:1 ethanol:oil molar ratio; 200 rpm) and in the study of Cruz *et al.* [11] which evaluated the pre-treatment of soapstock acid oil with a FFA content of 65.5 wt.% (4 – 5 wt.% of enzyme; 35 °C; methanol:oil molar ratio 1.5:1; 200 rpm) steady state was reached after approximately 7 h with FFA reductions of 80 – 90 % [11, 13]. At the end, FAME content in the final product was around 68 wt.% [11].

Choi *et al.* [28] showed that it might be possible to perform enzymatic esterification without the addition of enzyme. In such study, a FAME content of 87 % could be obtained after 12 days via *in situ* lipase-catalyzed only with addition of methanol [28]. Rice bran is known to contain several types of lipases (phospholipases, glycolipases, esterases) which might improve the economic sustainability of RBO pre-treatment aiming bioenergy production [29]. In agreement, in further studies with RBO, the presence of *in situ* lipase should be considered.

7.2.4.2 Alkaline transesterification of oil mixtures

The pre-treatment processes had as main goal to make RBO suitable for biodiesel production (acidity lower than 2 mg KOH.g⁻¹). As it was possible to observe, the pre-treatment reduces the acidity of the oil converting FFA in FAME (commonly known as biodiesel). Simultaneously, some triglycerides are converted into FAME by transesterification, although it is inferred that the amount of alcohol present in the mixture was not enough to the complete conversion of triglycerides. The remaining fraction of the oil composed by triglycerides should be converted into FAME by transesterification reaction.

For biodiesel production, only pre-treated oil from homogenous acid esterification was considered, due to the low content of FAME in the final product (and consequently a high content of triglycerides). Besides that, performing alkaline transesterification for enzymatic pre-treated oil was not very interesting because at the end of the biodiesel production it is necessary to wash the product with water (process avoided when employing enzymatic pre-treatment process). Among the pre-treated oils, the RBO pre-treated at 65°C with 4 wt.% of catalyst (best condition) was thus submitted to alkaline transesterification (the highest FAME content after esterification pre-

treatment). In this case the acidity of pre-treated oil was $3.7 \text{ mg KOH.g}^{-1}$. Consequently, alkaline transesterification cannot be performed directly using this raw material. The pre-treated RBO was blended with SFO (low acidity) to produce a mixture with an AV lower than 2 mg KOH.g^{-1} , as previously referred (section 7.2.3.4). From the transesterification reaction, results a final product with a FAME content of 88 wt.%. Despite that high value, it is still lower than that required for use as automotive fuel ($> 96.5 \text{ wt.}\%$). However, alkaline transesterification reaction allowed to increase FAME content in the final product in around 42 wt.%.

Chromatographic analysis showed that the methyl esters profile contains mostly C18:1, oleic acid methyl ester (42 wt.%), and C18:2, linoleic acid methyl ester (33.9 wt.%). Table 7.9 shows the FAME found in biodiesel from rice bran oil.

Table 7.9. Characteristics of biodiesel produced from RBO by alkaline transesterification

Methyl Ester of the following fatty acids	FAME (wt.%)	[30]
Palmitic (C16:0)	18.7	16.4
Oleic (C18:1)	42.1	45.2
Linoleic (C18:2)	33.9	36.0
Others (< 2wt.% each)	5.30	2.40

Most of the biodiesel is constituted by monounsaturated fatty acids (61.3 wt.%), followed by polyunsaturated fatty acids (35.1 wt.%). Saturated fatty acids only corresponded to 3.6 wt.% of the biodiesel composition. The relatively high content of polyunsaturated acids in the biodiesel might lead to a low stability of the biodiesel product, since those are more susceptible to oxidation (higher number of reactive sites when compared to monounsaturated fatty acids [31]). Linolenic acid is reported as the fastest to be oxidized, followed by linoleic and oleic acid [32]. For biodiesel produced from RBO, 75 wt.% of the mass is constituted by linoleic and oleic acid and for this reason it is expected that biodiesel might have some problems related to its oxidation stability. Nonetheless, oxidation stability parameter does not only depend upon FFA composition since the presence of natural antioxidants in the oil might present a relevant influence on the oxidation stability of the final biodiesel [31, 33].

7.2.5 CONCLUSIONS

The results from the present study showed that the high acid value of rice bran oil (47 mg KOH.g^{-1}) could be effectively reduced either by homogenous acid esterification (65°C or 55°C , 4 wt.% of catalyst during 8 h) or enzymatic esterification (35°C , 5 wt.% catalyst during 24 h).

In terms of reaction conditions, using an homogeneous acid catalyzed process, the increase of catalyst concentration from 2 to 4 wt.% for the temperatures of 55°C or 65°C was beneficial for the free fatty acids reduction whereas at 45°C no benefits occurred. Temperatures higher than 45°C improved the reaction rate during the first hours; after 8 h, differences were however small.

Using the biocatalysed process, better results were obtained (higher FAME content in the final product) being possible to reduce the acidity from around 47 mg KOH.g^{-1} to 11 mg KOH.g^{-1} after only 60 min.

At the end of both processes (homogeneous and heterogenous esterification), evidences of transesterification were found. Those were very expressive in the case of the biocatalysed process, in which the FAME content of the product reached 87 wt.%.

The transesterification reaction of pre-treated oil obtained by acid esterification (65°C with 4 wt.%) blended with SFO allowed to produce a biodiesel with 88 wt.%.

This study showed that acid rice bran oil might be used as raw material for biodiesel production and that the biocatalysed conversion of free fatty acids and glycerides might be an interesting alternative process aiming FAME production without the need of a pre-treatment. Further studies should also consider *in situ* lipase-catalyzed processes.

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7.3 OLIVE POMACE BY-PRODUCT FOR ENERGY PRODUCTION

ABSTRACT

In the present study, biodiesel production from olive pomace oil was evaluated, a topic of special interest for Portugal, one of the main European producers of olive. Enzymatic and acid esterification as well as glycerolysis were studied as alternative processes to enable the recovery of the acid oil ($4.4 - 50.6 \text{ mg KOH.g}^{-1}$). Olive pomace oil was obtained from fresh olive pomace ($\sim 18 \text{ wt.}\%$ of oil) and from olive pomace after being stored 4 months. The air storage of the olive pomace during 2 weeks led to an increase in the acidity of the oil extracted of almost 2 fold. The results from the enzymatic esterification (35°C , 24 h, 200 rpm) showed a clear influence of enzyme concentration (1 – 5 wt.%, relative to oil) in the final acidity of the oil. The assay with a methanol:oil molar ratio of 6:1 and an enzymatic concentration of 3.5 wt.% was selected as the best condition, being the acidity reduction 85%; also, the pre-treated oil showed a FAME content of 84 wt.%. The enzymatic route proved to be a good option to produce biodiesel, independently of the original acidity of olive pomace oil. The use of a neutralization step (lower FFA content), before enzymatic pre-treatment, improved FAME production during esterification reaction. Acid esterification and glycerolysis pre-treatment did not bring relevant benefits mostly due to the relatively low original acidity of the oil. The results indicate olive pomace as having potential to be used as a sustainable raw material for biodiesel production and enzymatic esterification as an efficient process to reduce the high acidity of the oil after 4 months of storage.

7.3.1 SCIENTIFIC RELEVANCE

By-products such as olive pomace are seen as potential low cost feedstocks for biodiesel production [1]. In the Mediterranean region there is a large production of olive oil for human consumption from which an industrial by-product is obtained, the olive pomace, presenting a relevant oil content (on average 5 – 8%, wet basis) [2]. As previously highlighted (Chapter 6), in Portugal, the olive production forecast for 2016 was around 8.58×10^5 ton [3]. At the traditional olive press process, Azbar, Bayram [4] indicates that 1 ton of olives leads to around 400 kg of solid waste (olive pomace), 200 kg of olive oil and 600 kg of wastewater containing residual solids and oil [58]. It is therefore advisable to develop studies regarding the recovery of such material, in particular, for biofuels production.

The present work evaluated the use of olive pomace oil for bioenergy production. Considering the acidity of the raw material, different pre-treatment processes were performed, namely, acid and enzymatic esterification as well as glycerolysis. Afterwards, transesterification was conducted aiming to obtain a high final biodiesel yield.

7.3.2 SPECIFIC OBJECTIVES

The following specific objectives were established:

- To characterize olive pomace by-product (oil content and moisture);
- To study the effect of the storage on the acidity of the olive pomace oil;
- To study the viability of olive pomace oil as raw material for biodiesel production;
- To produce biodiesel by alkaline transesterification using as raw material the pre-treated oils.

7.3.3 MATERIALS AND METHODS

7.3.3.1 *Materials*

Olive pomace was supplied from an olive oil company in Northern Portugal ("DouroSol Company"). Petroleum ether (LabChem $\geq 90\%$) was used for chemical oil extraction (Soxhlet). Methanol (Fischer Scientific $\geq 99\%$) was used as the acyl acceptor for esterification and transesterification reactions. The homogeneous catalyst used was sodium hydroxide 97% - powder (reagent grade, Aldrich). For enzymatic reaction the catalyst was lipase *Thermomyces lanuginosus* (Lipolase 100L, activity $\geq 100,000$ U/g) from Sigma-Aldrich. All the other reagents were of analytical grade.

7.3.3.2 *Extraction of olive pomace oil*

The oil extraction was performed using a 1 L Soxhlet extractor. First, the raw material was crushed in a mortar and placed inside the thimble. Then, the thimble was dipped in petroleum ether solvent for 6 h (equivalent to 14 turns of the solvent in the extractor). After the extraction, the solvent was removed in a rotary evaporator at close to 70 °C. Several extraction cycles were conducted until the necessary amount of oil for the study was obtained. The procedure performed was based on NP EN ISO 659 (2002).

In order to evaluate the effect of the storage in the acidity from the olive pomace oil, the extraction was carried out with the fresh olive pomace and after 15, 60, 120 and 365 days of air storage at room temperature.

7.3.3.3 *Analytical methods*

The moisture and oil content of the olive pomace by-product were determined according to NP 12880 (2000) and NP EN ISO 659, respectively. Oxidation stability and acidity were determined in the extracted olive pomace oil. The oxidation stability and acidity of the olive pomace oil were determined by accelerated oxidation in agreement with EN 14112 using a Rancimat equipment (Metrohm) and by a titrimetric method according to EN 14104, respectively.

The fatty acid methyl ester content of the pre-treated oils (enzymatic, acid esterification, or glycerolysis) and of the final biodiesel product was determined according to EN 14103 (2003). The procedure employed has been previously described in section 7.1.3.2. In order to evaluate whether

or not significant differences existed between the results obtained from the different processes, the analysis of variance (ANOVA) was performed with the support of the Excel® software (Analysis ToolPak).

7.3.3.4 *Pre-treatment of olive pomace oil*

Pre-treatment was conducted once the oil acidity was higher than 1 wt.% (see section 7.3.4.1). This step is necessary when biodiesel production is made by homogeneous alkaline transesterification, since the catalyst will react with the FFA present, leading to soap formation [5]. All the steps employed for olive pomace oil pre-treatment are summarized in Figure 7.6.

- *Homogenous acid esterification*

The olive pomace oil subjected to esterification had an acid value of 4.4 ± 0.3 mg KOH.g⁻¹. The same oil was used in the glycerolysis pre-treatment. The amount of sample (acid oil) used for each experiment was 25 g.

For the acid esterification reactions, the following reference conditions were selected according to the literature [6]: 65 °C, 2.0 wt.% H₂SO₄ catalyst, 6 h and 9:1 molar ratio of methanol to acid oil.

The setup of the batch reactor and the monitoring of the reaction (0.5 mL of sample from the reaction mixture collected at different time intervals - 0, 15, 30, 60, 120, 180 and 360 min) was made according to the procedures described in the literature [6]. At the end of the esterification reactions the pre-treated oils were washed to remove the excess of catalyst and dried (Figure 7.6 – Purification step) for further determination of the final acid value of the product, as well as, of the fatty acid methyl ester (FAME) content.

- *Glycerolysis*

The best conditions and the laboratory apparatus used in the study by Costa *et al.* [7] were implemented (T = 200 °C, 3:1 molar ratio of glycerol:oil, 6 h). The glycerolysis was carried out using 25 g of acid oil and 0.5 mL samples were collected at different time intervals (0, 15, 30, 60, 90, 120, 189, 360 min) to evaluate the progression of the reaction [7].

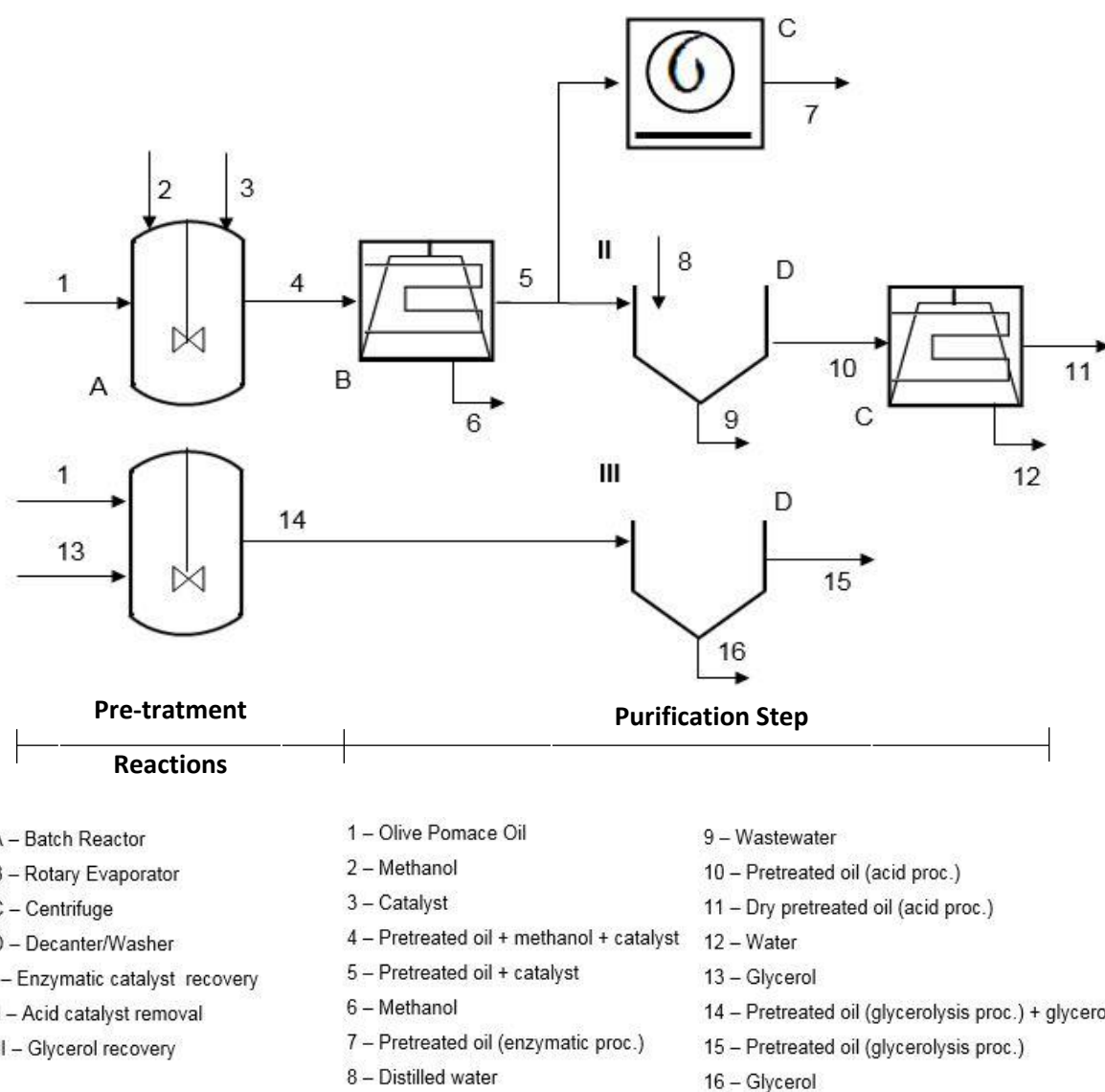


Figure 7.6. Flow sheet for olive pomace oil pre-treatment: I – Heterogeneous esterification; II – Homogeneous esterification; III – Glycerolysis pre-treatment;

- *Heterogeneous esterification*

The oil extracted from fresh olive pomace showed low acidity. However, due to constraints related with the availability of fresh olive pomace, it was not possible to obtain fresh oil and thus the enzymatic processes were conducted using oil with an acidity of $50.6 \pm 0.4 \text{ mg KOH.g}^{-1}$, extracted from olive pomace oil stored approximately for 130 days.

For heterogenous esterification, the crude olive pomace oil was added to a batch reactor placed in an orbital shaking incubator (Agitorb 200IC) where methanol and the enzyme were added. In this

case, the enzymatic catalyst catalyzes both the transesterification of triglycerides and the esterification of FFA. Accordingly, the two processes were promoted by varying the reaction conditions, namely methanol amount.

For esterification, the methanol:oil molar ratio was 2:1 [8]. The following conditions were used: 250 rpm stirring, 24 h, 35 °C, according to previous studies and recommendations of the catalyst supplier [8, 9]. Methanol addition was performed stepwise (8 steps) after being verified that one single addition inhibited the enzyme.

Five enzyme concentrations were studied, namely 1, 2, 3, 3.5 and 5 wt.% (relative to oil's weight), added at the beginning of the esterification reaction [9, 10]. Different enzyme concentrations were evaluated aiming to determine the best reaction conditions, considering the acidity reduction and also the economic viability of the process (namely enzyme's costs). For the optimum enzyme concentration (which allowed to obtain the lowest acid value for the pre-treated oil) a higher methanol:oil molar ratio was also evaluated (9:1) with the objective of understanding if the amount of methanol was limiting the acidity reduction.

Table 7.10 resumes the experimental conditions employed. Samples were collected from the reactor at different times: 15, 30, 60, 120, 240, 480 and 1440 minutes, to monitor the reaction progression. At the end (Figure 7.6 – Purification step), the excess of methanol was recovered in a rotary evaporator (T = 65 °C; 200 mbar, 30 min) and the remaining fraction was centrifuged during 5 min at 3500 rpm with the objective to remove the catalyst.

Table 7.10. Reaction conditions considered for the enzymatic process

Assay	Temperature (°C)	Catalyst (wt.%)	Alcohol:oil molar ratio for esterification
1	35	1.0	2:1
2	35	2.0	2:1
3	35	3.0	2:1
4	35	3.5	2:1
5	35	5.0	2:1
6	35	3.5	2:1

The experiment was repeated for the condition which enabled the higher FFA reduction to confirm the results.

In addition, a neutralization step was performed before the enzymatic pre-treatment. The main objective was to evaluate if the acidity reduction had an impact on the performance of the enzymatic process. Neutralization was tested for the product from the best experimental conditions based on the results previously obtained.

Neutralization was made by washing the olive pomace oil with a NaOH solution (750 ppm), followed by two washings with water (1:1 V/V of oil:NaOH solution/water per wash) [9]. Following, enzymatic esterification (first step) and transesterification reaction (second step) were performed. For enzymatic esterification the following conditions were adopted: methanol:oil molar ratio of 2:1 (8 steps addition) at 35 °C. Samples of 0.5 mL were collected at different times (3, 60, 180 and 420 min) for monitoring the reaction. The final reaction time was established considering that the steady state for enzymatic esterification is reached after 7 h, according to literature [9].

7.3.3.5 *Transesterification*

For biodiesel production, the methanol:oil molar ratio was calculated considering an oil molecular weight of 880 g.mol⁻¹. This value was calculated based on fatty acids profile determined (section 7.3.4.1). Synthesis of biodiesel was performed according to the procedures described in section 7.1.3.4. The transesterification reaction using homogenous catalysts was performed using the products obtained from homogenous esterification and the glycerolysis pre-treatment, which showed an acid value near 1 wt.%, compatible with the process requirements.

For the pre-treated oil from enzymatic esterification, the transesterification step was performed using a methanol:oil molar ratio of 6:1. A different methanol:oil molar ratio (9:1) for transesterification reaction was also tested for a catalyst concentration of 3.5 wt.%. Transesterification reaction was developed during approximately 17 h (24 h after the beginning of the esterification reaction).

For neutralized and non-neutralized oil, enzymatic transesterification was performed using a methanol:oil molar ratio of 3:1 (divided in five steps). The FAME content was measured during 24 h at different times (60, 180, 420 and 1440 min). In this case, a lower methanol:oil molar ratio was adopted (comparing with the adopted for enzymatic transesterification of the non-neutralized oil) aiming to reduce the reaction costs.

7.3.4 RESULTS AND DISCUSSION

7.3.4.1 Raw materials properties

The results concerning the characterization of both the by-product and the extracted oil are present in Table 7.11. According to the literature, olive pomace shows some amount of oil (8 — 12 wt.%) and high moisture (25 — 35 wt.%) [11-13]. For olive pomace oil, oxidation stability and acid value were measured immediately after the production of the olive pomace and after 4 months of air storage.

Table 7.11. Characterization of olive pomace and olive pomace oil

Olive Pomace		Olive Pomace Oil			
		Fresh		After 4 months	
Oil content (wt.%)	Moisture content (wt.%), wet basis	Oxidation stability (h)	Acid value (mgKOH.g ⁻¹)	Oxidation stability (h)	Acid value (mgKOH.g ⁻¹)
17.7 ± 0.3	26.8 ± 0.1	12.7 ± 0.6	4.4 ± 0.3	2.14 ± 0.06	50.6 ± 0.4

For this olive pomace, the amount of oil was higher than that reported in the literature [13], a positive aspect concerning the potential of this by-product for biodiesel production. The moisture of 26.8 wt.% was in the range referred by the literature [14]. Since the objective was to recover this by-product for bioenergy purposes, the high moisture of olive pomace can be a drawback, since it increases the costs of transport and accelerates pomace degradation.

Oxidation stability found for fresh olive pomace oil was around 13 h. The oxidation stability is related with the fatty acids profile (susceptible to oxidation) and with the presence in the oil of free fatty acids, as well as, of natural oxidants (phenolics compounds) [15]. The oil stability is directly affected by the presence of polyunsaturated fatty acids because they are highly susceptible to natural oxidation [5]. According to the literature, olive oil pomace is mostly constituted by oleic acid (55 — 83 wt.%), a monounsaturated acid, thus, the oil is known to be more susceptible to chemical degradation [16].

The results of the oxidation stability of 12.7 ± 0.6 h can be considered high when compared with other raw materials used for biodiesel production like canola oil (5.64 h) or soybean oil (6.09 h) [17]. This fact can be related both with the composition and the presence of phenolic compounds [5]. After 4 months the oxidation stability of the oil decreased severely, perhaps due to the natural degradation occurred [18, 19].

The acidity determined in the oil extracted from the fresh olive pomace was $4.4 \text{ mg KOH.g}^{-1}$, value that was much lower than the usually reported in the literature (44 mg KOH.g^{-1}) [1]; however, Rajaeifar *et al.* [20] studied an olive pomace oil with 5.75 wt.% (12 mg KOH.g^{-1}) of acidity, lower than the usually reported but still higher than that obtained in the present study.

In order to evaluate the impact of storage, olive pomace oil was extracted after two weeks of storage; the extracted oil presented around $8.5 \text{ mg KOH.g}^{-1}$ of acidity, corresponding almost to the double of the verified for the fresh oil. Figure 7.7 shows the evolution of the olive pomace oil acidity during the storage in air.

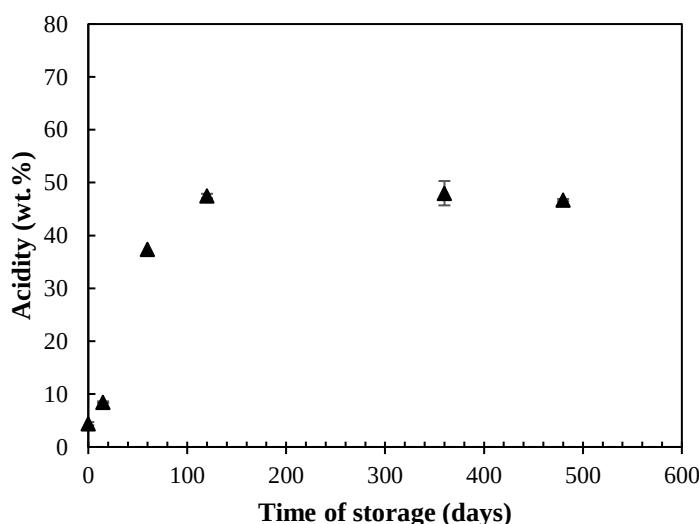


Figure 7.7. Acidity evolution of olive pomace oil (errors bars show maximum and minimum values) for different storage periods, in air.

Acid value of fresh oils (storage for 2 days) are low, but acidity continues increasing with storage time at the facilities where the olive oil is extracted. After two months of storage, the oil acidity was very high (approximately 40 mg KOH.g^{-1}). According to the results, after 4 months the acidity of the extracted oil seems to stabilize. After one year, the acidity of the oil extracted was around 50 mg KOH.g^{-1} . The observed high acidities make inviable using the conventional homogeneous alkaline transesterification for biodiesel production [6].

The results obtained showed the relevance of revising the storage conditions to obtain an olive pomace oil with high quality (low acidity and high oxidation stability), in particular if biodiesel production is aimed. Unfortunately, since most olive pomace oil shows low quality, the study of pre-treatments to decrease the acidity is essential. In Portugal, olive harvest is performed from the end of November up to February. Recovering the oil present in the olive pomace, usually performed by

chemical reaction, is possible only at the end of the harvest season. Most of the olive industry employs mechanical means to make oil extraction, leading to the production of olive pomace that is forward to other industrial facilities. Sometimes olive pomace is directly burn in boilers to produce heat in the olive industry. The majority of olive pomace oil produced in Portugal is exported to Spain [21].

7.3.4.1 Pre-treatment by homogeneous esterification

This pre-treatment was performed to the fresh pomace oil (2.2 wt.%). Figure 7.8 shows the acidity evolution during the homogenous acid esterification reaction which includes the influence of the inorganic acid used as catalyst (H_2SO_4). The results reveal that this pre-treatment was not able to reduce most of the acidity of the olive pomace oil. According to the Figure 7.8, the decrease of acidity is more pronounced for the first 30 minutes. After that, esterification rate is low, remaining the oil's acidity almost unchanged. At the end of the reaction, after purification of the product (removal of sulphuric acid by water washing), the oil acidity was 1.2 wt.% (a reduction of 45%), a threshold value for applying the alkali transesterification.

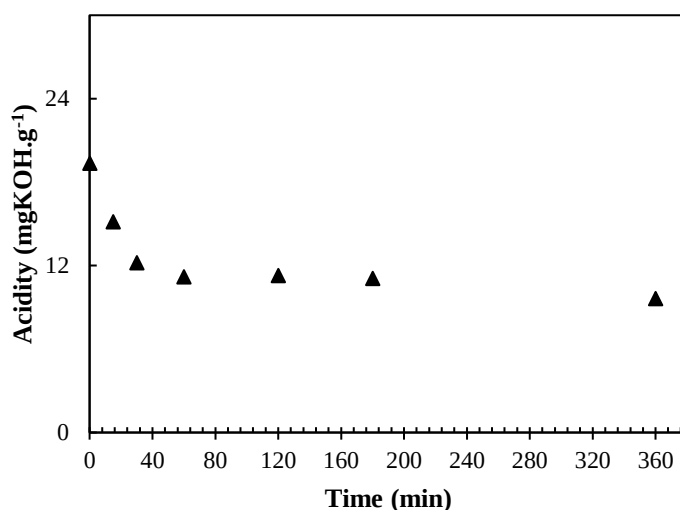


Figure 7.8. Evolution of the acidity during homogenous acid esterification of olive pomace oil (65 °C, 9:1 methanol:oil molar ratio, 2 wt.% of catalyst concentration).

The extraction of the oil from fresh olive pomace has high potential because an oil with a low content of FFA is obtained. However, the collection of fresh olive pomace at its producers is

expected to be unviable from economical point of view. Figure 7.9 shows the product after the reaction.

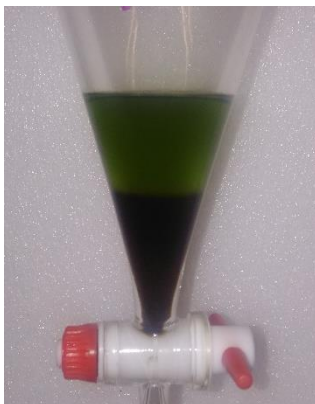


Figure 7.9. Separation of the lipid fraction (upper layer) obtained after acid esterification of olive pomace.

7.3.4.2 Glycerolysis

Glycerolysis was monitored for six hours, as shown in Figure 7.10. According to the results, no visible decrease of acidity occurred during the reaction. At the end of the reaction, after separation of the two fractions (glycerol and pre-treated oil) the acidity of the oil was $4.1 \text{ mg KOH.g}^{-1}$. This fact reveals that the acidity reduction was only $0.3 \text{ mg KOH.g}^{-1}$, thus the pre-treatment was not effective.

The beginning of the glycerolysis reaction is associated to a slightly increase of acidity which can be related with the presence of moisture (reversibility of the glycerolysis reaction). In fact, the presence of moisture associated with the high temperature employed during glycerolysis reaction might lead to the decomposition of TG, DG or MG leading to the production of FFA. For this reason, it is important to remove water previously from the reaction mixture.

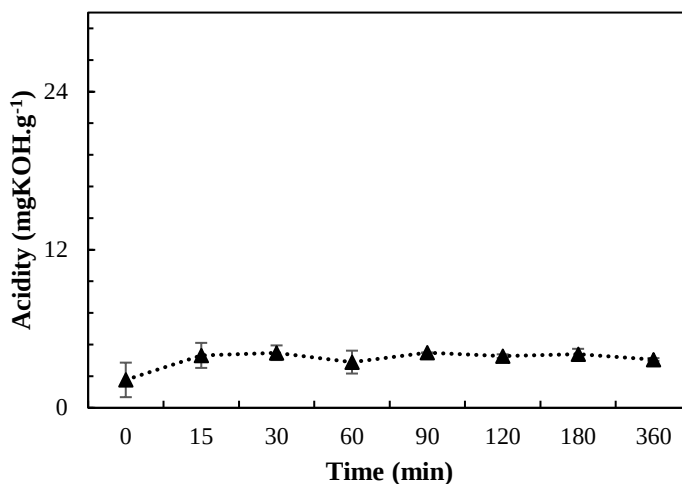


Figure 7.10. Evolution of the acidity during olive pomace oil glycerolysis (200°C, 3:1 glycerol:oil molar ratio). Error bars show maximum and minimum values obtained.

Figure 7.11 shows the separation of the pre-treated oil from glycerol by settling.



Figure 7.11. Separation of pre-treated oil (upper layer) after glycerolysis.

Taking into consideration the results obtained with glycerolysis reaction and acid esterification, using fresh olive pomace oil, it becomes evident that these two pre-treatments were not efficient to reduce the acidity, at this low level of acidity.

The following experiments were performed with olive pomace that was stored for four months.

7.3.4.3 *Heterogenous esterification and transesterification*

Enzymatic esterification can be considered a simpler and environment-friendly process because the pre-treated oil can easily be purified at the end of the process, avoiding the need of a washing step.

Figure 7.12 shows the evolution of the olive pomace oil acidity during the enzymatic esterification. The results can be directly compared for all enzyme concentrations, since the acidity is only due to the presence of FFA.

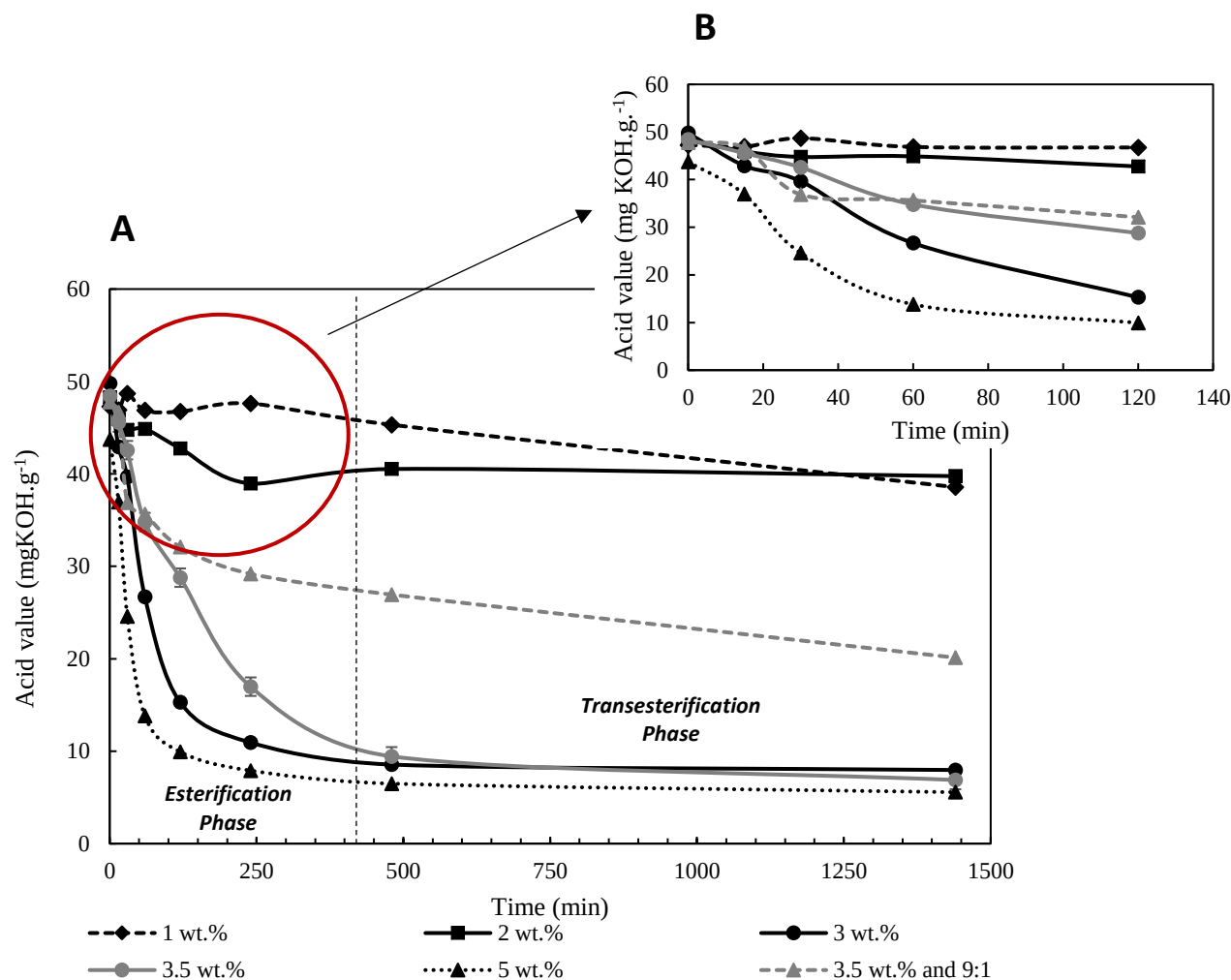


Figure 7.12. Acidity reduction during heterogeneous enzymatic esterification at 35 °C, with stepwise alcohol addition; A - during 24 hours; B - for the first 2 hours. Error bars show maximum and minimum values obtained for the “best condition” (assay 4).

According to the results, catalyst concentration clearly influences the conversion rate of enzymatic esterification. For enzyme concentration below 3 wt.% (1 and 2 wt.%) the reaction rate is very low compared to that observed at higher enzyme concentrations. In fact, for an enzyme concentration of 1 wt.% and 2 wt.%, after 24 h the acidity reduction was only around 7% (the pre-treated oil showed an acid value of 47.3 mgKOH.g⁻¹). Also, at those concentrations, the reduction of acidity is mostly obtained at the first 120 min. After that, the acidity tends to stabilize.

For an enzyme concentration higher than 2 wt.%, enzymatic reaction progresses much faster in the first 8 h of reaction. For the catalyst concentration of 3 wt.%, 3.5 wt.% and 5 wt.% (methanol:oil molar ratio of 6:1), after 8 h the steady state was reached, with the acidity reduction being between 80% and 85%. At the end of the reaction, after purification, the acidity reduction varied between 83% and 86%. After purification, the acidity of the pre-treated oil was very similar to the original acidity of the oil (50.6 mg KOH.g⁻¹).

For a catalyst concentration of 1 wt.% and 2 wt.%, the low acidity reduction seems to be mostly due to the enzyme added was possibly denatured by the addition of methanol, being this effect mostly felt at the low catalyst concentrations (1 wt.% and 2 wt.%). At higher enzyme concentrations the effect of enzyme inhibition/denaturation is not so pronounced, since the amount of enzyme available still enables the reaction progression.

In the study developed by Choi *et al.* [22], for the esterification of acid rice bran oil (40 °C; ethanol:oil molar ratio of 1:5) the steady state was reached after approximately 10 h with a final FAME content higher than 80 wt.% for a catalyst concentration between 10 wt.% and 15 wt.%. For Cruz *et al.* [9], which developed an enzymatic esterification pre-treatment for acid oil from soapstock (FFA content of 65.5 wt.%; 4 – 5 wt.% of enzyme; 35 °C; methanol:oil molar ratio 1.5:1; 200 rpm), steady state was reached after 7 hours. According to Costa *et al.* [23], steady state was achieved after 8 h for an enzymatic esterification of rice bran oil (initial acidity of 47 mg KOH.g⁻¹) performed at 35 °C using a 2:1 methanol:oil molar ratio for FFA conversion (and 6:1 molar ratio of methanol:oil for triglycerides conversion) with 5 wt.% of catalyst [23]. Table 7.12 shows the final acid value obtained for each assay and the respective FAME content.

Table 7.12. Characteristics of the product (acid value and FAME) obtained after heterogeneous esterification and transesterification

Assay	Acid value (mg KOH.g ⁻¹)	FAME content (wt.%)
1 (1 wt.% and 6:1*)	50.2	6.4
2 (2 wt.% and 6:1*)	47.2	9.9
3 (3 wt.% and 6:1*)	8.4	82.4
4 (3.5 wt.% and 6:1*)	7.5	84.0
5 (5 wt.% and 6:1*)	7.3	87.6
6 (3.5 wt.% and 9:1*)	19.6	35.2

*methanol molar ratio adopted for enzymatic transesterification

In agreement with the results it is possible to conclude that the assay 5 (enzyme concentration of 5 wt. %) allowed to achieve the best results: the lowest acidity and the highest FAME content. However, the final acidity obtained using 3.5 wt. % of enzyme was very similar to the obtained using the highest catalyst concentration of 5 wt.%. Therefore, taking into consideration the high cost associated to the enzyme, the best catalyst concentration selected was 3.5 wt. % (assay 4). For this enzyme concentration, a higher methanol:oil molar ratio was evaluated at the transesterification reaction which did not bring any benefit (higher FAME content). In fact, the increase of methanol:oil molar ratio to 9:1 had a negative effect leading to the inhibition/denaturation of enzyme, as previously reported, affecting the reaction's yields, since the final FAME content found in the oil was lower than the obtained for assays 4 or 5. Accordingly, the methanol:oil molar ratio of 2:1 for esterification reaction and 6:1 for transesterification reaction were considered the best conditions for the overall process.

Considering the initial acidity of the oil, 25 wt. % of FFA, it was expected finding the same percentage in the final product as FAME. However, at the end of the reaction, FAME content for assays 3, 4 and 6 was higher than 25 wt.% (approximately 30 – 60 min after the beginning) which shows that both esterification and transesterification reaction occurred. Figure 7.13 shows the linear relationship between acid value and FAME content. To establish such relation, it was considered that when the acid value of the pre-treated oil is null, the FAME content is maximum (100 wt. %). Although with few data, the linear relationship shows the expected inverse trend, meaning the conversion of FFA into FAME.

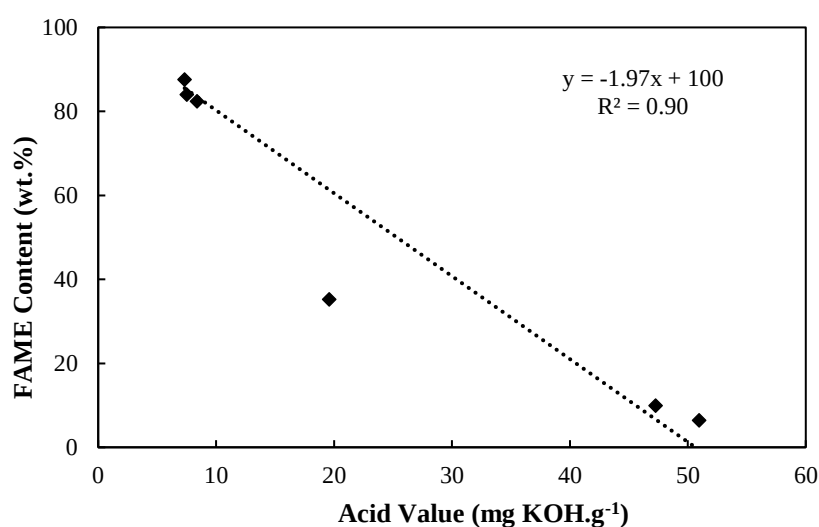


Figure 7.13. Relation between acid value and FAME content of the final product and linear correlation obtained.

For the optimum enzyme concentration selected (3.5 wt. %), the evolution of FAME content for non-neutralized and neutralized olive pomace oil during enzymatic reaction was compared (as described before in section 7.3.3.4). Figure 7.14 shows the FAME content evolution for each assay (with and without neutralization).

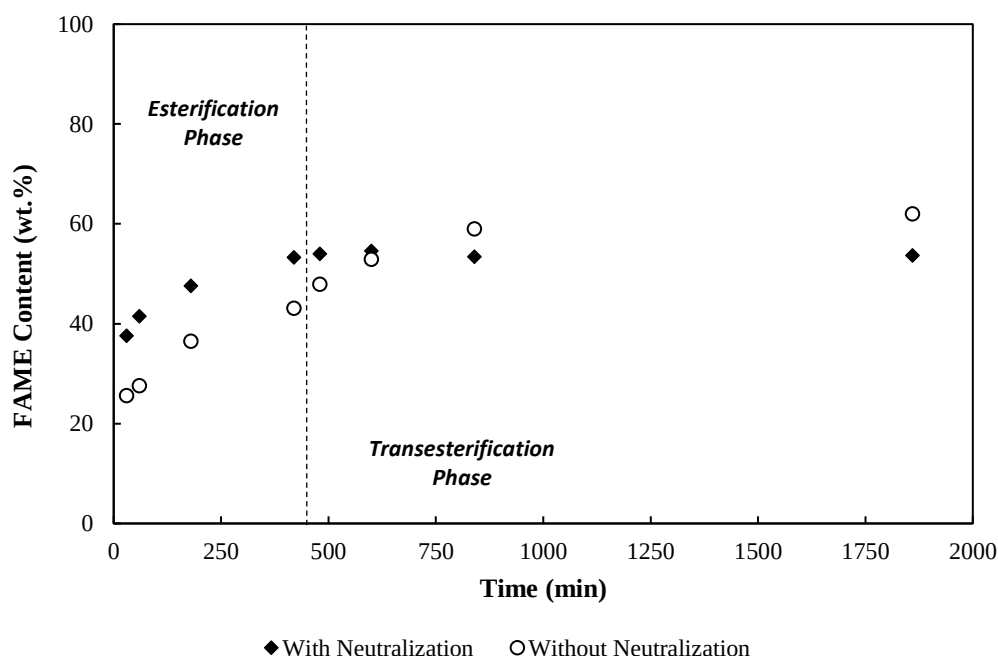


Figure 7.14. FAME content evolution during enzymatic esterification and transesterification for olive pomace oil neutralized with NaOH and oil without neutralization.

At the esterification phase, due to the lower initial acidity, the production of FAME is more pronounced than that verified for the oil without neutralization. This fact can be related with the lower availability of FFA (neutralized oil), which made possible a faster conversion of FFA into FAME (for the same enzyme concentration). According to Haar *et al.* [24], for the esterification reaction, an increase of FFA content in the substrate leads to a reduced FFA conversion.

According to the results obtained, for oil neutralized with NaOH (lower acidity) FAME content was 38 wt.% after only 30 min. Without neutralization, FAME content was 26 wt.%.

After 7 h (at the end of esterification reaction), FAME content obtained for neutralized and untreated oil was 53 wt.% and 43 wt.%, respectively.

For the transesterification phase, the differences of FAME concentration between untreated oil (crude oil) and neutralized oil were very small. After 8 hours, the FAME in neutralized oil tends to

stabilize (53 wt.%). For untreated oil, the FAME content keeps increasing until 62 wt.%. The difference in FAME for these two oils at the final of transesterification can be related with the availability of methanol.

The FAME content obtained with the untreated oil (62 wt.%) was lower than that on assay 4 (84 wt.%), despite the same enzyme concentration. For the untreated oil the methanol:oil molar ratio was 3:1, and for assay 4 it was 6:1. For the transesterification reaction, each mol of oil requires three mol of alcohol to produce FAME. However, usually, the amount of alcohol added to the reactional mixture is above the stoichiometric amount, to ensure that the equilibrium is pushed towards the products.

7.3.4.1 Alkaline transesterification of pre-treated oils

Biodiesel production was done using the pre-treated oils from the acid esterification and glycerolysis reactions. Table 7.13 presents the composition profile of methyl esters for biodiesel produced by alkaline transesterification reaction and in for the product after the enzymatic esterification.

Most of the biodiesel from olive pomace oil was constituted by the oleic acid methyl ester (monounsaturated acid – C18:1). The FAME profile found was in agreement with the reported in the literature [11].

Table 7.13. Fatty acid methyl ester profile for biodiesel produced using the different feedstocks

Methyl ester of the following fatty acids	Feedstock		
	Enzymatic esterification product	Acid esterification product	Glycerolysis product
Palmitoleic (C16:1)	10.3 ^a	10.1 ^b	10.4 ^a
Stearic (C18:0)	3.40 ^a	3.30 ^b	3.40 ^a
Oleic (C18:1)	71.9 ^a	71.3 ^b	72.0 ^a
Linoleic (C18:2)	11.6 ^a	12.7 ^b	11.5 ^a
Others (< 1 wt.% each)	2.90 ^a	2.60 ^b	2.70 ^c

^{a,b,c} Mean values with different letters in the same row vary significantly ($p \leq 0.05$).

Although the results show that some differences occur among FAME products, those were not considered relevant.

According to standard EN 14214, the minimum FAME content acceptable for commercial biodiesel is 96.4 %. For the biodiesel produced from acid esterification and glycerolysis, the FAME content

obtained was 81% and 85%, respectively. For the best condition selected for enzymatic esterification followed by transesterification, the FAME content was 84 wt.% (assay 4).

7.3.5 CONCLUSIONS

The olive pomace oil showed high potential to be used as raw material for biodiesel production. The original acidity verified for this oil was higher than the threshold value of 2 mgKOH.g⁻¹ for direct application of alkaline transesterification (4.4 – 50.6 mg KOH.g⁻¹). Acid esterification and glycerolysis of the fresh oil did not affected significantly the original acidity of the olive pomace oil. Usually, olive pomace oil is only recovered after some time of storage, and, according the present study, the acidity greatly increases when the product is stored in the air (increase from 4.40 mg KOH.g⁻¹ until approximately 50 mg KOH.g⁻¹ after 4 months of storage). Thus, the recovery of olive pomace oil as energy source seems to be viable without any pre-treatment only when the oil is extracted from very fresh pomace.

Enzymatic esterification (methanol:oil molar ratio of 2:1) and transesterification (methanol:oil molar ratio of 6:1) using 3.5 wt.% of enzyme showed to be an efficient process to reduce the acidity of the oil from 50.6 mg KOH.g⁻¹ to 7.5 mg KOH.g⁻¹ (85% of reduction). The final product had a FAME percentage of 84 wt.%.

The reduction of the FFA by neutralization allowed an initial high conversion into FAME; however, at the end of the process (after transesterification reaction), FAME content was higher for the oil without neutralization than with neutralization , mostly due to the amount of methanol available.

Based on all the results obtained, this by-product has potential to be used as a sustainable raw material for biodiesel production and the enzymatic esterification and transesterification demonstrated to be an efficient, perhaps a more eco-friendly process, to convert all the lipids into FAME.

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7.4 WASTE COFFEE GROUNDS, GRAPE POMACE WASTE AND BREWERY SPENT GRAIN – A PRELIMINARY CHARACTERIZATION AIMING AT BIOENERGY PRODUCTION

ABSTRACT

In Portugal there are several wastes or by-products with potential for bioenergy production. Grape pomace, waste coffee grounds or brewery spent grain are produced in large amounts, however, the majority of these by-products is either not recovered or only used for animal feed. The preliminary characterization here presented allows to identify these wastes/by-products as good sources for oils. The oils extracted (chemical extraction) from them showed high acidity, which makes them unsuitable for biodiesel production through the traditional route. In agreement, to enable the use of these oils for biodiesel production, pre-treatment steps are required. Overall, it is necessary to develop integrated solutions to enable sustainable material management practices for those materials, and, consequently, decrease the costs from biodiesel production.

7.4.1 SCIENTIFIC RELEVANCE

Beyond the wastes and by-products referred before (soapstock – section 7.1, rice bran – section 7.2, olive pomace – section 7.3), in Portugal, other types of agro-industrial wastes were identified as having high potential for bioenergy production, for the reasons that will be exposed from now on.

The production of wine leads to the generation of high amounts of grape pomace, a type of waste which results from the wine processing. In 2016, the production of grape pomace in Portugal was estimated in 2.0×10^5 ton. Almost 18 wt.% of grape pomace corresponds to grape seeds; on the other hand, studies indicate that grape seeds can be recovered for bioenergy production [1, 2]. Grape pomace is mainly used for the production of wine spirit (called “aguardente”). After that, in most of cases, grape pomace is spread on the soil.

Recent data shows that coffee is one of the most commercialized products in the world, where the global consumption exceeded 9.3 billion kg in 2016, growing at an annual rate of 1.5% [3, 4]. It is estimated that 1.4 billion coffee's cups are prepared everyday [5]. After coffee extraction with hot water, the remaining solid waste is known as spent coffee grounds (SCG). From 1 kg of soluble coffee 2 kg of wet SCG are obtained. It is estimated that 8 – 9.8 million ton of coffee are produced every year which means that, according its oil content, it may be obtained 1.3 billion L of biodiesel from coffee oil [6]. In Portugal (2016), the average of coffee consumption was around $11.8 \text{ g.inhab}^{-1}.\text{day}^{-1}$, and most of the SCG produced is landfilled [7, 8], which clearly demonstrates that it is fundamental to conduct a more correct management of this waste.

Other relevant by-product in the Portuguese context is the brewery spent grain (BSG). The global beer production was estimated as 1.96 billion hectoliters in 2016 [9]. In Europe, the second largest beer producer in the world, there are approximately 6500 brewery companies (producing 383 million hectoliters of beer). During beer production, different stages are performed and BSG results from the saccharification of malted grains. BSG represents 85% of the by-products obtained in the brewery process; 100 L of beer lead to the production of around 45 kg of BSG [9]. Based on this information it can be estimated a global yearly production of 87 million ton of BSG [9]. According to Eurostat, in 2018, Portugal produced 7.22×10^8 liters of beer, which might correspond to 3.3×10^8 kg of BSG. According to the beer producers mostly of BSG produced is forwarded for animal feed.

The present study shows a preliminary characterization of the mentioned by-products/wastes. The main objective was to assess their potential for bioenergy production.

7.4.2 SPECIFIC OBJECTIVES

To characterize spent coffee grounds, grape pomace and brewery spent grains in order to evaluate their potential as alternative raw materials for bioenergy production, considering their moisture, oil content and its acidity.

7.4.3 MATERIALS AND METHODS

7.4.3.1 *Materials*

Grape pomace was donated by a wine producer from Cinfães, at North of Portugal. Waste coffee grounds was collected from a restaurant near Porto. Brewery spent grain was provided by a local beer producer at the Porto metropolitan area. The seeds from grape pomace were separated manually, after all the mass be dried.

Petroleum ether (LabChem $\geq 90\%$) was used for chemical oil extraction (Soxhlet). Methanol (Fischer Scientific $\geq 99\%$) was used as the acyl acceptor on the esterification and transesterification reaction. The catalyst used was the sodium hydroxide powder 97% (reagent grade, Aldrich). All the other reagents were of analytical grade.

7.4.3.2 *Extraction of oil*

The oil extraction was performed using a Soxhlet extractor (100 ml) as referred in section 7.3.3 (NP EN ISO 659 (2002)).

7.4.3.3 *Analytical methods*

The moisture in the grape pomace, grape seeds (red and white), SCG and BSG was determined according to the NP 12880 (2000). The acid value of the oils extracted by the chemical process before referred was measured by a titrimetric method, according to EN 14104.

7.4.4 RESULTS AND DISCUSSION

Grape pomace from the two collected varieties, respectively red pomace grape and white pomace grape, are characterized in Table 7.14.

Table 7.14. Preliminary characterization of grape pomace (red and white varieties)

	Red Grape Variety		White Grape Variety	
	Grape pomace	Grape seeds	Grape pomace	Grape seeds
Oil Content (wt.%)	5.9 ± 0.0	12.0 ± 0.2	8.5 ± 0.4	11.2 ± 0.8
Oil acid value (mg KOH.g ⁻¹)	-	4.5 ± 0.7	-	4.6 ± 0.6
Seeds/grape pomace (% dry matter)	31.5		28.7	

The moisture content was determined only on the red grape variety, being 63.2 ± 0.2 %, due to the low amount of the white grape sample. The value obtained is in the range referred by the literature (65 – 76%) [10, 11]. Grape pomace is reported to be comprised by 70 wt.% grape skins, 12 wt.% stalks and 18 wt.% seeds [12]. In the study developed by Gafoor *et al.* [13], the amount of seeds and skin varies from 38% to 52% of the total dry matter [13], values above those in Table 7.14, practically similar in both red and white varieties. According to the literature, the oil content of grape pomace varies from 5.47 wt.% to 8.66 wt.%, and the grape seeds might present an oil content from 12.4 wt.% to 16.0 wt.% [14]. The seeds from this work shown in Figure 7.15 are in agreement with the literature. The amount of oil in the grape seeds depends on the variety and maturity of the grape [15].

The acid value found on the oils from the two varieties of grape seeds studied was similar. According to the study developed by Hussein *et al.* [16], oil with an acidity of 5.3 mg KOH.g⁻¹ was obtained from fresh grape seeds. Usually, the acid value parameter is used as a limiting quality parameter for industrial use by the conventional process of biodiesel production. In this case, the results obtained showed an oil with relatively low acidity, and, thus, potential to be used for bioenergy production.

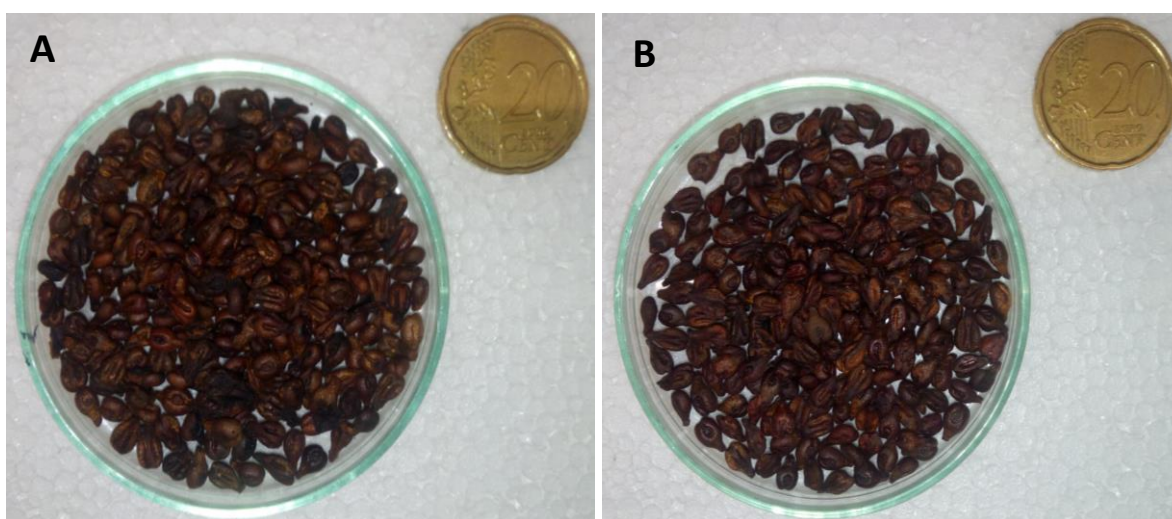


Figure 7.15. Grape seeds obtained from grape pomace (A – red variety; B – white variety).

The preliminary SCG and BSG characterization is presented in Table 7.15.

Table 7.15. Preliminary characterization of spent coffee grounds and brewery spent grain

	Spent Coffee Grounds	Brewery Spent Grain
Oil content (wt.%)	8.9 ± 0.3	12.6 ± 0.6
Oil acid value (mg KOH.g⁻¹)	15 ± 1	33 ± 1
Moisture (wt.%)	40.1 ± 0.0	69 ± 1

SCG shows a high moisture content. According to the literature, SCG has moisture between 55 wt.% and 70 wt.%. [6, 17], range above the value found in the characterized product. For SCG, moisture content depends directly on the operating conditions of the coffee machine used to obtain the drinkable coffee. Also, the time of storage influences the moisture. The high moisture content in SCG leads to the faster deterioration of this product (natural decomposition) [6]. The oil content was slightly lower than the values reported in the literature (10 – 15 wt.%), although this value directly depends on the coffee variety [6].

The acid value of $14.5 \text{ mg KOH.g}^{-1}$ was higher than the values reported in the literature in the range of $4.01\text{--}6.39 \text{ mg KOH.g}^{-1}$ [18]. The difference is related with the quality of the SCG, due to the sample (Figure 7.16) deterioration during the time of storage.

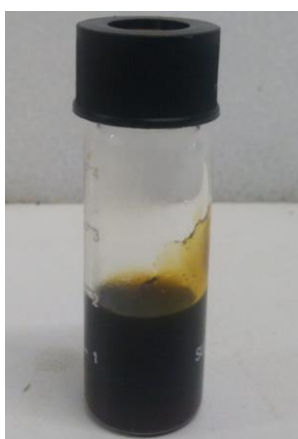


Figure 7.16. Oil extracted from spent coffee grounds.

Figure 7.17 shows a BSG sample that gave a high moisture content of 69% and around 13 wt.% of oil. Moisture percentage agrees with the values reported in the literature of around 70 wt.%, a major drawback when the recovery of this by-product is considered (high costs with transport and high susceptibility to “natural” deterioration) [9]. Several studies showed that oil in the brewery spent grains can vary between 3.9 wt.% and 13.3 wt.% [9]. The value obtained in our sample was close to the highest ones reported and slightly lower than that found for olive pomace (17.7 wt.%), rice bran (18.9 wt.%) or grape seeds (12.0 wt.%), revealing the potential of this type of waste for biodiesel production. According the study developed by Mallen *et al.* [9], the oil extracted from brewery spent grain had an acid value of $32.6 \pm 0.1 \text{ mgKOH.g}^{-1}$, value very similar to the obtain in our study ($33 \pm 1 \text{ mgKOH.g}^{-1}$)



Figure 7.17. Brewery spent grain sample analyzed.

The results suggested that SCG, BSG and grape pomace may be interesting sources of oil, despite its percentage varied between 9 wt.% and 12 wt.%. Due to the fact that all them showed an acidity above the threshold value for homogeneous alkaline transesterification (2 mgKOH.g^{-1}) [19], requires pre-treatment processes or other alternative routes. Anyway, the use of these oils for biodiesel production should be considered and be a subject of future investigations.

7.4.5 CONCLUSIONS

The recovery of oils from wastes/by-products may be important to reduce costs on the bioenergy production processes as well as to better manage those materials, in the light of the principles of circular economy, in order to provide new economic opportunities, thus contributing to long-term competitiveness. The type of wastes and by-products here characterized are produced in large scale

in Portugal, and some of them are disposed of or, in the best scenario, used as animal food or organic amendment. Such alternatives add little value to the producer or, in some cases, represent significant economic and environmental costs for the society, that must look these wastes/by-products as more valuable raw materials, for example as a source of oils. Despite the fact that the oil percentage found in spent coffee grounds, in brewery spent grain and grape pomace it is not appreciable when compared with the conventional oilseed crops. Although, one must look at them from a perspective of developing integrated solutions enabling more sustainable materials management practices as well as adding value to the economic sector.

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CHAPTER 8 CONCLUSIONS AND FUTURE WORK

8.1. CONCLUSIONS

The main conclusions of this work are according the type of raw material studied aiming bioenergy production, i.e., non-edible oils or oils recovered from wastes and by-products.

8.1.1. NON-EDIBLE CROPS

8.1.1.1. Agronomic Studies

According to the literature review on more than 300 oilseed plant species, only 40 of them have potential for biofuel production. Based on the seeds' oil content and considering the characteristics of the evaluated non-food crops, the following 4 were selected:

- *Ricinus communis*;
- *Crambe abyssinica*;
- *Jatropha curcas*;
- *Simmondsia chinensis*.

An overview and assessment of the agronomic conditions required by each crop was compiled, providing a good basis of agronomic information for future studies. Among them, *Crambe abyssinica*, an annual non-edible oilseed crop, was selected for cultivation in Portugal under the scope of the present study.

Cynara cardunculus, a perennial crop with Portuguese native species, producing seeds with high oil content, is already cultivated in Portugal with the main purpose of obtaining flowers for the production of an aqueous extract used as milk coagulant in the manufacture of traditional sheep milk cheese. Thus, further agronomic studies with *Cynara cardunculus* were developed aiming at obtaining and recovering the seeds and biomass, in order to open a window for the full recovery of this resource.

The agronomic studies showed that:

Crambe abyssinica

- *Crambe abyssinica* might be cultivated in Portugal resulting in seeds with relatively high oil content (> **26 wt.%**) and acceptable yields (742 kg.ha⁻¹);
- The first season showed the best growing cycle, evidencing that weather conditions, namely temperature and soil moisture, influence not only seeds yield but also oil yield;
- Mild temperatures until flowering and high water availability allowed achieving good emergence rates on shorter periods (between 19 and 43 days);
- The prevalence of cold weather or high temperatures during the germination affected negatively the germination rate of the seeds;
- The viability analysis of scaling crambe production in Portugal should consider the integrated use of all the biomass resource;
- Under the conditions of the agronomic study carried out, the life cycle assessment for the production of 1 ton of crambe seeds showed the consumption of diesel (field preparation) and electricity (irrigation) as causing most of the environmental impacts;
- The substitution of mineral diesel for biodiesel and the application of organic manure might be a way of improving the environmental performance of the system previously referred;

Cynara cardunculus

- *Cynara cardunculus* can be cultivated in Portugal aiming also seeds production and recovery. The seeds showed a relatively high oil content (from around **22 wt.%** to **27 wt.%**) and seeds yield was close to 189 kg.ha⁻¹;
- The low seeds' yields obtained for cardoon are mainly justified by the use of a wild variety and low plant density employed;
- In 2019 lower seeds' yields were obtained, with the amount of seeds per capitula being significantly lower than those verified in 2018. The decrease of water availability mostly during the Winter-Spring 2019 season might justify the lower yields obtained;
- The percentage of viable seeds seem to be the parameter that better reflects the climatic variation verified in the years (2018 – 48.5%; 2019 – 29.7%);
- The results suggest that the cut of the flower does not affect the characteristics of the seeds

(seeds' oil content, percentage of viable seeds and 1000 seeds' weight);

- The seeds' oil content was not affected by cardoon ecotype;
- The scale-up of cardoon production should be focused on the integral use of all the biomass resources;
- The agronomic study of cardoon has shown that cultivating this crop with low inputs is possible, at least when compared with other oilseeds' crops, although requiring to enhance and stabilize seeds' yields for which the use of a cultivated variety is proposed.

8.1.1.2. Nutritional Value Assessment of Biomass Wastes and By-products

The nutritional assessment of cardoon and crambe biomass revealed that these materials have good nutritional properties:

- Seeds showed a high content of protein (**16 – 20 wt.%**) and oil (**> 22 wt.%**);
- After the extraction of the oils, the remaining solid fraction (meal and presscake) generally shows a high protein content (**18 – 25 wt.%**), within the typical optimum level of protein for animal feed (16 – 22 wt.%);
- Biomass from cardoon heads showed a protein content around **7 wt.%**, similar to the values found in the cereal grains;
- Biomass from the crambe stem (straw) showed low content of protein (around **4 wt.%**) and high content of fibers, comparable to what is verified in other crops like rape straw, barley straw, wheat straw or oat straw;
- Overall, the nutritional value assessment showed that the different biomass samples from *Crambe abyssinica* and *Cynara cardunculus* crops are rich in protein, oil and fiber, having potential to be used as animal feed.

8.1.1.3. Non-Edible Oil Refining and Biodiesel Production

The oil extracted from crambe seeds showed a high phosphorus content. The presence of phosphorus in the oil affects biodiesel quality, increasing the potential generation of pollutants in the exhaust gases. For this reason, water and chemical degumming were applied to refine crambe raw oil and it was possible to conclude that the best condition was the dose of 0.80 vol.% from a solution with 25 wt.% of acid (H_3PO_4) that allowed to obtain biodiesel with 3.8 ppm of phosphorus.

Biodiesel produced by homogenous alkaline transesterification using the crambe oil extracted from the seeds, showed good quality. The results suggest that:

- Almost all the properties evaluated are in agreement with the European biodiesel quality standard EN 14214;
- Three kinds of biodiesel were obtained from the crambe oils used, ranked in decreasing quality: biodiesel from Portuguese refined oil > biodiesel from Portuguese crude oil > biodiesel from Brazilian commercial oil;
- Biodiesel produced from the refined oil presented the highest quality, due to lower content of contaminants, showing the relevance of using a purification step;
- The high oxidation stability of the crambe biodiesel was identified as an important advantage of using this feedstock.

Biodiesel produced by enzymatic transesterification using oil from the Portuguese crambe seeds (acid value below 2 mg KOH.g⁻¹), with one step addition of methanol, has shown to be a good alternative process to the conventional route of biodiesel production using *Crambe abyssinica* oil as raw material (from different origin). The results showed that:

- It was possible to obtain 84 ± 5 wt.% of FAME after 2 hours, a value that using the predictive model established might be increased to almost 99 wt.% after 24 hours using a methanol:oil molar ratio of 6:1 and 8 wt.% of enzyme;
- Biodiesel from crambe oil seems to be technically viable and may constitute a promising complementary alternative to the conventional edible oils generally used for biodiesel production in Portugal.

Biodiesel produced from cardoon oil, using either alkaline or enzymatic catalysts, showed relatively high purity (around 87 wt.%), although below that required by the standard EN 14214 (96.5 wt.%). Further studies with this oil should be performed aiming at the full characterization of the biodiesel produced.

8.1.2. RECOVERY OF WASTES/BY-PRODUCTS

8.1.2.1. Oil Pretreatment and Biodiesel Production

Among several types of wastes/by-products identified in Portugal as having high potential for bioenergy production, soapstock, rice bran and olive pomace were selected for further studies.

For acid oil from soapstock the results showed the following:

- By homogeneous acid esterification and glycerolysis reactions, an acid oil from soapstock resulting from soapstock chemical treatment ($128.8 \text{ mg KOH.g}^{-1}$) can be efficiently pre-treated, allowing to obtain a final acid value close to 10 mg KOH.g^{-1} (using both processes);
- After alkaline transesterification, biodiesel with 90 wt.% purity could be obtained;
- Acid esterification was an efficient and easy process for reducing the acidity of acid oil using mild temperatures (65°C); however, the purification process generates wastewater which requires further treatment;
- Glycerolysis was also an efficient process to reduce oil acidity and does not require purification; however, the reaction conditions, and namely the high temperatures, increase the costs of the process and this might reduce its economic viability.

For rice bran oil the results showed that:

- Rice bran showed an oil content of $18.9 \pm 0.5 \text{ wt.}\%$;
- The oil had high acidity of 47 mg KOH.g^{-1} , which could be effectively reduced either by homogenous acid esterification (65°C or 55°C , 4 wt.% of catalyst during 8 h) or enzymatic esterification (35°C , 5 wt.% catalyst during 24 h);
- For the homogenous acid esterification, increasing the temperature from 45°C to 55°C or 65°C , significantly increases the rate of the esterification reaction during the first two hours. However, after 8 h, similar results are obtained;
- Under the best conditions (65°C and 4 wt.% of catalyst), after 60 min of reaction, the acidity tends to stabilize, showing no relevance in increasing the reaction time;

- The biocatalysed process allowed to obtain better results (higher FAME content in the final product), with an acidity reduction of 70 % (from 47 mg KOH.g⁻¹ to 11 mg KOH.g⁻¹) in 60 min;
- After 24 hours, the enzymatic process allowed to reach an acidity reduction of 82 % (final acidity of 7 mg KOH.g⁻¹) and a FAME content of 87 wt.%, thus clearly revealing the conversion of free fatty acids and glycerides into FAME.
- The initial FFA content of the rice bran oil represents only around 23 wt.% of oil's mass, although at the end of esterification reaction FAME was higher than 23 wt.% which leads to conclude that esterification and transesterification reactions occurred simultaneously and, in this case, transesterification was extensively promoted.

The study developed with acid olive pomace oil revealed that:

- Fresh olive pomace presented about 18 wt.% of oil;
- The air storage of the olive pomace during 2 weeks led to an increase in the acidity of the oil extracted of almost 2 fold. After two months of storage, the oil acidity was very high (approximately 40 mg KOH.g⁻¹). According to the results, after 4 months the acidity of the oil seems to stabilize (around 50 mg KOH.g⁻¹).
- The acidity of olive pomace oil could be reduced from 50.6 mg KOH.g⁻¹ to 7.5 mg KOH.g⁻¹ (85% of reduction) by enzymatic esterification, using a methanol:oil molar ratio of 6:1 and an enzymatic dose of 3.5 wt.%, giving a pretreated oil having a FAME content of 84 wt.%.
- Similar acidity reduction (7.3 mg KOH.g⁻¹) was achieved using an enzymatic dose of 5 wt.%, giving a pretreated oil having a FAME content of 87.6 wt.%.
- Acid esterification and glycerolysis pretreatment, conducted using "fresh oil", did not bring relevant benefits mostly due to its relatively low acidity (4.4 ± 0.3 mg KOH.g⁻¹).

8.2. FUTURE WORK

The present work covered a large spectrum of subjects, therefore opening many research lines for future studies. The main subjects considered to be important to further explore, are presented as follows:

- Cultivating *Crambe abyssinica* in large scale with fertilization and irrigation systems, aiming to study the seeds' yields at an industrial level;
- Cultivating *Cynara cardunculus* using seeds from a domestic ecotype with the minimum agronomic inputs in marginal lands, aiming to obtain significant values for stabilized seeds' yields;
- Making agronomic studies on other alternative non-edible oilseed crops in Portugal, namely *Ricinus communis*, *Jatropha curcas* or *Simmondsia chinensis* to see whether or not they might be considered relevant alternatives to the conventional raw materials used for biodiesel production;
- Analysing the presence of antinutrients in biomass wastes/by-products resulting from the crops production and oil extraction;
- Determining the nutritional value of grape pomace, waste coffee grounds and brewery spent grains after oil extraction;
- Conducting a full characterization of the oils recovered, from grape pomace, spent coffee grounds and brewery spent grains;
- Identifying and quantifying the natural antioxidants in rice bran oil;
- Studying *in situ* lipase-catalyzed processes for biodiesel production using acid oils;
- Identifying and quantifying the natural antioxidants in cardoon oil;
- Determining the germination capacity of cardoon seeds according to the degree of seeds maturation;
- Characterizing biodiesel obtained from *Cynara cardunculus* L., using a domestic ecotype cultivated in Portugal;
- Optimizing the transesterification of cardoon oil using alkaline and enzymatic catalysts;
- Assessing the economic viability of the enzymatic transesterification reaction.