UNIVERSIDAD POLITÉCNICA DE MADRID

ESCUELA TÉCNICA SUPERIOR DE

INGENIEROS DE MINAS Y ENERGÍA

**BIODIESEL FROM MICROORGANISMS
AND OTHER ALTERNATIVE
FEEDSTOCKS: PRODUCTION,
COMPOSITION AND PROPERTIES**

PhD Thesis

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OTHER ALTERNATIVE FEEDSTOCKS:
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RESUMEN

La creciente demanda de energía, la dependencia de los combustibles fósiles y las preocupaciones sobre el cambio climático alientan la búsqueda de fuentes de energía sostenibles. Los biocombustibles, respaldados por políticas actuales de Europa y Estados Unidos, tienen un papel destacado en la presente transición energética. En concreto, el biodiésel puede ser una alternativa al diésel y, actualmente, muchos países lo utilizan en mezclas con dicho combustible. Sin embargo, estudios recientes han señalado la falta de sostenibilidad del biodiésel cuando se produce a partir de aceites como la soja, la palma, la colza y el girasol y, además, algunos fabricantes de automóviles están preocupados por aumentar el porcentaje de biodiésel de mezcla debido a la posible reducción en la calidad del combustible. Todo ello implica que las propiedades del biodiésel y su relación con su composición sea un tema de interés relevante.

Esta tesis estudia nuevas fuentes de biodiésel y evalúa sus propiedades, con el objetivo final de mostrar alternativas sostenibles a los aceites actuales utilizados para la producción de biodiésel, aumentando la diversidad de fuentes de energía, reduciendo la dependencia de los combustibles fósiles y promoviendo el desarrollo de una economía circular.

Las principales propiedades del biodiésel se estiman a partir de su composición, proponiendo nuevas ecuaciones para estimar la estabilidad a la oxidación, el comportamiento en frío y la lubricidad de los ésteres etílicos de ácidos grasos (FAEEs), un tipo de biodiésel que utiliza bioetanol, en lugar del metanol comúnmente utilizado que, actualmente, tiene un origen fósil.

Los microorganismos son probablemente, entre las materias estudiadas, los que tienen un mayor potencial para convertirse en la principal fuente de biocombustible en un futuro cercano. En esta tesis, se modifica genéticamente la bacteria *Escherichia coli* para cambiar su composición de ácidos grasos libres mejorando las propiedades finales del biodiésel obtenido a partir de dichos ácidos.

Además, esta tesis examina un proceso supercrítico para la producción de FAEEs a partir de grasa animal con alto contenido de ácidos grasos libres, uno de los mayores problemas de las materias primas residuales. Se evalúa, a su vez, la degradación de los compuestos poliinsaturados, un problema común de este tipo de procesos.

Asimismo, se han obtenido FAEEs a partir de aceite de semilla de uva, opción de gran potencial en países productores de vino como España. Se ha caracterizado y evaluado el potencial como biocombustible de la macroalga *Chaetomorpha cf. gracilis*, recogida en humedales de Cuba y posible alternativa a plantas terrestres que ocupan terreno cultivable. Por último, se ha producido biodiésel de plantas del desierto de Túnez y se han analizado sus propiedades, una alternativa interesante debido a su baja necesidad de agua y su capacidad para crecer en tierras áridas.

ABSTRACT

The increasing demand for energy, the dependence on fossil fuels and the concerns about climate change encourage the search of sustainable energy sources. Biofuels are believed to have an important role in the coming energy transition and are supported by both European and American current policies. Biodiesel has already been proved to be an alternative to diesel and is currently used by many countries in biodiesel-diesel blends. However, recent studies have pointed out the non-sustainability of biodiesel when it is produced from current oils like soybean, palm, rapeseed and sunflower. Moreover, some car manufacturers are worried to increase the percentage of biodiesel blends because of the possible reduction in fuel quality, hence, the properties of biodiesel and their relation with its composition are an important matter that must be carefully considered.

This thesis studies new biodiesel sources and assesses their properties, with the ultimate goal of showing sustainable and feasible alternatives to the current oils used for the production of biodiesel, aiming to improve the sustainability of the energy sources, reduce the dependence on fossil fuels and develop knowledge to move toward a circular economy.

Main properties of biodiesel made from different sources are estimated from their composition, proposing new equations to estimate the oxidation stability, cold flow performance and lubricity of fatty acid ethyl esters (FAEEs), a type of biodiesel that uses bioethanol, instead of commonly used methanol, which currently has a fossil origin.

Of all the studied sources, microorganisms have the hugest potential to become sources of biofuels. In this thesis, the bacteria *Escherichia coli* is genetically modified to change its fatty acid composition improving the final properties of the biodiesel obtained from these fatty acids.

Moreover, this thesis examines a supercritical process for FAEEs production from animal fat with high content of free fatty acids, one of the biggest problems of waste feedstocks. The degradation of polyunsaturated compounds, a common problem of this kind of process, is assessed.

Besides, FAEEs from grape seed oil, with a huge potential in wine producing countries as Spain, have been evaluated. The macroalga *Chaetomorpha cf. gracilis* grown in Cuban

wetlands, an alternative to avoid the excessive use of land, has been characterized to assess its potential as a biofuel and, lastly, biodiesel from desert plants from Tunisia has been produced and its properties analyzed, an interesting alternative because of their low need of water and their ability to grow in arid lands.

DISSEMINATION

Undergraduate Thesis Projects (UTPs)

UTP1: Alonso, Elena (2014). Biodiésel de ésteres etílicos de ácidos grasos: correlación composición-propiedades. (Supervised by David Bolonio). Mark: 9.

UTP2: Rebaque, Virginia (2015). Estudio de las propiedades de biodiesel obtenido a partir de aceites de la república tunecina. (Supervised by David Bolonio). Mark: 10 MH.

UTP3: Fernández, Marina (2016). Hydrolysis of lignocellulosic material to sugars as feedstock for growing microorganisms. (Supervised by David Bolonio). Mark: 10.

UTP4: García, Inés (2016). Caracterización de FAMEs y aceites con las técnicas analíticas GC-MSD y GC-FID para su evaluación como potenciales fuentes de energía (Supervised by David Bolonio). Mark: 10 MH.

UTP5: Martín, Víctor (2016). Biodiésel de ésteres metílicos y etílicos de ácidos grasos a partir de materias primas de cuba: composición y propiedades. (Supervised by David Bolonio). Mark: 10 MH.

UTP6: Rueda, Paula (2017). Análisis de Ciclo Vida comparativo de la producción de biodiesel a partir de *Citrullus colocynthis* y Palma. (Supervised by David Bolonio and Israel Herrera). Mark: 9.7.

UTP7: Aramburu, Jone (2018). Análisis de ciclo de vida del biodiesel obtenido a partir de grasa animal en España. (Supervised by David Bolonio and María Jesús García Martínez). Mark: 9.5.

Master's Theses (MTs)

MT1: Bolonio, David (2013). Biodiésel obtenido de la bacteria *Escherichia coli*. (Supervised by Laureano Canoira). Mark: 10.

MT2: Ge Chuansheng (2014). Oxidation stability and cold performance of biodiesel. (Supervised by David Bolonio and Laureano Canoira). Mark: 9.5.

MT3: Timón, Natalia (2017). Desulfuración inducida por ultrasonidos de combustibles de pirólisis. (Supervised by David Bolonio and Laureano Canoira). Mark: 10.

MT4: Fernández, Marina (2018). Life cycle analysis of grape seed oil biofuel in Spain. (Supervised by David Bolonio and María Jesús García). Mark: 10.

International Congresses (ICs)

IC1: Energy and Environment Knowledge Week (E2kW 2014)

Related MT: 3

Organizer: Energy and Environment Science and Technology Campus (CYTEMA)

Venue: Toledo (Spain)   Date: 30-31 October, 2014

Title: Desulfurization of pyrolysis fuel produced from waste lube oils, tyres and plastics

Category: Author and oral presentation

IC2: The Energy & Materials Research Conference (EMR2015)

Related UTPs: 1, 2

Related MTs: 1, 2

Organizer: Formatex Research Center

Venue: Madrid (Spain)   Date: 25-27 February, 2015

Title: Estimation of Cold Flow Performance and Oxidation Stability of Fatty Acid Ethyl Esters from Lipids Obtained from *Escherichia coli*

Category: Author and oral presentation

IC3: International Congress and Expo on Biofuels and Bioenergy

Related UTPs: 7

Related research stay: University of Graz (16 March – 16 June, 2015)

Organizer: OMICS International

Venue: Valencia (Spain)   Date: 25-27 August, 2015

Title: Production of biodiesel from animal fat using supercritical ethanol

Category: Author and poster presentation

Best poster award

Publication: <http://dx.doi.org/10.4172/2090-4541.S1.003>

IC4: Green and Sustainable Chemistry Conference

Related UTPs: 2, 4, 6

Organizer: Elsevier

Venue: Berlin (Germany)   Date: 3-6 April, 2016

Title: Biofuels (FAEEs) from rare oils of the Tunisian Republic

Category: Author and oral presentation

IC5: 5th World Bioenergy Congress and Expo

Related UTPs: 2, 4

Organizer: OMICS International

Venue: Madrid (Spain)   Date: 29-30 June, 2017

Title: Fatty acid methyl esters (FAMES) obtained from rare seeds of Tunisia: *Ibicella lutea*, *Peganum harmala*, *Smyrniolum olusatrum*, *Onopordum nervosum* and *Solanum elaeagnifolium*

Category: Author and oral presentation

IC6: IX International Renewable Energy Conference, Energy Saving and Energy Education (CIER 2017)

Related UTPs: 4, 5

Organizer: The Study Centre for Renewable Energy Technologies (CETER)

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Title: Green-filamentous macroalgae *Chaetomorpha* cf. *gracilis* from Cuban wetlands as a feedstock for diesel engine fuel improver

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IC7: 7th Euro Biotechnology Congress

Related research stay: Joint Bioenergy Institute (Berkeley) (1 August, 2016 – 1 January, 2017)

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Title: Improved lipid biosynthesis in *E. coli* through heterologous expression of a plant thioesterase

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Book chapter

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Papers

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Related IC: 2

Published paper 2: Al-Lal, A. M., Bolonio, D., Llamas, A., Lapuerta, M., & Canoira, L. (2015). Desulfurization of pyrolysis fuels obtained from waste: Lube oils, tires and plastics. *Fuel*, 150, 208-216.

Related MT: 3

Related IC: 1

Published paper 3: Lapuerta, M., Sánchez-Valdepeñas, J., Bolonio, D., & Sukjit, E. (2016). Effect of fatty acid composition of methyl and ethyl esters on the lubricity at different humidities. *Fuel*, 184, 202-210.

Related UTPs: 1

Published paper 4: Houachri, T., Bolonio, D., F. Ortega, M., García-Martínez, M. J., Llamas, A., Al-Lal, A. M., El Gazza, M., & Canoira, L. (2017). Geographical variability of the composition and properties of fatty acid methyl esters from *Citrullus colocynthis* in Tunisia. *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects*, 1-9.

Related UTPs: 2, 4, 6

Related IC: 4

Published paper 5: Houachri, T., Bolonio, D., Llamas, A., Rodríguez-Fernández, J., El Gazzah, M., Mittelbach, M., Magín, L., & Canoira, L. (2018). Fatty acid methyl and ethyl esters obtained from rare seeds from Tunisia: *Ammi visnaga*, *Citrullus colocynthis*, *Datura stramonium*, *Ecballium elaterium*, and *Silybum marianum*. *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects*, 40(1), 93-99.

Related UTPs: 2, 4, 6

Related IC: 4

Published paper 6: Bolonio, D., Marco Neu, P., Schober, S., García-Martínez, M. J., Mittelbach, M., & Canoira, L. (2018). Fatty acid ethyl esters from animal fat using supercritical ethanol process. *Energy & Fuels*, 32(1), 490-496.

Related UTP: 7

Related research stay: University of Graz (16 March – 16 June, 2015)

In publication process

Unpublished paper 1: Fatty acid methyl esters (FAME) obtained from seeds of Tunisia: *Ibicella lutea*, *Onopordum nervosum*, *Peganum harmala*, *Silybum marianum*, *Smyrniolum olusatrum* and *Solanum elaeagnifolium*.

Related UTPs: 2, 4

Related IC: 5

Unpublished paper 2: Green-filamentous macroalgae *Chaetomorpha cf. gracilis* from Cuban wetlands as a feedstock to produce alternative fuel: A physicochemical characterization.

Related UTPs: 4, 5

Related IC: 6

Unpublished paper 3: Tuning the free fatty acids composition of *Escherichia coli* to obtain optimum biodiesel properties.

Related IC: 7

Related research stay: Joint Bioenergy Institute (Berkeley) (1 August, 2016 – 1 January, 2017)

Unpublished paper 4: Fatty acid ethyl esters (FAEEs) obtained from grape seed oil: a fully renewable biofuel.

Related MT: 4

1. INTRODUCTION

1.1 THE NEED OF ALTERNATIVE BIODIESEL FEEDSTOCKS

The increasing global demand for energy and stronger environmental concerns have made necessary the search for new and sustainable sources of energy. The use of biofuels, especially in the transport sector, has proved to be an option to reduce the use of oil. This sector accounts for 27.8 % of the total energy consumption [Key World Energy Statistics, 2016], with a contribution from biofuels of 3.5 % in 2014 [Energy Efficiency Indicators, 2014]. Biodiesel represents the 33 % of global biofuel production [Renewables global status report, 2016], having increased 8.5 times from 2005 to 2015 [World biodiesel production by year, 2015]. Despite this promising growth, further support for advanced biofuel research, development and demonstration is still needed to reach the share of 27 % of world transportation fuel by 2050 [Eisentraut et al., 2011], an international target to avoid around 2.1 gigatonnes (Gt) of CO₂ emissions per year, which is around 20 % of the annual global total emissions of fossil fuels [Global Carbon Emissions, 2015].

Different legislations and targets are set worldwide and the European Union, specifically, aims to have 10 % of the transport fuel of every EU country come from renewable sources such as biofuels by 2020. The production of these biofuels must fulfill very strict requirements to reduce greenhouse gas emissions without adversely affecting the environment or social sustainability [COM/2016/0767]. The most important criteria are: 1) The greenhouse gas (GHG) emission saving, taking into account all life cycle emissions, shall be at least 50 % for biofuels produced in installations in operation on or before 5 October 2015, 60 % in installations starting operation from 5 October 2015, 70 % for biofuels produced in installations starting operation after 1 January 2021; 2) Biofuels cannot be grown in areas converted from land with previously high carbon stock such as wetlands or forests; 3) Biofuels cannot be produced from raw materials obtained from land with high biodiversity such as primary forests or highly biodiverse grasslands.

In 2015 in Europe, more than twice as much diesel was consumed for transport compared to gasoline [Eurostat, 2017] while in 2016, the blending of bioethanol and biodiesel in transport fuels was respectively 3.3 and 5.8 percent (energy basis), and thus well below the 10 percent

target for 2020 [EU Biofuels Annual, 2017]. Focusing on biodiesel, rapeseed oil is still the dominant biodiesel feedstock in the EU accounting for 47 % of total consumption in 2016 while palm oil was the second-most important feedstock accounting for 18 % [EU Biofuels Annual, 2017]. Both rapeseed and palm oil struggle to accomplish GHG savings (38 and 19 % according to default values set by BIOGRACE [BIOGRACE, 2018]) and have been criticized by numerous studies which point to wrongly use edible oils for biofuel purposes, the extensive occupation of land and the consequences of deforestation [Gilbert, 2012a; Gilbert, 2012b].

Based on the previous information, there is an important and urgent need of alternative feedstocks for biodiesel that fulfill sustainability requirements and are economically feasible, especially in European countries, where biodiesel could reduce the high dependence of foreign oil.

1.2 TRADITIONAL AND NEW BIODIESEL SOURCES

Biodiesel is a mixture of fatty acid alkyl esters, commonly produced by the reaction of methanol or ethanol and triacylglycerides in the presence of an alkali catalyst, a process called transesterification. In the middle of 1990s, the cost of feedstock accounted for 60–75 % of the total cost of biodiesel fuel [Krawczyk, 1996], but more recent studies point out that about more than 85 % of the costs of production are due to feedstock costs [Zhang et al., 2003; Hass et al., 2006]. Therefore, the choice of the feedstock determines the economic feasibility of the biodiesel production and the sustainability of the process. The most commonly used oils for the production of biodiesel are soybean, palm, rapeseed and sunflower [Woiciechowski et al., 2016; EIA, 2014]. All of them are expensive, edible and require arable land to effectively grow, which encourage the necessity of looking for other sources. The most promising alternatives are:

- Microorganisms have recently attracted great attention as a source of biodiesel [Meng et al., 2009]. Probably their most important advantage compared to other options is that only microorganisms can produce enough quantity of biofuels to wholly replace oil fuels for the transport sector [Chisti, 2007]. Land occupation by plants set up a limit on the total biofuel production but microorganisms grown in tanks and fed through carbon dioxide (CO₂) from the atmosphere could overcome that problem [Parmar et al., 2011].

- Non-edible plant oils or oils from non-arable¹ land plants: *Jatropha curcas* has led the search of non-edible oils from drought-resistant plants, capable of surviving in abandoned and fallowed agricultural land [Fairless, 2007; FAO, 2008] but more research is needed to fully commercialization and reduction of costs. [Wang et al., 2011]. There has been an extensive debate over an increase in food prices caused by the production of biofuels. Despite a general scientific consensus that there is little direct evidence of biofuels causing high food prices [Zhang et al., 2010; Gilbert et al., 2010; Ajanovic, 2011], media news has collected different studies [Tangermann, 2008; Malins, 2017] influencing public opinion on this subject [Fung et al., 2014]. However, with or without food competition, occupation of arable land and consumption of water during the production of biofuels is an important environmental and social problem when using plants in biofuel production [Escobar et al., 2009; Requena et al., 2011]. Economic globalization and development needs cropland that is shrinking in availability and triggers deforestation [Döös, 2002; Lambin et al., 2011], which has been recently pointed to cause 17% of global greenhouse gas emissions [Overmars et al., 2014].
- Marcoalgae or seaweed are an interesting and scarcely studied alternative to terrestrial plants, with ease adaptability to different growing conditions, can grow either in freshwaters or marine-waters, avoiding the use of land [Aresta et al., 2005] or can be harvested in ponds, occupying non-arable land.
- Waste feedstocks: Many have been proposed for biodiesel production [Sajjadi et al., 2016] and the most commonly used until now have been used cooking oil (UCO) and waste animal fats [EIA, 2017; EU Biofuels Annual, 2017]. The use of these types of feedstock eliminates the need to dispose them, and can make a major, although limited, contribution to the supply of biodiesel. Their probably most important advantage is their cost, which is negligible, but important challenges remain in their economic feasibility to use as biofuel sources, mainly because logistic or production issues.

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¹ The term “arable land” is used throughout the thesis with the meaning of “used land or suitable for growing crops”.

The search and consideration of different biodiesel sources does not have the goal to find the best and only feedstock, but to increase diversity, which is the key to a sustainable production. Moreover, in the course of biodiesel production, there are byproducts useable in industry. The transition from refineries to biorefineries should integrate the production of low value biofuels with high value biobased chemicals [Bozell et al., 2010] and only based on that synergy, future sustainable production chains of biofuels and high value chemicals from biomass will be established [Cherubini, 2010].

1.3 BIODIESEL FROM MICROORGANISMS

Oleaginous microorganisms are defined as microbes with a content of lipids higher than 20% [Meng et al., 2009] and can produce what is commonly named as single cell oils (SCO). There are many kinds of microorganisms that can be classified (for the purpose of biofuel production) into microalgae, bacteria, yeasts and filamentous fungi (or mold).

Microalgae and cyanobacteria are sunlight-driven cell factories that can convert CO₂ to oil, which has made them the first choice for microbial biofuel production. Several microalgae have already been tested in photobioreactors obtaining high oil yields such as *Schizochytrium sp.* (50-77 dry wt %) and *Nitzschia sp.* (45–47 dry wt %) [Chisti, 2007]. Microalgae grow extremely rapid doubling their biomass within 24 h [Meng et al., 2009]. and demand less water and nutrients for their growth as compared to terrestrial crops [Rashid et al., 2014]. Despite these advantages, the scale-up applications of microalgae biofuels have encountered some technical limitations, especially during the harvesting [Milledge et al., 2013] and the oil extraction processes [Halim et al., 2012] where there is still no universal technology for efficient and low-cost production [Rashid et al., 2014]. Cyanobacteria have, in general, lower lipid content but introduce several advantages as lipid feedstock compared to microalgae: easier to genetically manipulate and increase their lipid content, higher growth rate and simpler cell wall, which aids in lipid extraction [Silva et al., 2014]. As remarkable examples, *Synechococcus sp.* PCC7942 was studied for biodiesel production, reporting a lipid content of 29 dry wt. % [Silva et al., 2014] and *Anabaena sphaerica* MBDU 105, which contains 19 dry wt % content of lipids [Anahas et al., 2015].

Bacteria (except cyanobacteria), yeast and fungi use biomass, instead of CO₂, as the carbon source for biodiesel. Bacteria, compared to microalgae, accumulate lower percentage of

lipids, for example: *Rhodococcus opacus*, one of the best candidates for triacylglycerides (TAGs) production, has been reported to produce 38 dry wt % of TAGs [Kurosawa et al., 2010]. As a positive side, bacteria have a superiority in the production of biodiesel with highest growth rate (to reach huge biomass only need 12–24 h) and easy culture method [Meng et al., 2009].

Several yeast species have also been studied for their lipid accumulation: *Cryptococcus curvatus* can accumulate large amounts of oil, up to 60% of the cell's dry weight [Thiru et al., 2011] and *Rhodotorula graminis* has been reported to produce a lipid content 54% dry. wt. [Galafassi et al., 2012].

Exploitation of oleaginous filamentous fungi for biodiesel has gathered strength from recent studies focused on poly-unsaturated fatty acid production (PUFA), such as arachidonic acid and γ -linolenic acid [Sakuradani et al., 2009]. As an advantage over microalgae or yeast cells, the harvest of fungal cells can be easier because of their filamentous growth [Xia et al., 2011]. Among the major lipid producers there is *Mucor circinelloides* with a 46% dry wt. lipid content or *Mortierella isabellina* with a reported 50–55% dry wt. oil [Papanikolaou et al., 2004].

Single cell oils are a real and very promising alternative for biodiesel production but work in this area has been still very limited [Li et al., 2008] and, as mentioned before, major technological challenges must be addressed to scale-up production and to have an economical and sustainable process. In addition, along with technology improvements, genetically modified microorganisms have already proved themselves to improve yields and efficiency of biofuel production processes [Peralta-Yahya et al., 2012]. Up to now, most of the synthetic biology work is based on amenable hosts for metabolic engineering like *E. coli* or *S. cerevisiae* which have been successfully used to test different approaches [Rungtaphan et al., 2014; Steen et al., 2014] and can be used as models to eventually use more complex but convenient organisms for biofuel production [Wijffels et al., 2013].

1.4 BIODIESEL FROM NON-ARABLE LAND PLANTS

Scarcity of land resources and water has encouraged searching alternatives to the traditional plants used for biodiesel production. Suitable land for agriculture is very difficult to quantify,

but recent studies [Fischer et al., 2012; Alexandratos et al., 2012; Zabel et al., 2014] show the quandary of arable land availability joined to the increasing global population. Accordingly, 91% of all suitable arable land is already occupied by agriculture when today's protected and densely forested areas are preserved in the future. In the USA, only 2% of currently suitable land is not yet used or protected/dense forest. In China, currently almost all the arable land suitable for agriculture has been under cultivation [Yang et al., 2009]. Africa has the highest potential for expansion of agricultural land, having 20% of the agriculturally suitable area currently not used for agriculture or is statistically not recorded in the data of currently used agricultural land [Ramankutty et al., 2008].

New sources of biofuels should therefore be able to grow effectively in non-arable lands to not compete with food crops, and ideally they have a need of minimum amounts of water. Many plants have been considered [Atabani et al., 2013], some of the most promising [Balat, 2011; Ahmia et al., 2014] are:

- Jatropha (*Jatropha curcas*) is a plant that originated in Mexico and now grows widespread in tropical and subtropical areas in Latin America, Asia, and Africa [Dias et al., 2012]. Jatropha seeds generally contain toxic components but produce 27–40% oil. Unlike other potential oil crops, jatropha is so attractive that it has been planted in large scale (>10 million ha globally) even without the agronomic improvement and evaluation needed for adaptation as a useful crop [Singh et al., 2014; BiofuelDigest, 2014]. However, studies with large-scale plantations of jatropha indicated that the growth performance and oil yield of this plant were far below than expected. Therefore, in order for jatropha to meet the demands as a renewable energy resource, this plant needs to be well domesticated [Fairless, 2007; McKeon, 2016].

- Castor (*Ricinus communis* L.) is an important industrial crop. The major producing countries are Brazil, China, India and the countries of the former Soviet Union [Koutroubas et al., 1999]. Castor seeds are poisonous to humans and animals since they contain the toxic protein ricin [Ogunniyi et al., 2006]. Worldwide, castor is cultivated on 12 600 km² with an annual seed production of 1.14 Mt and an average seed yield of 902 kg/ha [Sailaja et al., 2008]. Moreover, it can be grown on marginal lands, usually unsuitable for food crops [Berman et al., 2011]. Castor oil composition differs

significantly from other oils, containing approximately 80–90% of 12-hydroxy-9-cis-octadecenoic acid (ricinoleic acid), which has been considered one of the main drawbacks for this oil to become a favorable option in biodiesel fuel formulations [Knothe, 2008; Canoira et al., 2010]. The methyl ester of ricinoleic acid causes very high viscosity, exceeding the maximum values for kinematic viscosity in both the European (EN) and American (ASTM) biodiesel standards, but this could be overcome using several improvers successfully tested in previous studies [Boshui et al., 2010].

- Karanja (*Pongamia pinnata*) is mainly found native in India, Northern Australia, Fiji and in some regions of Eastern Asia [Karmee et al., 2005]. Karanja oil is considered non-edible and its toxicity has been confirmed by previous studies [Natanam et al., 1989]. The plant is highly tolerant of salinity and alkalinity, being able to grow on seashores and doing well on most soils (sandy, stony to clayey) [Murphy et al., 2012], in addition, it shows high tolerance against drought bearing temperatures up to 50 °C [Halder et al., 2014]. In India, karanja oil is one of the potential oils with a yearly production of 135,000 million tones [Bobade et al., 2012], out of which only 6% is being presently utilized [Kumar et al., 2013]. As a slight drawback, it has been reported that the conventional alkali-catalyzed route of biodiesel production from karanja oil does not work out effectively, because an often high free fatty acids (FFAs) content [Naik et al., 2008], making necessary an acid-catalyzed pre-esterification.

- Jojoba (*Simmondsia chinensis*) is a perennial shrub that grows naturally in a number of desert and semi-desert areas, mainly, the Sonora desert (Mexico) and in the South-West of US (Colorado desert). It is also cultivated in some countries, such as Argentina (2.0 kt/yr), Israel (1.1 kt/yr) or USA (1.0 kt/yr) [Canoira et al., 2006; El Bassam, 2010]. Plantations that were established with selected higher yielding clones are capable of producing up to 900 kg/ha [Undersander et al., 1990] and its seeds contain 45 and 55 wt % of inedible oil [Shah et al., 2010]. Unlike vegetable oils and animal fats, jojoba oil is not a triacylglyceride but a mixture of long chain esters (97–98 wt %) of fatty acids and fatty alcohols [Borugadda et al., 2012]. Biodiesel from jojoba oil has been successfully produced and its properties fulfill the standards with the exception of the viscosity, which exceeds the limit [Canoira et al., 2006].

1.5 BIODIESEL FROM MACROALGAE

The search for oil sources that do not occupy arable-land has presented macroalgae as a promising feedstock for biofuels. Macroalgae are classified into three major groups based on their photosynthetic pigmentation variations: red (*Rhodophyta*), brown (*Phaeophyta*) and green (*Chlorophyta*) [Chen et al., 2015]. There are several reviews on biofuel production from algae, but they usually focus on microalgae utilization [Brennan et al., 2010], mainly because several studies have shown higher oil yields in microalgae than macroalgae [Afify et al., 2010; Maceiras et al., 2011]. Nevertheless, biofuel obtained from macroalgae has an important potential [Aresta et al., 2005; Maceiras et al., 2011]. Many species grow in beaches or wetlands (not appropriate for terrestrial plants), they are treated as waste [Maceiras et al., 2011] and their collection has been pointed to have a big potential for biodiesel production [Maceiras et al., 2011]. The average photosynthetic efficiency of macroalgae is 6–8%, which is much higher than that of terrestrial biomass (1.8–2.2%) [Ross et al., 2008]. Macroalgae are fast growing marine and freshwater plants that can grow to considerable size (up to 60 m in length). Growth rates of marine macroalgae far exceed those of terrestrial biomass, mainly due to no water limitations [Gellenbeck et al., 1983]. Moreover, many species of macroalgae have not been studied so more macroalgae with bigger oil content could be found and, improving farming methods [Ross et al., 2008], they could become a viable source for biodiesel production.

1.6 BIODIESEL FROM ALTERNATIVE FEEDSTOCKS

The first step in any path to the future is wiser use of the energy resources. This includes elimination of obvious waste, higher energy conversion efficiency, substitution for lower energy intensity products and processes, recycling, and more energy-modest lifestyles [Lior et al., 2012]. The use of waste feedstocks for biofuel production would never satisfy the total demand of energy consumption but their use is essential for a clean, sustainable and circular economy [Willson et al., 2010; Stahel, 2016].

Wastes are defined as secondary products with little or no economic value, understanding secondary products as products of a process that have inelastic supply with demand where even if the market value of a secondary product increases, one would not expect more of it

to be produced from that process [ICF, 2015]. Many different wastes have been studied for biodiesel production, such as tobacco seed oil [Giannelos et al., 2002], olive cake [Hepbasli et al., 2003], vegetable oil soapstock [Haas, 2005], palm fatty acid distillate [Chongkhong et al., 2007], UCO [Chhetri et al., 2008], tall oil, coffee oil [Oliveira et al., 2008], municipal wastewater sludges [Mondala et al., 2009], waste fish oil [Yahyaee et al., 2013], waste animal fats [Canoira et al., 2008; Banković-Ilić et al., 2014; Altun et al., 2014], and soybean oil deodorizer distillate [Yin et al., 2016]. Among all of them, the most successful wastes for biodiesel production have been UCO and animal fats which respectively counted for 1) 12.6 and 10.22 % of the total biodiesel production of US in 2016 [Monthly Biodiesel Production Report, 2017] and 2) 18 % and 8 % of the total biodiesel consumption in the EU in 2015 [EU Biofuels Annual, 2017].

Nevertheless, some problems must be addressed to achieve a worldwide use of waste feedstocks in biodiesel production. For example, in the case of UCO and other feedstocks the collection infrastructure and logistics management must be optimized to lower the cost [Araujo et al., 2010]. On the other hand, the slaughter industry is generally well managed for product control and handling procedure, but there is a biosafety issue related to animal fats that could come from the contaminated animals [Greene et al., 2007]. In addition, low cost oils and fats often contain large amounts of FFA that cannot be converted to biodiesel using an alkaline catalyst [Canakci et al., 2001]. A proposed solution is to follow an acid-catalyzed process (using, for example, sulfuric or p-toluenesulfonic acid) but this entails slower reaction rate, and the requirement of high catalyst concentration and high temperature. Moreover, the separation of the catalyst and leaching of the catalyst can be serious issues with homogeneous acid catalysts [Kulkarni et al., 2006; Canoira et al., 2008]. As an alternative, it is possible to perform the reaction without catalyst, following a process of high pressures and temperatures, using supercritical alcohol [Saka et al., 2001]. Although it is an interesting technical option, some concerns exist over the degradation of products and the consequent loss of material [Imahara et al., 2008], together with the need of optimization to lower the current high operating costs [Marchetti et al., 2008].

1.7 PROPERTIES OF BIODIESEL

The properties of a biodiesel fuel are determined by: 1) The production process (see Table 1), which must be performed correctly to make a biodiesel in accordance with the standards and 2) The structure of its component fatty esters (see Table 2), therefore, depend directly on the raw material used for the production, unlike bioethanol fuel [Knothe, 2005; Ramos et al., 2009].

Table 1: Biodiesel properties that depend on the production process

Property	ASTM D6751		EN 14214	
	Limit	Test	Limit	Test
Acid number	0.50 mg KOH/g	D664	0.50 mg KOH/g	EN 14104
Alcohol control	0.2 wt % methanol max	EN 14110	0.20 wt % methanol max	EN 14110
	130 °C flash point, min	D93		
Carbon residue on 10% distillation residue, max	0.050 wt %	D4530		
Cold soak filtration time (CSFT), max ^a	360 s	D7501		
Copper strip corrosion, max	No 3	D130	class 1	EN ISO 2160
Ester content			96.5 % min	EN 14103
Free glycerin, max	0.020 wt %	D6584	0.02 wt %	EN 14105 EN 14106
Group I metals (Na + K), max	5 mg/kg	EN 14538	5.0 mg/kg	EN 14108 EN 14109 EN 14538
Group II metals (Ca + Mg), max	5 mg/kg	EN 14538	5.0 mg/kg	EN 14538
Monoacylglycerides, diacylglycerides and triacylglycerides, max	^b MG 0.40 wt %	D6584	MG 0.70 wt % DG 0.20 wt % TG 0.20 wt %	EN 14105
Phosphorous, max	0.001 wt %	D4951	4.0 mg/kg	EN 14107 EN 16294
Sulfated Ash, max	0.020 % mass	D874	0.02 % mass	ISO 3987
Sulfur, max (by mass)	Two grades: S15 15 ppm S500 0.05%	D5453	10.0 mg/kg	EN ISO 20846 EN ISO 20884 EN ISO 13032
Total contamination, max			24 mg/kg	EN 12662
Total glycerin, max	0.240 wt %	D6584	0.25 wt %	EN 14105
Water, max	0.050 vol %	D2709	500 mg/kg	EN ISO 12937

^a360 max for Grade 2B. 200 max for Grade 1B.

^bMonoacylglycerides for Grade 1B

Table 2: Biodiesel properties that depend on the composition

Property	ASTM D6751		EN 14214	
	Limit	Test	Limit	Test
Cetane number, min	47	D613	51.0	EN ISO 5165
Cloud Point	Report	D2500	Location and season dependant	EN 23015
Cold filter plugging point		D6371	Location and season dependant	EN 116
Density		D1298	860-900 kg/m ³	EN ISO 3675 EN ISO 12185
Distillation temperature (vol % recovered)	90 %: 360°C max	D1160		
Flash point, min	93°C	D93	101°C	EN ISO 2719 EN ISO 3679
Iodine value, max			120 g I ₂ /100g	EN 14111 EN 16300
Kinematic viscosity	1.9-6.0 mm ² /s	D445	3.5-5.0 mm ² /s	EN ISO 3104
Linolenic acid methyl ester, max			12.0 wt %	EN 14103
Lubricity, max	520 µm	D 6079	460 µm	EN ISO 12156-1
Oxidation stability, min	3 h	EN 14112 EN 15751	8 h	EN 14112
Polyunsaturated methyl esters, max			1.00 wt %	EN 15779

EN 14214 in Europe and ASTM D6751 in the USA are the two major specifications establishing the quality requirements for alkyl ester-based biodiesel fuels for transport. Although European and American standards are similar, EN 14214 is slightly stricter, because ASTM D6751 is intended for biodiesel to be used in blends with fossil diesel, while B100 accomplishing the EN 14214 could be used unblended in a diesel engine (if the engine has been adapted to operate on B100). In a summary, the most important properties are:

- Cetane number: main parameter of the combustion of a diesel-fuel, indicates the ignition quality: The higher the cetane number, the shorter the autoignition time [Meher et al., 2006]. ASTM D6751 and EN 14214 set a minimum of 47 and 51 respectively. With respect to its relation with the biodiesel composition, the longer the fatty acid carbon chains and the more saturated the molecules, the higher the cetane number [Ramos et al., 2009]. Moreover, fatty acid methyl esters (FAMES) have slightly lower cetane number than their corresponding fatty acid ethyl esters (FAEEs) [Knothe et al., 2005; Lapuerta et al., 2010b].

- Density: key fuel property, which directly affects the engine performance characteristics. Cetane number and heating value are related to the density [Tat et al., 2000]. In addition, diesel fuel injection systems deliver the fuel by volume, so the changes in the fuel density will influence engine output power due to a different mass of fuel injected [Bahadur et al., 1995]. EN 14214 sets a minimum and maximum of 860 and 900 kg/m³. Unlike the cetane number, the longer the fatty acid carbon chains and the more saturated the molecules, the lower the density. Moreover, FAMES have slightly higher density than FAEEs [Lapuerta et al., 2010b].

- Oxidation stability: one of the major issues affecting the use of biodiesel because of its content of polyunsaturated methyl esters [Knothe et al., 2006]. EN 14214 sets a lower limit of 8 h as the minimum induction period, while the ASTM D6751 sets a limit of 3 h. To increase the resistance of biodiesels against autoxidation, it is usually treated with oxidation inhibitors (antioxidants) [Mittelbach et al., 2003].

- Cold filter plugging point (CFPP): indicates the cold flow performance and is generally considered a more direct and reliable indicator of low-temperature operability than other measurements such as cloud point (CP) and pour point (PP) [Echim et al., 2012]. At the CFPP temperature, the fuel will contain solids of sufficient size to render the engine inoperable due to the fuel plugging [Dunn et al., 1995]. EN 14214 sets limits depending on the season and country (see Table 3). The shorter the fatty acid carbon chains and the more unsaturated the molecules, the lower the CFPP and, hence, the better the cold flow performance [Knothe, 2008]. Thus, biodiesel fuels with good cold flow performance have normally poor oxidation stability and vice versa. Additionally, FAEEs have better low temperature properties than FAMES [Lee et al., 1995; Knothe et al., 2008].

- Kinematic viscosity: main reason why vegetable oils are not directly used in diesel engines and must be transesterified to esters. High viscosity causes poor fuel atomization during the spray, increases the engine deposits, needs more energy to pump the fuel and wears fuel pump elements and injectors [Kinast, 2003].

Table 3: Country specific CFPP requirements according to various national versions of EN 14214

Country	Standard	Season	Dates of season	Max CFPP
France	NF EN 14214	Summer	1st April - 31st October	0 °C
		Winter	1st November - 31st March	-15 °C
Germany	DIN EN 14214	Summer	15th April - 30th September	0 °C
		Winter	16th November - 28th February	-20 °C
		Spring	1st March - 14th April	-10 °C
		Autumn	1st October - 15th November	-10 °C
Italy	UNI EN 14214	Summer	March 16th - November 14th	0 °C
		Winter	November 15th - March 15th	-10 °C
Spain	UNE EN 14214	Summer	1st April - 30th September	0 °C
		Winter	1st October - 31st March	-10 °C
United Kingdom	BS EN 14214	Summer	16th March - 15th November inclusive	-5 °C
		Winter	16th November - 15th March inclusive	-15 °C

Moreover, high viscosity also causes more problems in cold weather, because viscosity increases with decreasing temperature [Joshi et al., 2007]. EN 14214 sets a kinematic viscosity range of 3.5-5.0 mm²/s and ASTM D6751 of 1.9-6.0 mm²/s. The longer the fatty acid carbon chains and the more unsaturated the molecules, the higher the viscosity [Ramírez-Verduzco et al., 2012]. Additionally, FAMES have slightly lower viscosity than FAEEs [Knothe et al., 2015].

- Lubricity: necessary to reduce the friction between the engine components like fuel delivery and injection system, cylinder liners, etc. This property has taken an important role lately, because low blend levels of biodiesel can restore lubricity to (ultra-)low-sulfur petroleum-derived diesel (petrodiesel) fuels, which have poor lubricity but are now the main diesel fuels available due to stricter environmental standards [Knothe et al., 2005]. In the US the ASTM D975 limit is 520 μm and in Europe the EN 590 limit is 460 μm . The longer the fatty acid chain and the more unsaturated the molecules, the better the lubricity. Moreover, FAEs have better lubricity than FAMES [Lapuerta et al., 2016].

As all the above properties depend on the composition, many researchers have developed prediction models to optimize biodiesel manufacturing and obtain biodiesel with the best specifications [Sajjadi et al., 2016]. Moreover, these models open the possibility to screen new sources of biodiesel, using a minimum amount of sample to know the composition, the properties can be predicted, saving the time and money to actually measure them. There are, for example, thousands of algal species, and this diversity gives researchers many options for identifying production strains and provides sources for genetic information that can be used to improve these production strains [Hannone et al., 2010]. Prediction models are a necessary tool to help choosing the best biofuel source candidates.

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2. OBJECTIVES AND SCOPE

The principal aim of this thesis is to study new sources of biodiesel and to assess their properties (see Figure 1). The final purpose is to make sustainable biodiesel to reduce the dependence of the transport sector on fossil fuels. Five categories of biofuel feedstocks have been considered: 1) The bacteria *Escherichia coli* (*E. coli*) has been chosen as a model microorganism, it has been genetically modified to change and increase the production of free fatty acids that have been esterified to produce biodiesel; 2) The macroalga *Chaetomorpha cf. gracilis* oil has been physicochemically characterized to assess its potential as a biodiesel source; 3) Seed oils from desert or non-arable land plants have been transesterified to biodiesel; 4) Grape seed oil has been examined as a waste of the wine industry and 5) Animal fat has been selected as a feedstock with high free fatty acids to study the production of biodiesel following a supercritical method. The produced biodiesel fuels have been preferably made of fatty acid ethyl esters (FAEEs), because the use of bioethanol, instead of methanol, would make biodiesel a total renewable fuel. The properties of the aforementioned biodiesel fuels have been estimated from their composition and their crystallization onset temperature, assessing their quality to blend with diesel fuel. In the case of *E. coli*, these properties were improved through genetic engineering.

Figure 1: Objectives of the thesis

The specific objectives are:

1. Estimation of oxidation stability, cold flow performance and lubricity of biodiesel

Some properties of biodiesel depend directly on the composition and, therefore, the source used for its production. The prediction of properties from the composition is a very useful method to assess the potential of new biodiesel sources. Previous studies have proposed different equations to predict the oxidation stability [McCormick et al., 2007; Parke et al., 2008; Sarin et al., 2010a], the cold filter plugging point (CFPP) [Ramose et al., 2009; Sarin et al., 2010b; Sue et al., 2011; Wang et al., 2012], the cloud point (CP) [Imahara et al., 2006] [Lopes et al., 2007; Sarin et al., 2009; Agarwal et al., 2010; Su et al., 2011] and the pour point (PP) [Sarin et al., 2009; Agarwal et al., 2010; Su et al., 2011] but all of them, except the CP prediction of Lopes et al. (2007) [Lopes et al., 2007] deal with fatty acid methyl esters (FAMES), and there are currently no equations for the estimation of these properties for fatty acid ethyl esters (FAEEs). Therefore, the objective is to find a relation between composition and the induction time, parameter that represents the oxidation stability, and the cold flow performance points (CFPP, CP and PP) of FAEEs. Moreover, there are not equations to predict the lubricity of biodiesel, neither for FAMES or FAEEs, so new equations for this property will also be proposed.

2. Genetic change of the *E. coli* fatty acid composition to obtain optimum biodiesel properties

Microorganisms have a huge potential to become a source for biofuels, especially engineered strains with better efficiency and yields [Alper et al., 2009; Meng et al., 2009]. The bacteria *E. coli* is highly amenable to metabolic engineering for fuel production and an ideal host to test different approaches which subsequently can be adapted to other microorganisms for use in a consolidated bioprocess to generate advanced biofuels from biomass [Bokinsky et al., 2011]. Several studies have already proved the potential of *E. coli* to produce biodiesel (FAEEs) [Kalscheuer et al., 2006; Steen et al., 2010] or its precursors (fatty acids) [Lu et al., 2008; Steen et al., 2010; Zhang et al., 2011; Lennen et al., 2012; Zhang et al., 2012]. However, most of the studies focus on increasing the yield, and only some of them change the fatty acid composition of *E. coli* [Steen et al., 2010; Zhang et al., 2011; Lennen et al., 2012; Zhang et al., 2012] to improve the final properties of the biodiesel. Despite the different

approaches made, none of these studies uses prediction equations to estimate the properties of biodiesel from the composition to, later, tune the composition to get optimum properties and fulfill the requirements set by the international standards (EN and ASTM). Thus, the objective is to modify genetically *E. coli* to obtain optimum biodiesel properties. To this end, *E. coli* cells will be modified to express a leaderless version of the enzyme thioesterase I ('TesA), the transcription factor FadR and a plant acyl-ACP thioesterase (FatA).

3. Physicochemical characterization of the fuel from green-filamentous macroalgae *Chaetomorpha cf. gracilis*

Marine macroalgae potentially represents a significant source of renewable energy. Their average photosynthetic efficiency is 6–8%, which is much higher than that of terrestrial biomass (1.8–2.2%) [Ross et al., 2008]. Growth rates of marine macroalgae far exceed those of terrestrial biomass, mainly due to no water limitations [Gellenbeck et al., 1983]. Moreover, annual primary production rates (grams of carbon·m⁻²·yr⁻¹) are higher for the major marine macroalgae than for most terrestrial biomass [Ross et al., 2008]. To date, a few studies have proved the feasibility of biodiesel production from macroalgae [Aresta et al., 2005; Maceiras et al., 2011], but major improvements are still needed to have a process economically sustainable [Milledge et al., 2014]. Along with new advances in technology to grow algae and extract the desired components, one of the key steps to make the process more efficient is to find macroalgae species more suitable for biofuel production [Hannon et al., 2010]. There are thousands of macroalgae species [The Seaweed Site, 2018] and most of them have yet not been characterized as potential source of biofuel. Therefore, the objective is to carry out a physicochemical characterization of the fuel obtained from the green filamentous macroalga *Chaetomorpha cf. gracilis* and assess its potential as a biofuel feedstock, studying the oil productivity and the content of co-products that could improve the economics of the entire system.

4. Biodiesel from desert plants

The development of high lipid content microorganisms or engineered strains for biodiesel production would be a potential and promising way in the future [Meng et al., 2009], but immediate solutions are needed to combat climate change and reduce the dependence of fossil fuels. By 2020, the EU aims to have 10% of the transport fuel of every EU country

coming from renewable sources [European Commission, 2018]. Biodiesel can have an important role in this, and oil from plants or waste feedstocks are the only viable and current option. While palm and rapeseed are still the dominant sources in the EU (accounting for 47 and 18 %, respectively), both oils have been criticized by numerous studies which point to the extensive occupation of land and the consequences of deforestation [Gilbert, 2012a; Gilbert, 2012b]. The objective is to assess the biofuel potential of scarcely studied plants that can grow in non-arable land and do not require big amounts of water, thus not competing with food supplies. The investigated plants (*Ammi visnaga*, *Citrullus colocynthis*, *Datura stramonium*, *Ecballium elaterium*, *Silybum marianum*, *Ibicella lutea*, *Onopordum nervosum*, *Peganum harmala*, *Smyrniolum olusatrum* and *Solanum elaeagnifolium*) were collected in arid parts of Tunisia, their oil content is assessed and their biodiesel properties will be estimated using equations from the literature and from this thesis.

5. Biodiesel from grape seed oil

Waste-to-Energy (WtE) technologies offer the promise of using organic wastes for beneficial energy use, while reducing the quantities of waste that are disposed or released to the environment. Many types of wastes have been studied as potential biofuel sources [Giannelos et al., 2002; Hepbasli et al., 2003; Haas, 2005; Chongkhong, 2007; Chhetri et al., 2008; Demirbas, 2008; Oliveira et al., 2008; Mondala et al., 2009; Yahyaee et al., 2013; Banković-Ilić et al., 2014; Yin et al., 2016] and several studies have pointed out their advantages compared to edible plant oils [Canakci, 2007; Gui et al., 2008; Skaggs et al., 2017]. In this context, the objective is to study the production and properties of biodiesel, specifically fatty acid ethyl esters (FAEE), obtained from grape seed oil and bioethanol coming from the residues of the wine industry. Both grape seeds and the surplus of wine are secondary products that can be considered wastes because of their little economic value and that their supply only depend on the production of wine, having an inelastic supply with demand [ICF, 2015]. This process would have a limited but substantial impact, especially in countries with high wine production, such as Italy, France and Spain, the world biggest producers [Wine Institute, 2015]. Moreover, current biodiesel is made of FAMES and the methanol needed during the transesterification has a fossil origin. FAEEs from grape seed oil and bioethanol would result in a fully renewable waste-derived biofuel.

6. Biodiesel from animal fat using supercritical ethanol process

A key factor to make biodiesel a real alternative to diesel fuel is to reduce its cost. Biodiesel price has always been higher than the diesel one [AFDC, 2017] and many studies describe biodiesel as a viable alternative but only if the cost of oil feedstock is reduced, which accounts for more than 80 % of the total estimated production costs [Haas et al., 2006]. There are large amounts of low cost oils and fats that could be converted to biodiesel. The problems with processing these low cost oils and fats is that they often contain large amounts of free fatty acids (FFAs) that cannot be converted to biodiesel using alkaline catalyst [Meher et al., 2006]. Currently, this problem is overcome through a two-step esterification process. In the first step, the FFAs are converted to FAMES by an acid catalyzed pretreatment and, in the second step, transesterification is completed by using alkaline catalyst [Canakci et al., 2001; Canoira et al., 2008]. An alternative to this process is the use of high pressure and temperature reactions. Reactions in supercritical methanol or ethanol do not require catalyst and are an interesting alternative especially dealing with high FFAs feedstocks [Demirbas, 2009; Chen et al., 2012] and when integrated to power cogeneration [Anitescu et al., 2008; Deshpande et al., 2010]. A worrying problem of supercritical reactions for biodiesel production is the degradation of unsaturated compounds, which can produce undesirable products and a reduction of yield [He et al., 2007; Imahara et al., 2008; Vieitez et al., 2011]. The objective is to study the production of biodiesel (FAEEs) from a waste with high FFAs content (animal fat), comparing the traditional catalyzed process with the supercritical direct transesterification and the two-step process of hydrolysis and esterification. Yields and degradation of polyunsaturated compounds will be assessed with changes in the reaction temperatures, ethanol:animal fat molar ratio and reaction time.

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3. ESTIMATION OF KEY BIODIESEL PROPERTIES

3.1 ESTIMATION OF COLD FLOW PERFORMANCE AND OXIDATION STABILITY OF FATTY ACID ETHYL ESTERS FROM LIPIDS OBTAINED FROM *ESCHERICHIA COLI*

3.1.1 CONTEXT

This study is focused on biodiesel, a fuel comprised of monoalkyl esters of long-chain fatty acids. Besides being a renewable fuel, biodiesel has important advantages compared to ordinary diesel: it contains significantly lower sulfur and no aromatic hydrocarbons, it has higher cetane number and oxygen content, showing better combustion efficiency and reducing sooting tendency, it has higher flash point and it is considered a biodegradable, non-toxic and harmless substance. The main disadvantages are its reduced heating value, poorer cold flow performance and limited oxidation stability [Bozbas, 2008]. However, the key factor for the economic feasibility of biodiesel is the reduction costs. It has been found that feedstock alone represents 75 % of the overall biodiesel production cost [Atabani et al., 2012]. Therefore, selecting the best feedstock is crucial to ensure low biodiesel cost. The option lies in considering non-edible oils as a feedstock for biodiesel. In fact, some studies point out that biodiesel from microorganisms (bacteria, yeast, fungi or microalgae) seems to be the only renewable biofuel that would have the potential to displace petroleum-derived transport fuels without adversely affecting the problem of land use and food supply [Chisti, 2008; Li et al., 2012]. Moreover, these microorganisms can produce biofuels derived from lignocellulosic or waste feedstocks [Yang et al., 2013]. Algae offer the ability to produce biofuel feedstocks and precursors directly from CO₂ and sunlight; however, algae also require land use and a significant amount of water which could be a problem in arid regions. Moreover, significant genetic engineering challenges remain before algal-based technologies can be practical, including large-scale cultivation, harvesting, and product separation [Huffer et al., 2012].

From this point, two alternatives seem to be the most viable: first, the improvement through genetic modification of microorganisms to be able to accept lignocellulosic feedstocks and produce triacylglycerides with a good efficiency, for later carrying out the transesterification

process [Bokinsky et al., 2011; Li et al., 2008]. Second, to make the microorganisms produce directly fatty acid ethyl esters (FAEEs) from lignocellulosic materials [Bokinsky et al., 2011]. This second way could reduce cost due to the elimination of the transesterification stage. From the second process mainly FAEEs are produced because the toxicity of methanol for many life forms makes the production of fatty acid methyl esters (FAMES) more difficult. From the first process both FAMES and FAEEs can be obtained. In addition, methanol is currently produced from natural gas, it is a highly toxic and hazardous compound, and its use requires special precautions. Thus, FAME-based biodiesel is not an entirely renewable product since the alcohol component is of fossil origin and only the use of bioethanol for production of FAEE-based biodiesel would result in a fully sustainable fuel [Farrell et al., 2006; Kalscheuer et al., 2006]. Therefore, from both processes and considering environmental and health issues, the most feasible compound for biodiesel formulation is FAEE.

Furthermore, the development of a host suitable for manufacturing biodiesel from microorganisms with good efficiency is a challenge. Many requirements are needed, some of them are: 1) To be able to metabolize all sugars arising from upstream processes. Glucose and xylose are the main sugars derived from lignocellulosic feedstocks, but other sugars may be present, including cellobiose and arabinose. 2) To produce the biofuel under anaerobic conditions, avoiding the need to aerate large reactor volumes. 3) To be able to modify the profile of FAEEs obtained to control the properties of the biodiesel [Huffer et al., 2012; Knothe, 2005]. Although in the future probably algae will provide other paths, the genetic improvements must be made first in a more amenable host for metabolic engineering. *Escherichia coli* (*E. coli*) seems to be the most promising host for these studies. *E. coli* is able to use both pentose and hexose sugars, and is highly amenable to metabolic engineering for fuel production. Additionally, while the genetic engineering is greatly facilitated by the tractability of *E. coli*, the approaches described can be readily adapted for other microorganisms for use in a consolidated bioprocess to generate advanced biofuels from biomass [Bokinsky et al., 2011].

A crucial challenge of all these studies is the characterization of the fuel produced by bacteria. The concentration obtained in FAEEs from *E. coli* reaches by now only 1 g/L [Duan et al.,

2011; Steen et al., 2010]. This amount makes difficult to test the biodiesel to know its physical properties. In this thesis, this problem is addressed and two of the most limiting properties of biodiesel are studied: cold flow performance and oxidation stability. In fact, most of the biodiesel fuels produced at industrial scale are upgraded by adding oxidation stabilizers and cold-flow depressors, despite the cost of these additives, which becomes a significant part of the final cost of biodiesel fuels.

The behavior of biodiesel at low temperature is an important quality criterion. This is because partial or total crystallization of the fuel may cause blockage of the fuel lines and filters, leading to fuel shortage, problems of starting or driving and engine damage due to inadequate lubrication [Atabani et al., 2012]. Among the cold properties of liquid fuels three have received special attention: the cloud point (CP), the pour point (PP) and the cold filter plugging point (CFPP). All these tests require a minimum amount of sample of 45 mL. The CP is the temperature of a liquid specimen when the smallest observable cluster of crystals first appears upon cooling (ASTM D2500 and EN 23015). The PP is defined as the lowest temperature at which movement of the test specimen is observed (ASTM D7346 and ISO 3016). The CFPP is the highest temperature, expressed in multiples of 1 °C, at which a given volume of fuel fails to pass through a standardized filtration device in a specified time when cooled (ASTM D6371 and EN 106).

Oxidation stability is one of the most important properties of biodiesel and affects biodiesel primarily during extended storage. Degradation by oxidation yields oxidation products that may compromise fuel properties, impair fuel quality and engine performance. Oxidation cause a tendency to form deposits on engine parts such as injectors and critical fuel pump components and can cause polymerization-type reactions that produce high molecular weight insoluble sediments and gums [Pullen et al., 2012]. Rancimat method is used to test the oxidation stability, recording the induction time (IT) when the maximum rate change in the slope of the water conductivity is produced. This method requires an amount of 7.5 g (EN 15751). Although it may seem a small quantity compared to the 45 mL needed for the cold properties tests, considering an efficiency of 1 g/L for the bacterial cultures would mean to obtain 7.5 L of product, many times an impossible task in genetic laboratories.

From the previous information, it can be inferred that there is a necessity of a method to estimate both the cold flow performance and the oxidation stability of biodiesel using a little amount of product (<1 g). The purpose of this part of the thesis is, first, to have a fast, easy and non-expensive way to assess the properties of FAEEs produced via classical transesterification process and second to be able to assess the properties of FAEEs produced in minimum amounts through genetic studies.

Two approaches have been carried out (Figure 2). First, correlations between composition and properties have been generated. Gas chromatography with flame ionization detector (GC-FID) is used to analyze the fatty acid composition, where only some milligrams are needed. There are some studies concerning these correlations between composition and properties [Su et al., 2011; Ramos et al., 2009] but there is a great interest in the development of accurate models to predict the properties of biodiesel [Saxena et al., 2013]. Researchers have found accurate correlations between composition and CP [Imahara et al., 2006] but PP and CFPP are more difficult to correlate [Coutinho et al., 2010]. Oxidation stability has been studied also in previous works [McCormick et al., 2007; Park et al., 2008; Sarin et al., 2010b], but no correlation between oxidation stability and composition concerning FAEEs has been found. Second, a correlation between the crystallization temperature obtained with differential scanning calorimetry (DSC) and the three cold flow properties is proposed. DSC experiments only need about 6 mg of product. A previous work studied the correlation between FAMES crystallization temperatures obtained through DSC test and CP, PP and CFPP [Dunn, 1999], but no work was found concerning FAEEs.

FAEEs present some advantages over FAMES as a slight increase in the heating value and cetane number and an improvement in the cold properties [Mittelbach et al., 2007; Lapuerta et al., 2010b]. Only a few studies compare properties of FAME and FAEE [Zajac et al., 2008] which conclude that FAME and FAEEs are similar and both of them able for utilization as diesel engine fuels or as components of blends with diesel fuel.

3.1.2 EXPERIMENTAL SECTION

Materials

Rapeseed and camelina oils were supplied by the Laboratory of Fuels and Petrochemistry of the Gomez-Pardo Foundation, soybean, crude palm kernel oil and animal fat were supplied by Combustibles Ecológicos Biotel SL, refined coconut oil was purchased from Acros Organics (CAS No. 8001-31-8), linseed oil was purchased from Fisher Scientific (CAS No. 8001-26-1), sesame oil was purchased from Vitasia, sea-buckthorn oil was purchased from Seabuckwonders and waste frying oil was supplied by the catering service of the ETS Ingenieros de Minas y Energía of the Universidad Politécnica de Madrid.

Figure 2: Development of an indirect method to predict the cold flow properties (CP, PP and CFPP) and the oxidation stability of biodiesel (FAEEs) using only milligrams of a sample (composition and DSC) and the application of the method to measure the properties of biodiesel obtained from *E. coli*

Absolute ethanol was purchased from Probus SA. Sodium, used to make sodium ethoxide to catalyze the transesterification reaction, was purchased from Sigma-Aldrich. Sulfuric acid used in the esterification and in the acid catalysed transesterification was purchased from Panreac. p-Toluensulphonic acid, used as catalyst for the esterification reaction of the crude oils, was of synthesis grade, and it was purchased from Scharlau. The 4 Å molecular sieve was of technical purity, and it was purchased from Scharlau. Magnesium silicate Magnesol D-60 was kindly supplied by The Dallas Group of America. Methanol was of synthesis grade, and it was purchased from Scharlau and potassium hydroxide was purchased from Merck. Calcium oxide of reagent grade, used to eliminate the water after the esterification process, was purchased from Sigma-Aldrich. Diethyl ether of reagent grade was purchased from Scharlau.

Hexanoic, octanoic, decenoic, dodecanoic, tetradecanoic, hexadecanoic, octadecanoic and (Z)-octadec-9-enoic acids were purchased from Sigma-Aldrich. All acids had a minimum purity of 98 %. The fatty acids were used to make pure FAEEs.

The *E. Coli* BL21 (DE3) strain was provided generously by the Centre for Plant Biotechnology and Genomics (GBGP), the LB medium was made through their components: Bacto Tryptone Peptone purchased from Becton Dickinson (BD), Yeast Extract purchased also from BD and sodium chloride purchased from Merck (PhEur). The antibiotic ampicillin was purchased from Sigma Aldrich. The thionyl chloride to derivatize the bacterial extract was purchased from Scharlau.

Degumming, esterification and transesterification of oils, fats and acids

The process followed to make biodiesel from the oils and fats was previously described in the literature [Canoira et al., 2008; Llamas et al., 2012] but slight changes were made due to the change in the final product from FAMES to FAEEs. First, the acidity of all the oils was measured to separate them into two groups depending on the results. Cameline oil and animal fat have an acidity of 4.6 and 11.7 mg KOH/g respectively so a previous esterification process was necessary. The rest of oils have acidities less than 2 mg KOH/g and the transesterification process was carried out directly.

In the case of animal fat, phosphoglycerides (also called gums) were present, so previously a degumming process was carried out. The fatty raw material was heated to 95 °C with stirring at 500 rpm. When the fat was at this temperature, 85 wt % orthophosphoric acid (0.2 vol % of the fat) was added to the reactor, and it was stirred for 30 min. The reaction mixture was centrifuged at 3000 rpm for 15 min, and the gums were separated at the bottom of the centrifuge tubes.

For the esterification step the oil/fat was heated to 70 °C with stirring at 600 rpm. p-Toluenesulfonic acid (mass ratio oil/p-toluenesulfonic acid, 200:1) dissolved in ethanol (molar ratio of ethanol-oil, 6:1) was added to the reactor, and the reaction mixture was kept at 70 °C with stirring at 600 rpm for 4 h. At the end of the reaction, freshly calcined calcium oxide (mass ratio oil-calcium oxide, 50:1) was added to the reactor at 70 °C with stirring, to neutralize the acid catalyst and to eliminate the water produced in the esterification, forming insoluble calcium hydroxide. The reaction mixture was cooled at room temperature and transferred to centrifuge tubes to eliminate the calcium salts. The resultant mixture of oil and ethanol was used for the next transesterification step without any further treatment, only with adjustment of the amount of ethanol.

In the transesterification process, the oil or the mixture of oil and ethanol from the esterification process (in the case of camelina oil and animal fat) was heated to 70 °C with stirring at 600 rpm. Sodium ethoxide (mass ratio oil-sodium ethoxide, 100:2) dissolved in ethanol (molar ratio of ethanol-oil, 6:1) was added to the reaction mixture. After 3 h the reaction mixture was cooled to room temperature in the reactor. It was decanted in the centrifuge tubes, centrifuged at 3000 rpm for 5 min, and the upper biodiesel phase was separated from the lower G-phase. The biodiesel was transferred to a rotary evaporator to eliminate the excess ethanol. Finally, the biodiesel was treated with 2 wt % Magnesol D-60 at 77 °C, with stirring at 800 rpm for 30 min, leaving the adsorbent decanting overnight, and filtering it afterward through a 0.45 µm filter.

The FAEE profiles of the biodiesel fuels obtained with this procedure are reported in Table 4. All the biodiesel fuels meet the standard EN 14214 having an ester content above 96.5 wt %.

Table 4: FAEE profiles of the biodiesel fuels

FAEE, wt %	Rapeseed	Palm	Soybean	Coconut	Linseed	Sesame	Camelina	Sea-buckthorn oil	Animal fat	Waste frying oil	^c Blend 1	^d Blend 2	^e Blend 3	^f Blend 4	^g Blend 5	^h Blend 6
C8:0	0.00	0.00	0.00	14.10	0.00	0.00	0.00	0.00	0.00	0.00	4.86	7.65	7.05	9.87	4.23	0.00
C10:0	0.00	0.00	0.00	8.75	0.00	0.00	0.00	0.00	0.00	0.00	2.97	4.46	4.37	6.12	2.62	0.00
C12:0	0.00	0.31	0.00	32.17	0.00	0.00	0.00	0.00	0.00	0.00	19.31	24.59	16.08	22.52	9.65	0.19
C14:0	0.00	1.01	0.00	20.49	0.95	0.00	0.00	0.73	2.29	0.44	7.46	11.00	10.24	14.63	6.15	0.99
C16:0	5.04	45.98	12.81	10.88	6.90	9.54	6.90	29.77	25.18	14.17	30.44	21.12	8.89	9.69	8.09	30.35
C16:1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	27.66	3.16	2.37	0.00	0.00	0.00	0.00	0.00	0.00
C18:0	9.22	9.73	6.52	2.93	4.46	5.88	2.88	1.34	16.15	3.93	6.67	5.38	2.91	3.39	2.89	7.62
C18:1	59.55	33.53	29.37	8.31	21.34	40.27	17.37	28.74	43.89	66.40	22.21	18.23	12.84	12.22	14.65	28.65
C18:2	17.74	9.14	46.06	2.37	17.27	43.13	33.47	7.84	7.87	10.33	6.07	7.01	17.92	6.84	24.14	12.39
C18:3	6.59	0.00	4.92	0.00	49.08	0.34	26.03	3.92	0.63	0.77	0.00	0.57	13.01	14.72	18.22	19.63
C20:0	0.51	0.30	0.32	0.00	0.00	0.00	1.33	0.00	0.00	0.00	0.00	0.00	0.66	0.00	0.93	0.18
C20:1	1.36	0.00	0.00	0.00	0.00	0.84	12.01	0.00	0.84	1.59	0.00	0.00	6.00	0.00	8.41	0.00
Ester content, wt %	98.78	97.56	98.72	100.00	99.27	97.58	96.79	100.00	98.15	97.68	98.44	98.97	98.39	99.78	97.75	98.24
^a U _{FAEE}	85.24	42.67	80.35	10.68	87.69	84.58	88.88	68.16	56.39	81.46	28.28	25.81	49.78	33.78	65.42	60.68
^b N _c	19.92	19.03	19.73	14.44	19.87	19.87	19.89	18.77	19.37	19.72	16.77	16.01	17.16	16.07	18.25	19.37

^aU_{FAEE}: total content of unsaturated FAEEs, wt %

^bN_c: weighted-average number of carbon atoms in the FAEE (N_c)

^cBlend 1: 62 wt % palm and 38 wt % coconut biodiesel

^dBlend 2: 4 wt % rapeseed, 31 wt % palm, 5 wt % soybean and 59 wt % coconut biodiesel

^eBlend 3: 50 wt % coconut and 50 wt % camelina biodiesel

^fBlend 4: 70 wt % coconut and 30 wt % linseed biodiesel

^gBlend 5: 30 wt % coconut and 70 wt % camelina biodiesel

^hBlend 6: 60 wt % palm and 40 wt % linseed biodiesel

The production of pure ethyl esters from pure acids was made through an esterification process described in the literature [Kemp, 1967] similar to the esterification process described above but using sulfuric acid as catalyst (2.5 vol %) and molecular sieve to dry the ester.

Production of FAEEs from *E. coli*

E. coli BL21 (DE3) strain was cultivated in baffled flasks at 37 °C, rotating at 150 rpm for 16 h. Ampicillin was added for plasmid selection (100 mg/L) and LB medium for growth. This medium is an optimum medium but there are studies that make *E. coli* grow in lignocellulosic material which would be a perfect feedstock for the production of sustainable biodiesel [Bokinsky et al., 2011; Steen et al., 2010].

Two different methods for the production of biodiesel were used. In the first method, cells were pelleted by centrifugation (5 min, 13,000 × g) and treated with 5 M KOH in methanol: water (1:1, v/v). After neutralization with acetic acid, fatty acids were extracted with petroleum ether as described [Aldai et al., 2006]. Once the extraction was completed the derivatization to FAEEs was done using thionyl chloride dissolved in ethanol [Canoira et al., 2003].

In the second method, an acid transesterification was made because it has been reported that the use of an acid could facilitate to break down the cell wall of the organism and other sources of fatty acids such as membrane lipids could be converted to biodiesel [Wahlen et al., 2011]. This method was followed with slight changes in the way to perform the reaction. Lyophilized *E. coli* biomass was directly transesterified with ethanol using sulfuric acid as catalyst. The reaction vessel was placed in an ultrasound bath at 80 °C (Elmasonic S40 (H)) for 15 h. Phase separation of the products was accomplished by washing the ethanol solution with chloroform and water followed by centrifugation. FAEEs partitioned with chloroform in the lower, organic phase, were then analysed by GC-FID.

The FAEE profiles obtained with the two methods were determined through GC-FID using the parameters described in Section 3.1.2 and it is shown in Table 5.

Table 5: FAEE profile of biodiesel from *E. coli*

FAEE, wt %	<i>E. coli</i> (1 st method)	<i>E. coli</i> (2 nd method)
C12:0	8.98	6.69
C14:0	12.51	14.90
C16:0	31.08	32.40
C16:1	22.85	23.82
C18:0	3.42	7.13
C18:1	21.16	15.06
U _{FAEE}	44.01	38.88
N _c	17.79	17.81

Gas chromatography

EN 14103 method was used for quantification of ester content in biodiesel. A Hewlett Packard 5890 Series II gas chromatograph equipped with FID detector and split/splitless injector was used for the analysis. An HP-Wax column (30 m x 0.32 mm id x 0.15 µm) of polyethylene glycol was used for separation.

The following analytical conditions were used for the analysis: injector temperature: 210 °C; split ratio: 80:1; injection volume: 1 µL; column flow rate (He): 1 mL/min constant flow mode; FID temperature: 240 °C; H₂ flow rate: 40 mL/min; air flow rate: 400 mL/min; oven program: 200 °C hold 9 min, to 230 °C at 20 °C/min, hold 10 min; calibration standard: solution of methyl heptadecanoate in heptane (5 mg/mL); sample preparation: 250 mg of sample in 10 mL vial with 5 mL of methyl heptadecanoate solution.

Cold flow properties tests

Both the CP and PP were determined with the Automatic Cloud and Pour Point Analyzer CPP 97-6. The CFPP was measured with the CFPP analyser TLF (ISL-ATPEM). The experimental errors of the CP, PP and CFPP are <3 °C, <3 °C and ±1 °C respectively.

The crystallization onset temperature (COT), defined as the point in the thermogram when the first exothermic peak begins, was measured using DSC (Q20 TA-Instruments). This temperature was chosen because previous studies with FAMES showed a good linear correlation between this temperature and the CP, PP and CFPP [Lee et al., 1995; Dunn,

1999]. Six milligrams of each of the studied FAEEs were added to a 40 μ L sealed vial under a nitrogen flow rate of 40 mL/min. The temperature program consisted on two ramps. First, the sample was heated to 70 $^{\circ}$ C and kept at this temperature during a period of 5 min. Second, the sample was cooled from 70 $^{\circ}$ C to -80 $^{\circ}$ C. Once this temperature was reached, the sample was kept at -80 $^{\circ}$ C for 5 min. The process was repeated twice to verify repeatability. The rate of temperature was chosen as 5 $^{\circ}$ C/min following previous studies in FAMEs which reported a good combination of resolution characteristics and timeliness [Dunn, 1999; Foon et al., 2006; Giraldo et al., 2013]. Figure 3 shows the thermogram obtained with the analysis of the palm biodiesel and how the COT is chosen.

Oxidation stability test

The Rancimat method described in EN 15751 was used to assess the oxidation stability. This method is listed as the oxidative stability specification in ASTM D6751 and EN 14214. A minimum IT at 110 $^{\circ}$ C of 3 h is required for ASTM D6751, whereas a more stringent limit of 8 h is specified in EN 14214. The experimental error of each measurement is: $0.09 \cdot \text{mean} + 0.16$.

Figure 3: Thermogram of the palm FAEE and the crystallization onset temperature (COT)

3.1.3 RESULTS AND DISCUSSION

Experimental results of cold flow properties

The results of the cold properties tests of the biodiesel fuels are reported in Table 6. The relation between the percentage of unsaturated compounds (U_{FAEE}) and the cold properties can be observed comparing Tables 4 and 6. Camelina, linseed and rapeseed biodiesel fuels, with the highest percentage of unsaturated FAEEs (over 85 wt %), present the lowest values for CP, PP and CFPP. For example, the CFPP of these three biodiesel fuels is always lower than -10 °C and would be acceptable according to the standard EN 14214 for grades A to D in temperate climates. Nevertheless, cold properties not only depend on the amount of unsaturated FAEEs, but also on the length of the carbon chain of the esters.

Table 6: Results of cold flow properties and oxidation stability tests

Biodiesel	Cloud point (°C)	Pour point (°C)	Cold filter plugging point (°C)	Crystallization Onset Temperature (°C)	Induction time (h)
Rapeseed	-5	-16	-11	-8.8	4.12
Palm	9	9	10	6.9	7.73
Soybean	-1	-5	-5	-3.2	0.71
Coconut	-2	-9	-5	-5.1	8.60
Linseed	-7	-11	-15	-7.7	0.58
Sesame	0	-3	-6	-2.6	2.33
Camelina	-4	-6	-12	-5.0	0.65
Sea-buckthorn	-	-	-	-4.1	5.40
Animal fat	9	10	4	8.5	7.30
Waste frying oil	-1	-3	-2	-2.9	5.00
Blend 1	5	3	1	2.9	8.17
Blend 2	1	-2	0	-1.6	7.67
Blend 3	-	-	-	-	2.9
Blend 4	-	-	-	-	3.2
Blend 5	-	-	-	-	1.6
Blend 6	-	-	-	-	2.2

This effect is clearly shown in the coconut biodiesel that, despite having the lowest percentage of unsaturated FAEEs (10.68 wt %), it has low cold flow temperatures, similar to soybean biodiesel, because of the high amount of short chain esters. Palm biodiesel presents the opposite effect, having a medium percentage of unsaturated FAEEs (42.67 wt %) and a high average length of carbon chains ($N_c = 19.03$), with the highest CFPP (10 °C).

Correlation between composition and cold flow properties

These two properties, percentage of unsaturated FAEEs and average length of carbon chain, have been used to correlate composition and cold properties [Su et al., 2011]. Biodiesel usually has a high percentage of unsaturated FAEEs. The lack of biodiesel with low percentage of unsaturated FAEEs makes difficult the search for a correlation between composition and properties. This is why Blends 1 and 2 were prepared. Both blends have a significant amount of coconut biodiesel (38 and 59 wt % respectively) what causes that the resulting percentage of unsaturated FAEEs and the length chain are lower than for the rest of biodiesel fuels (except for the coconut one), thus spreading out the values of the two parameters used for the correlation.

The three multiple linear correlations found are:

$$CP = -81.62 - 0.45 \times U_{FAEE} + 5.87 \times N_c \quad R^2 = 0.89 \quad Eq. 1$$

$$PP = -125.04 - 0.62 \times U_{FAEE} + 8.61 \times N_c \quad R^2 = 0.76 \quad Eq. 2$$

$$CFPP = -103.47 - 0.59 \times U_{FAEE} + 7.30 \times N_c \quad R^2 = 0.86 \quad Eq. 3$$

The data from Tables 4 and 6 were used to obtain equations 1, 2 and 3. Table 6 shows the results of CP, PP and CFPP for 11 FAEEs (11 points). The main results of the linear regression parameters are described in Table 7. Box-Tidwell test [Box et al., 1962] was used to assess if a transformation of the predictors was necessary but in the three cases the test was not relevant (p-value > 0.05) so no transformation was made. The assessment of the significance of the parameters was done through the Student's t-test for the individual parameters and with the F-test for the whole model. Both tests show significant results (p-value < 0.05). The best correlation (using the multiple R-squared value for comparison) was found with the CP reaching a $R^2 = 0.89$.

Table 7: Correlation parameters between the composition and cold flow properties or induction time (IT)

Property	Coefficient	p-value for Student's t-test	p-value for Box-Tidwell test	R ²	p-value for F-test
CP					
intercept	-81.62	0.00025	N/A	0.89	0.00017
^a U _{FAEE}	-0.45	0.00005	0.24747		
^b N _c	5.87	0.00015	0.40546		
intercept	1.96	0.00015	N/A	0.97	4.3E-08
^c COT	0.94	4.3E-08	N/A		
PP					
intercept	-125.04	0.00237	N/A	0.76	0.00355
U _{FAEE}	-0.62	0.00109	0.47361		
N _c	8.61	0.00198	0.75096		
intercept	-0.62	0.00228	N/A	0.97	5.4E-08
COT	1.40	5.4E-08	N/A		
CFPP					
intercept	-103.47	0.00095	N/A	0.86	0.00043
U _{FAEE}	-0.59	0.00017	0.20579		
N _c	7.30	0.00067	0.32922		
intercept	-1.70	0.00143	N/A	0.81	0.00014
COT	1.19	0.00014	N/A		
IT					
intercept	9.50	4.9E-08	N/A	0.97	3.75E-08
^d MU _{FAEE}	-0.03	0.0106	0.23856		
^e BAPE	-0.14	1.9E-06	0.44012		
intercept	4.45	0.00023	N/A	0.94	0.00116
BAPE	-0.04	0.00116	N/A		

^aU_{FAEE}: total content of unsaturated FAEEs, wt %

^bN_c: weighted-average number of carbon atoms in the FAEE (N_c)

^cCOT: crystallization onset temperature

^dMU_{FAEE}: sum of monounsaturated esters (C16:1+C18:1), wt %

^eBAPE: bis-allylic position equivalents (C18:2+2×C18:3), wt %

Correlation between crystallization onset temperature (COT) and cold flow properties

Generally, in the thermograms there are two major and distinctive exothermic peaks (indicating crystallization) present in the cooling thermograms. The first exothermic peak in the thermogram, occurring at higher temperature, is the crystallization peak for saturated ethyl esters while the second exothermic peak at lower temperature is the result of crystallization of unsaturated ethyl esters.

The COT of pure ethyl esters is quite similar to the melting point of these compounds [Mittelbach et al., 2007], but always a bit lower as it can be shown in Table 8. However, for blends of saturated and unsaturated FAEEs the exact value of the COT is difficult to predict. A previous study [Foon et al., 2006] reported that the position of the first crystallization peak depends very much on the content of saturated esters. As the percentage of saturated esters decreases, the crystallization temperature of the first exothermic peak also decreases accordingly.

Samples with high percentages of saturated esters tend to crystallize at higher temperature than samples with low percentages of saturated esters. Nevertheless, the use of the COT values of pure FAEEs for the prediction of the COT of blends of FAEEs is not accurate and, therefore, measurement of the COT is required for every biodiesel.

Table 8: Crystallization onset temperature (COT) and melting point of pure FAEEs

FAEE	COT	Melting point (°C)
C6:0	<-80.0	-67.6
C8:0	-56.8	-43.1
C10:0	-26.4	-20.0
C12:0	-6.1	-1.8
C14:0	4.4	12.3
C16:0	20.4	21.5
C18:0	30.2	32.0
C18:1	-56.6	-32.0

The COTs for all the biodiesel fuels measured with DSC are listed in Table 6. Acceptable linear correlations have been found between the COT and the three cold properties test: CP, PP and CFPP, as shown in Figures 4, 5 and 6. The confidence interval for the mean

and the prediction intervals for single values are drawn both with a 95 % of confidence level. The equations obtained are:

$$CP = 1.96 + 0.94 \times COT \quad R^2 = 0.97 \quad Eq. 4$$

$$PP = -0.62 + 1.40 \times COT \quad R^2 = 0.97 \quad Eq. 5$$

$$CFPP = -1.7 + 1.19 \times COT \quad R^2 = 0.81 \quad Eq. 6$$

The data from Tables 4 and 6 were used to obtain equations 4, 5 and 6. Table 6 shows the results of COT for 12 FAEEs (12 points). The statistical analysis is shown in Table 7. The coefficients of COT show statistical significance with p-values of the Student's t-test lower than 0.05 and the F-test is also positive for the three linear correlations.

Both the graphic representation and the R^2 show that the CFPP correlates worse with the COT, possibly because of the effect of other variables as viscosity and density of the biodiesel on this property.

Estimation of oxidation stability

The oxidation stability was assessed through the IT measured with the Rancimat test. Table 6 shows the IT of the biodiesel fuels obtained with the Rancimat procedure. Coconut, palm, animal fat and the blends 1 and 2 of biodiesel fuels have the highest values of IT, all of them above 7 h and therefore acceptable considering standard ASTM D6751 although only blend 1 is acceptable considering standard EN 14214. On the contrary soybean, linseed and camelina biodiesel fuels have the lowest values of IT (all below 1 h).

It can be noticed that these biodiesel fuels with low values of IT have high values of unsaturated esters (all above 80 % w/w) while the biodiesel fuels with high values of IT have lower values of unsaturated esters. A challenge that had to be addressed before doing the correlation is to find at least one oil with a significant content of palmitoleic acid because the bacteria *E. coli* has much more of this acid than any other common oil. That is the reason why sea-buckthorn berry oil was studied, since it has a content of this particular acid of 27.66 %. Also with the purpose of improving the correlation, Blends 3, 4, 5 and 6 were made to achieve a better distribution in the range of the polyunsaturated esters.

Figure 4: Correlation between crystallization onset temperature (COT) and cloud point (CP)

Figure 5: Correlation between crystallization onset temperature (COT) and pour point (PP)

Figure 6: Correlation between crystallization onset temperature (COT) and cold filter plugging point (CFPP)

The main conclusions of the statistical analysis were: 1) Monounsaturated esters affected IT mainly when the amount of linolenic acid ethyl esters was low. 2) The effect of the palmitoleic ester is similar to the effect of the oleic ester so an appropriate parameter for the correlation is the sum of the monounsaturated palmitoleic and oleic ethyl esters (MU_{FAEE}). 3) The effect of polyunsaturated esters can be measured with accuracy with the index proposed previously by Knothe [Knothe, 2002] that takes into account the bis-allylic positions of the esters ($BAPE = C18:2 + 2 \times C18:3$).

As a result of this analysis two equations are proposed to correlate the composition with the IT.

First, an equation for values of linolenic ester lower than 10 %:

$$IT = 9.50 - 0.03 \times MU_{FAEE} - 0.14 \times BAPE \quad R^2 = 0.97 \quad Eq. 7$$

Table 7 shows the statistical parameters of this multiple linear correlation that uses as variables the MU_{FAEE} and the BAPE. The p-values of the Student's t-test and Fischer test are lower than 0.05 so the parameters are statistically significant. From the Box-Tidwell test it can be derived that no transformation of the predictors is required. An R-squared of 0.97 shows that the chosen parameters approximate with a good accuracy the IT. If the coefficients affecting variables MU_{FAEE} and BAPE are compared, the presence of polyunsaturated compounds is observed to affect the final IT result about four times more than that of the monounsaturated ones.

Second, an equation for values of linolenic ester higher than 10 %:

$$IT = 4.45 - 0.04 \times BAPE \quad R^2 = 0.94 \quad Eq. 8$$

The data from Tables 4 and 6 were used to obtain equations 7 and 8. Table 6 shows the results of IT for 16 FAEEs (16 points). In this case the statistical analysis shows that the parameter MU_{FAEE} is not significant so an equation with only the variable BAPE is proposed. The values of the statistical tests of Equation 8 are presented in Table 7. The y-intercept and the coefficient of the BAPE show statistical significance with p-values of the Student's t-test lower than 0.05 as is shown in Table 7. F-test confirms also the significance of the model. Figure 7 shows the graphical representation of Equation 8. Confidence interval for the mean and the prediction intervals for single values are drawn both with a 95 % of confidence level. It is worth noting that this equation is very similar

to the equation that can be obtained through a linear regression using the data from Knothe [Knothe, 2002] and Rodríguez-Fernández [Rodríguez-Fernández, 2007].

Figure 7: Correlation between induction time (IT) and BAPE for values of C18:3 > 10%

Estimation of properties of FAEE obtained from *E. coli*

The correlations described in this thesis allowed to estimate the cold flow properties and the oxidation stability of samples of FAEE obtained from an *E. coli* culture. The differences in the composition obtained from the two methods lead to different predicted property values that are shown in Table 9.

The IT is almost the same but, using the second method, the cold flow properties are worse in an average of 3 °C. It can be concluded that the acid transesterification may increase the efficiency of the production of FAEEs using the phosphoglycerides of the cell membranes but this entails a worsening in the cold flow properties.

Table 9: Prediction of properties of biodiesel from *E. coli*

FAEE <i>E. coli</i>	Cloud point (°C)	Pour point (°C)	Cold filter plugging point (°C)	Induction time for oxidation stability (h)
1 st Method	3.0	0.9	0.5	8.18
2 nd Method	5.4	4.2	3.6	8.33

The lack of polyunsaturated compounds and the presence of a high percentage of monounsaturated compounds (C16:1 and C18:1) causes a good oxidation stability but moderate cold flow behavior. From these initial results, and using the equations proposed,

an optimum composition can be searched using genetic techniques. For example, the modification of the fatty acid synthesis of *E. coli* has been done successfully with the use of thioesterases [Voelker et al., 1994], achieving changes in the final fatty acid composition. Cold flow properties of biodiesel from *E. coli* have been improved (see Section 4) using these techniques that allow modifying the ester composition.

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3.2 EFFECT OF FATTY ACID COMPOSITION OF METHYL AND ETHYL ESTERS ON THE LUBRICITY AT DIFFERENT HUMIDITIES

3.2.1 CONTEXT

The improvement of common-rail systems in diesel engines has allowed the transport companies to develop new strategies to increase engine efficiency and reduce pollutant emissions. One of the strategies developed by the manufacturers is to use higher injection pressures to atomize the fuel, leading to better air mixing, faster evaporation and more efficient combustion process. Severely-loaded systems such as injectors or pressure pumps need fuels with high lubricating capacity to avoid any wear problem. However, almost all of the petroleum-based diesel fuels available in Europe and North America are now of ultra-low sulfur diesel (ULSD) type, to accomplish with the new environmental policies about limiting sulfur. The low sulfur diesel fuel has poor lubricity, caused by the removal of polar oxygen and nitrogen compounds, which happens during the process of sulfur species extraction. These polar compounds are adsorbed on the rubbed metal surfaces providing a protective layer that reduces adhesion and limit friction or wear [Barbour et al., 2000].

To restore the lubricity of these fuels, the use of additives or blends with another fuel of enhanced lubricity is required [Mozdzen et al., 1998]. For this reason, the influence of different compounds on the lubricity of diesel fuels has been tested and oxygenated compounds were ordered regarding their lubricity enhancing potential ($\text{COOH} > \text{CHO} > \text{OH} > \text{COOCH}_3 > \text{C=O} > \text{C-O-C}$) [Knothe et al., 2005]. Biodiesel, an alternative diesel fuel derived from the transesterification of vegetable oils or animal fats, can be used in blends with diesel to improve the lubricity, because biodiesel contains polar oxygenated compounds. Moreover, biodiesel is an environment-friendly option that can be cheaper than other kind of additives. Biodiesel-diesel blends have been widely studied and it is well known that biodiesel reduces the particulate matter and CO emissions and increases cetane number and flash point of diesel in blends [Candeia et al., 2007; Benjumea et al., 2008; Candeia et al., 2009]. In addition, biodiesel may not require engine modifications when used in blends up to 20 % (m/m) in diesel, as far as cold-flow properties are the required ones for the climate conditions [Dwivedi et al., 2014]. Biodiesel can be produced with methanol or ethanol resulting in fatty acid methyl esters (FAMES) or ethyl esters

(FAEEs), respectively, both compounds having inherent good lubricity. Generally, there are fewer studies of FAEEs because the lower price of methanol has made FAMEs to be widespread used and also because the base catalyzed production of FAEEs is more difficult than the production of FAMEs. During the course of reaction, emulsions are usually formed, which in the case of methanolysis easily break down but, in ethanolysis are more stable, probably because of the formation of monoacylglycerides [Zhou et al., 2003], and complicate the separation and purification of esters. Nevertheless, FAEEs have been industrially produced in places like Brazil and the development of new biotechnologies for the production of bioethanol can favor the use of ethanol, what would convert the biodiesel in a total renewable source of energy [Farrell et al., 2006]. The amounts of biodiesel needed to improve the lubricity of the diesel fuel are low. In fact, previous studies show how the reduction of lubricity tends to stabilize at concentrations higher than 1 % by volume [Karonis et al., 1999]. It is also interesting the effect on lubricity of the impurities of biodiesel, as free fatty acids or monoacylglycerols. Both compounds, although present in minimum amounts (≈ 100 ppm), are major contributing factors to improve the lubricity of blends of biodiesel with diesel [Knothe, 2005].

Lubricity is commonly measured through the high frequency reciprocating rig (HFRR) test due to the ability of providing a wide range of wear mechanisms [Lacey et al., 1998]. It has been proved recently that the HFRR is also suitable for evaluating the wear performance of fuels in more severe contact conditions than those that may exist in future fuel injection equipment [Hornby et al., 2013]. The test methods are specified in standards EN ISO 12156-1 and ASTM D6079 for Europe and US, respectively. In the US the ASTM D975 limit is 520 μm , in Europe the EN 590 limit is 460 μm , while the World Wide Fuel Charter recommends a maximum wear scar of 400 μm for diesel fuels to be commercialized in markets with advanced or highly advanced requirements for emission control and fuel efficiency [World Wide Fuel Charter, 2013].

Previous studies have shown that the lubricating properties of the fuel, hence the size of the wear scar in the HFRR test, are influenced by the relative humidity in the air, especially when low load is applied and oxidative wear is predominant [Lacey, 1993]. Because of this, the EN ISO standard proposes a correction of the measured wear scar depending on the vapor pressure, using the so-called humidity correction factor (*HCF*) which is set to 60 $\mu\text{m}/\text{kPa}$ for all the fuels, regardless of their composition. This unchanged factor has been proved to underestimate or overestimate lubricity when

dealing with different kind of fuels [Lapuerta et al., 2014] and depends on the fuel chemical composition.

This part of the thesis continues a previous study on lubricity [Lapuerta et al., 2014], in this case focusing on biodiesel fuels and components. *HCFs* and wear scars of FAMES and FAEEs are measured to study the influence of the biodiesel composition on the lubricity, taking into account the water content in the fuel and the effect of hygroscopy on the change of lubricity at different ambient humidities.

3.2.2 FUELS AND EXPERIMENTAL SCHEDULE

The following fuels were tested with the HFRR:

- Pure methyl esters: Methyl laurate, myristate and stearate were purchased from Sigma-Aldrich. Methyl palmitate was purchased from Alfa Aesar. The main characteristics of pure methyl esters are shown in Table 10.
- Synthetic ethyl esters were manufactured from pure fatty acids purchased from Sigma Aldrich and were transformed into the fatty acid ethyl esters through an esterification process described in the literature [Kemp, 1967]. The purity of the esters was tested following the EN 14103 method. A Hewlett-Packard 5890 series II gas chromatograph equipped with FID detector and split/splitless injector and an HP-Wax column (30 m × 0.32 mm i.d. × 0.15 µm) of polyethylene glycol was used for the analysis. Properties of these compounds are shown in Table 10 and results of the chromatographic analysis are shown in Table 11.
- Biodiesel fuel (FAME) from palm oil was a gift of the Antioquia University, and fulfils EN 14214. The fatty acid profile was analysed with gas chromatography as previously described and the results are provided in Table 12.
- Biodiesel fuels (FAEEs) from different kind of oils were also produced following a transesterification method described in the literature [Bolonio et al., 2015]. The composition was tested through gas chromatography and the results are shown in Table 13.

Every methyl or ethyl ester fuel was tested for a minimum of two humidities (or vapor pressures), using for that the equilibrium of the ionic compounds described in Table 14. The salts were purchased from Panreac and are of synthesis grade.

Table 10: Main properties of the methyl and ethyl esters

Fuel	Molecular formula	Carbon number in the acid chain	Average number of double bonds	Molecular weight (g/mol)	^a Melting point (°C)	^b Viscosity at 40 °C (mPa·s)	^c Density at 15 °C (kg/m ³)
Methyl							
Methyl Laurate	C ₁₃ H ₂₆ O ₂	12	0	214.11	4-5	2.08	873.28
Methyl Myristate	C ₁₅ H ₃₀ O ₂	14	0	242.13	18	2.84	868.18
Methyl Palmitate	C ₁₇ H ₃₄ O ₂	16	0	270.15	32-35	3.76	864.19
Methyl Stearate	C ₁₉ H ₃₈ O ₂	18	0	298.17	37-41	4.99	867.55
Ethyl							
Ethyl Caproate	C ₈ H ₁₆ O ₂	6	0	144.06	^d -67	-	878.56
Ethyl Caprate	C ₁₂ H ₂₄ O ₂	10	0	200.1	^d -20	1.60	873.34
Ethyl Laurate	C ₁₄ H ₂₈ O ₂	12	0	228.12	^e -2	2.22	867.96
Ethyl Myristate	C ₁₆ H ₃₂ O ₂	14	0	256.14	^a 11-12	2.99	867.65
Ethyl Palmitate	C ₁₈ H ₃₆ O ₂	16	0	284.16	^a 24-26	3.96	864.39
Ethyl Stearate	C ₂₀ H ₄₀ O ₂	18	0	312.18	^a 34-38	5.03	858.72
Ethyl Oleate	C ₂₀ H ₃₈ O ₂	18	1	310.18	^a -32	4.21	877.18
Ethyl Linoleate	C ₂₀ H ₃₆ O ₂	18	2	308.18	-	-	883.18

^a[Sigma Aldrich Corporation]; ^b[Pratas et al., 2010]; ^c[Lapuerta et al., 2010b];
^d[www.chemicalbook.com]; ^e[www.chemicalspider.com]

3.2.3 EXPERIMENTAL EQUIPMENT AND PROCEDURE

Lubricity was measured with a HFRR (PCS Instruments, London, U.K.) following the European EN ISO 12156-1 standard and the ASTM D6079 standard. The volume of the fuel samples was 2 mL and its temperature was set to 60 °C. A steel ball and a steel disk were completely submerged in the test fuel and put in contact with each other. The steel ball is held with a vibrator arm and loaded with 200 g mass. Then a vibration of 1 mm at a frequency of 50 Hz for 75 min causes a wear scar on both the ball and the disk. The wear scar on the ball, that represents the lubricating efficiency, was estimated by measuring the mean diameter of the scar (*MWSD*) obtained from the length of the wear scar in the axis parallel and perpendicular to the ball displacement using a stereomicroscope Optika SZR1 equipped with 100 × magnification lens.

All tests were made inside a climatic chamber where the ambient temperature and humidity were controlled with the use of salts. The climatic chamber is a specific cabinet produced by PCS instruments. Temperature and relative humidity are controlled and instantaneously displayed on the HFRR board. Prior to use, the test components were submerged into 10 min ultrasonic-baths, first two using toluene and a final cleaning bath with acetone.

Table 11: Composition of the synthetic ethyl esters

FAEE, wt%	C6:0	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2
C6:0	96.08							
C10:0		97.88						
C12:0	3.92		92.28					
C14:0				100			2.07	
C16:0		2.12			100	2.05	5.38	
C16:1							6.54	
C18:0						97.75		
C18:1			7.72				76.41	
C18:2							8.80	100
^a N _c	8.15	12.09	14.35	16	18	19.96	19.61	20
^b db	0	0	0.08	0	0	0	1.06	2

^aWeighted-average number of carbon atoms in the FAEE^bWeighted-average number of double bonds in the FAEE

Table 12: Fatty acid profile of the palm-oil biodiesel fuel

FAME, wt%	Palm
C10:0	0.22
C14:0	0.83
C16:0	42.52
C16:1	0.13
C18:0	4.77
C18:1	41.27
C18:2	10.09
C18:3	0.16
Ester content	100
^a N _c	18.05
^b db	0.62

^a Weighted-average number of carbon atoms in the FAME^b Weighted-average number of double bonds in the FAME

Table 13: Composition of the biodiesel fuels made of ethyl esters from different sources of oil

FAEE, wt %	Waste frying oil	Soybean	Sesame	Linseed	Camelina	Rapeseed
C14:0	0.44	0	0	0.95	0	0
C16:0	14.17	12.81	9.54	6.9	6.9	5.04
C16:1	2.37	0	0	0	0	0
C18:0	3.93	6.52	5.88	4.46	2.88	9.22
C18:1	66.4	29.37	40.27	21.34	17.37	59.55
C18:2	10.33	46.06	43.13	17.27	33.47	17.74
C18:3	0.77	4.92	0.34	49.08	26.03	6.59
C20:0	0	0.32	0	0	1.33	0.51
C20:1	1.59	0	0.84	0	12.01	1.36
Ester content	97.68	98.72	97.58	99.27	96.79	98.78
^a N _c	19.65	19.73	19.81	19.81	20.09	19.92
^b db	0.93	1.36	1.28	2.03	1.74	1.16

^a Weighted-average number of carbon atoms in the FAEE^b Weighted-average number of double bonds in the FAEE

Table 14: Ionic compounds used to reach different vapor pressure in the HFRR chamber

Ionic compound	Temperature (°C)	Relative humidity (%)	Vapor pressure (kPa)
Sodium hydroxide (NaOH)	25-28	8-9	0.25-0.35
Potassium acetate (KCH ₃ COO)	25-28	21-22	0.66-0.86
Magnesium chloride (MgCl ₂)	25-28	32-33	1.01-1.29
Potassium carbonate (K ₂ CO ₃)	25-28	42-43	1.33-1.68
Sodium bromide (NaBr)	25-28	55-56	1.74-2.10
Potassium chloride (KCl)	25-28	82-83	2.60-3.20

Experiments were repeated twice and repeatability was demonstrated to be less than 20 μm [Lapuerta et al., 2009], which is acceptable according to the European standard which has shown a repeatability of 63 μm and to the American standard that has a repeatability of 80 μm (slightly higher because of the non-existent correction for the ambient humidity).

Water content in the fuel samples was measured with a Karl Fischer 756 KF Coulometer from Metrohm, following the method described in standard EN 12937.

3.2.4 WATER CORRECTION FACTORS

The standard EN ISO 12156-1 proposes the following equation to calculate the wear scar of the samples, with a correction depending on the temperature and relative humidity of the air measured through the vapor pressure:

$$WS1.4 = MWSD + HCF(1.4 - \bar{p}_v) \quad (9)$$

Where $WS1.4$ is the wear scar normalized to a vapor pressure of 1.4 kPa and $\bar{p}_v$ is the mean vapor pressure during the test time. HCF is, according to the standard, a constant value of 60 $\mu\text{m}/\text{kPa}$, but a value depending on the composition of each fuel would predict more accurately the lubricating efficiency. This factor was obtained by differentiating Eq. (9) with respect to the vapor pressure and solving the equation for HCF :

$$HCF = \frac{dMWSD}{d\bar{p}_v} \quad (10)$$

The hygroscopy of a fuel depends on its composition and accounts for the amount of water which is absorbed from humid air (quantified with its humidity or vapor pressure).

In a previous study [Lapuerta et al., 2014] the effect of humidity on the wear scar was proposed to be an indirect effect of the hygroscopy of the fuel. Therefore, since each fuel has a different hygroscopy, depending on its composition, a constant value of 60 $\mu\text{m}/\text{kPa}$ would not be accurate to correct the effect of vapor pressure for all the fuels. The new parameters proposed to measure this effect are derived from the psychrometry theory:

- The water–air correction factor (WACF):

$$WACF = \frac{dMWSD}{dY_{H_2O/air}} = HCF \frac{dp_v}{dY_{H_2O/air}} \quad (11)$$

- The water-fuel correction factor (WFCF):

$$WFCF = \frac{dMWSD}{dY_{H_2O/fuel}} \quad (12)$$

- The hygroscopy (Hy) of the fuel:

$$Hy = \frac{dY_{H_2O/fuel}}{dY_{H_2O/air}} = \frac{\frac{dMWSD}{WFCF}}{\frac{dMWSD}{WACF}} = \frac{WACF}{WFCF} \quad (13)$$

Where $Y_{H_2O/air}$ and $Y_{H_2O/fuel}$ are the mass fraction of water vapor in the humid air and the mass fraction of water in the fuel respectively.

3.2.5 RESULTS AND DISCUSSION

Lubricity of FAMES and FAEEs

Humidity correction factor (HCF)

The change in the non-corrected wear scar through different vapor pressures between 0.25 and 2.75 kPa can be observed in Figure 8 and Figure 9. The relation between the *MWSD* and the vapor pressure is approximately linear and Eq. (10) can be applied to calculate the different *HCF*. The linear equations which best fitted the experimental results with the correction factors for all the methyl and ethyl esters are collected in Table 15 and Table 16. The R-squared value of the lines is always higher than 0.9 which proves a good linear correlation between the data. As can be seen in Figure 9 only a few lubricity tests could be performed at different humidities for the different fuels because not enough quantity was available for these fuels. Therefore, the accuracy in the *HCF* calculation could be affected.

Figure 8: *MWSD* of FAMES

No clear correlation was found between number of carbons and the value of *HCF* as it can be seen in Figure 10 but the *HCFs* of FAMES are always higher than those of FAEEs, for the same length of the carbon chain. In the case of FAEEs, generally an increase in the number of carbons of the fatty acid chain leads to a reduction in the *HCF*. Comparing the values of *HCF* obtained with the standard value of 60 $\mu\text{m}/\text{kPa}$ given by the EN 12156-1, all of them are below the standard.

Figure 9: *MWSD* of FAEEs

Table 15: *HCFs* of FAMEs

FAMEs	<i>MWSD</i> (μm) linear fit	<i>HCF</i> ($\mu\text{m/kPa}$)	R^2
C12:0	$45.83p_v+205.65$	45.83	0.9108
C14:0	$55.61p_v+202.94$	55.61	0.9538
C16:0	$48.00p_v+164.55$	48.00	0.9997
C18:0	$47.75p_v+153.95$	47.75	0.9643

Table 16: *HCFs* of FAEEs

FAEEs	<i>MWSD</i> (μm) linear fit	<i>HCF</i> ($\mu\text{m/kPa}$)	R^2
C6:0	$50.35p_v+188.20$	50.35	0.9911
C10:0	$42.67p_v+157.30$	42.67	0.9968
C12:0	$44.33p_v+116.92$	44.33	-
C14:0	$43.01p_v+116.02$	43.01	0.9982
C16:0	$40.72p_v+108.94$	40.72	-
C18:0	$40.22p_v+102.30$	40.22	-

Therefore, the use of *HCF* set by EN-12156-1 for the calculation of the corrected lubricity (*WSI.4*) would be wrong, overestimating or underestimating the wear scar produced by the fuels, depending on the vapor pressure when the lubricity test is carried out [Lapuerta et al., 2014].

Influence of the composition on the lubricity

The lubricity results (*WSI.4*) for pure FAMEs and synthetic FAEEs are reported in Figure 11 and Figure 12. To obtain these results the *HCF* values calculated in the previous section have been used. The Figure 11 shows a clear decreasing trend of the wear scar with the increase of the number of carbons in the fatty acid chain. This effect agrees with previous studies where it is shown that molecules with the same structure and functional group, improve their lubricity with the increase in their molecular weight [Knothe et al., 2005]. Especially in the case of FAEEs, a more sudden decrease of wear scar is observed between the caprylate and laurate ethyl ester while for shorter and longer carbon chain the wear scar seems to stabilize.

Figure 10: Variation of *HCF* with the number of carbons

Also, it can be noticed in Figure 11 that both methyl and ethyl esters remain below the maximum wear scar value that sets the EN 590 Standard.

Results show that FAEs have better lubricity than FAMES, probably because of their different molecular conformation. Short alkyl esters possess polarity in their head-groups that provide an amphipathic nature resulting in a head-to-head alignment of molecules [Sarin, 2012]. To the contrary, larger alkyl esters have non-polar head-groups that are able to neutralize the forces between more polar portions of the head-group aligning themselves in a head-to-tail arrangement with much larger molecular spacing. Such voluminous head-groups disrupt the spacing between individual molecules in the crystal structure causing rotational disorder in the hydrocarbon tail-group. In the case of FAEs, the length of the alcohol chain is still short enough to let the molecules organize in a head-to-head arrangement, producing better lubricity due to the length increase of the complete structure.

Figure 12 shows that the increase in the unsaturation causes slightly better lubricity, in accordance with previous studies [Geller et al., 2004]. It is important to indicate that this effect is opposite to the behavior of these compounds concerning their viscosity. The viscosity decreases with the number of double bonds, so between the three compounds depicted in the graphic, ethyl linoleate is the one with better lubricity but worse viscosity, confirming that viscosity and lubricity not always are correlated, especially in low-sulfur fuels [Barbour et al., 2000].

Figure 11: Normalized wear scar of FAMES and FAEEs

Lubricity of biodiesel fuels

Humidity correction factor (*HCF*)

Figure 13 shows the relationship between *MWSD* and vapor pressure for different biodiesel fuels from different oils. As can be seen in Figure 13, only four lubricity tests were carried out at different humidities for the different fuels because not enough quantity was available for these fuels.

Figure 12: Effect of the unsaturation of C₁₈ FAEEs on the lubricity

Therefore, the accuracy in the *HCF* calculation could be affected. An approximately linear relation was found between the vapor pressure and the *MWSD* (the R-squared of

all the estimation lines is near 1). The slope of the lines can be calculated using Eq. (2) to estimate the HCF s of the fuels. The best-fit equations together with the correction factors for the biodiesel fuels are collected in Table 17. It is important to emphasize that HCF values are very similar for all the fuels tested and oscillate between 38 and 46 $\mu\text{m}/\text{kPa}$. This range is similar to that previously observed for pure methyl and ethyl esters with carbon number higher than 14 (shortest ester composing the biodiesel fuels tested).

As previously noted for the individual FAMES or FAEEs, all the fuels have an HCF lower than 60 $\mu\text{m}/\text{kPa}$. Therefore, the use of the standard would overestimate or underestimate the wear scar produced, and individual values of HCF for each type of fuel would improve the precision in the estimation of the WS1.4.

Influence of the composition on the lubricity

The results of the lubricity of FAEEs from different fuels are reported in Table 18. To obtain these results the HCF values calculated in the previous section have been used. The table shows that all the biodiesel fuels accomplish the American (with normalized wear scars below 520 μm) and European (below 460 μm) standards.

Figure 13: $MWSD$ of biodiesel fuels

Table 17: *HCF* of biodiesel fuels

	Fuel	<i>MWSD</i> (μm) linear fit	<i>HCF</i> (μm/kPa)	R²
FAME	Palm	$53.87p_v+189.52$	53.87	0.9976
FAEEs	Waste frying oil	$42.23p_v+145.68$	42.23	0.9898
	Soybean	$38.16p_v+163.45$	38.16	0.9749
	Sesame	$41.17p_v+172.48$	41.17	0.9891
	Linseed	$43.94p_v+157.50$	43.94	0.9800
	Camelina	$46.58p_v+196.95$	46.58	0.9721
	Rapeseed	$45.44p_v+151.05$	45.44	0.9799

As can be observed in Table 13, the biodiesel fuels tested have different average number of carbon atoms and different average number of double bonds. These factors, together with the eventual impurities, are expected to have some influence on the lubricity of the fuels, as reported in other studies [Knothe et al., 2005; Geller et al., 2004]. Normalized wear scars are in general slightly lower than the values previously shown in Figures 11 and 12 for pure ethyl esters with more than 14 carbon atoms and different degrees of unsaturation. In any case, among the tested FAEEs, the normalized wear scars obtained are very similar and not differing in more than 60 μm, approximately, which is close to the minimum difference between tests to be considered significant in EN ISO 12156-1.

Table 18: Normalized wear scar of biodiesel fuels

	Fuel	<i>WSI.4</i> (μm)
FAME	Palm	264.93
FAEEs	Waste frying oil	204.79
	Soybean	216.87
	Sesame	230.12
	Linseed	219.02
	Camelina	262.16
	Rapeseed	214.67

Correlation for *WSI.4* and *HCF* for methyl and ethyl esters

As commented above, the biodiesel fuels tested have different average number of carbon atoms and different average number of double bonds and these factors are expected to have some influence on the lubricity of the fuels and on the effect of humidity. Therefore, two different correlations have been proposed to calculate the normalized wear scar (*WSI.4*) and the humidity correction factor (*HCF*) as a function of the carbon atoms number, the degree of unsaturation (that can be measured by the average number of double bonds) and the type of transesterification. To obtain these correlations all the results presented previously with pure methyl and ethyl esters and biodiesel fuels have been used. The following equations have been obtained to estimate the *WSI.4* (Eq. (14)) and the *HCF* (Eq. (15)) using these three parameters.

$$WSI.4 = 381.54 - 8.17N_c + 49.05db - 66.79(m - 1) \quad R^2 = 0.845 \quad (14)$$

$$HCF = 60.65 - 0.65N_c + 2.33db - 7.77(m - 1) \quad R^2 = 0.619 \quad (15)$$

Where:

N_c : Average number of carbon atoms.

db : Average number of double bonds.

m : Number of carbon atoms of the alcohol used for transesterification (1 if the transesterification was carried out with methanol and 2 if the transesterification was carried out with ethanol).

As can be observed in Eq. (14), as the average number of carbon atoms (N_c) increases, the normalized wear scar (*WSI.4*) decreases. In previous studies, fuels were shown to improve their lubricity as their molecular weight was increased [Knothe et al., 20005]. Therefore, the results obtained with Eq. (14) are in agreement with those previous studies. Regarding the type of transesterification, it can be seen in Eq. (14) that transesterification with ethanol, instead of methanol, has an enhanced effect in lubricity. This result is also in agreement with the results obtained in Figure 11. The correlation for *HCF* (Eq. (15)) has not such a good R-squared coefficient. The reason for this could be that the number of lubricity tests carried out at different humidities is not enough and this has a negative impact in the accuracy of the estimated *HCF*. As can be observed in Eq. (15), as the average number of carbon atoms increases and the average number of double bonds decreases, the *HCF* obtained becomes smaller than the *HCF* set by EN ISO 12156-1.

Therefore, the Standard overestimates the *HCF*. Furthermore, transesterification with ethanol contributes to decrease the humidity correction factor for every biodiesel fuel. The effect of the type of transesterification is more significant in the *HCF* than the effect of the carbon atoms number and the double bonds number. The results obtained with the two correlations are shown in Figures 14 and 15.

Figure 14: Correlation for the estimation of $WS_{1.4}$ and the experimental values

Influence of the water content in the fuel on the lubricity

In previous sections, the effect of humidity in the ambient air on the fuel lubricity was presented. In this section the effect of fuel water content in the fuel on its lubricity is analysed by addition of water to the fuels tested. Four pure methyl esters and a biodiesel fuel were selected to characterize the effect of the water content on the lubricity of the fuel. They were all tested under potassium acetate environment (vapor pressure from 0.66

to 0.86 kPa) and laboratory ambient pressure of 94.25 kPa. Figure 16 shows that two water additions were made to each fuel, of approximately 100 and 200 ppm. In all samples, an increase in water content produces an increase in wear scar.

The presence of water prevents the formation of the third body debris layer on the metal surface [Lancaster, 1990] and thus adhesive wear is not likely to occur to the same extent. According to Fillot [Fillot et al., 2007], this third body layer is often composed of particles detached from the rubbing surfaces which separate the surfaces in contact, avoiding direct interactions.

Figure 15: Correlation for the estimation of HCF and the experimental values

For this reason, water content present in the fuel cause a major abrasion and corrosion. $WACF$ can be obtained using the developed formula of Eq. (11) [Lapuerta et al., 2014] and $WFCF$ can be estimated using equation Eq. (12). Results are reported in Figure 17. Dots connected with solid lines represent saturated methyl esters, and isolated dot corresponds to the biodiesel fuel tested (with some degree of unsaturation).

It can be observed that the tendency is the same for $WACF$ and for $WFCF$. For the same type of chemical compound (in this case methyl esters), when the $WACF$ increases, the same does the $WFCF$ and vice versa. Therefore, water present in environment or water contained in the fuel affect in the same way to the fuel lubricity. This is consistent with the direct effect of the fuel composition on both the normalized wear scar and the humidity correction factor, as shown in equation (15), and it also proves that it is the water content in the fuels the main cause of the increase in the wear scar when humidity increases (Figure 18) [Lapuerta et al., 2014].

Figure 16: Influence of water content in the MWSD

The main implication of this conclusion is that the well-known benefits in lubricity of biodiesel fuels could be counteracted by their high hygroscopy, which tends to capture water from the ambient air and thus reduce their lubricating properties, as far as they are largely exposed to open atmospheres.

Figure 17: *WACF* and *WFCF* of pure methyl saturated esters and biodiesel fuel

Figure 18: Effect of water on lubricity

Using the two mentioned parameters, the hygroscopy of each ester can be calculated using Eq. (13) (Figure 19). In this case, the effect of the carbon number on the hygroscopy is minor, and variations are mainly attributed to inaccuracies in the determination of the effects of water in both: the fuel (*WFCF*) and the ambient air (*WACF*). Also, some effect of unsaturation to decrease hygroscopy can be appreciated. However, differences are small and the amount of data is not enough to guarantee the significance of this trend.

Figure 19: Hygroscopy of pure saturated methyl esters and biodiesel fuel

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4. TUNING THE FATTY ACIDS COMPOSITION OF *ESCHERICHIA COLI* TO OBTAIN OPTIMUM BIODIESEL PROPERTIES

4.1 CONTEXT

In the search of new biodiesel sources, lignocellulose biomass represents one of the most promising options [Duan et al., 2011]. The annual dry vegetative biomass production in terms of energy is approximately 2.2×10^{21} J [Broadmeadow, 2003], four times the total current consumption of energy. This biomass can turn into biofuels constructing non-native biosynthetic pathways of molecules in microbial hosts. The most important steps in this process are [Stephanopoulos, 2007]: 1) Biomass pretreatment, to reduce size and loosen up the lignin-cellulose material; 2) Digestion through cellulolytic enzymes to release the hydrolysis products, primarily six- and five-carbon sugars; 3) Metabolic engineering of microorganisms to transform sugars into fuel molecules or precursors of fuels. For this last step, the bacteria *Escherichia coli* (*E. coli*) has proved to be an ideal host to test different approaches that can be later readily adapted for other microorganisms that can be better adapted to a consolidated bioprocess to generate advanced biofuels from biomass. *E. coli* is easy to genetically modify through metabolic engineering and has been already used to industrially synthesize a diverse range of chemicals [Maeda et al., 2007; Atsumi et al., 2008; Wang et al., 2010; Peralta-Yahya et al., 2011] and, at laboratory scale, to produce a diverse range of fuel molecules [Bokinsky et al., 2011], highlighting major efforts to increase the production of free fatty acids (FFAs) [Steen et al., 2010], long hydrocarbon molecules with high energy density, ideal precursors of biofuels.

Fatty acid biosynthesis in *E. coli* is a highly regulated pathway, carried out by fatty acid synthase II (FASII). The first step is carboxylation of acetyl-CoA to form malonyl-CoA by expense of ATP. Then, the malonyl-CoA is activated to malonyl-ACP, preventing the growing fatty acid chain from degradation through anabolic reactions, and enters the fatty acid synthesis cycle. The cycle consists of a condensation, two reductions and a dehydration allowing the carbon chain to increase two carbons each cycle, until certain chain length is reached and the acyl-ACP can be used for membrane synthesis. Non-genetically modified *E. coli* produces around 27 mg/L of FFAs [Liu et al., 2010], but major titers can be achieved when the periplasmic enzyme thioesterase I (TesA) is

expressed as a cytosolic enzyme ('TesA), cleaving the thioester bond of fatty acyl-ACP, which causes the accumulation of FFAs mainly in the late exponential and in the stationary phase [Cho et al., 1995]. Along with this enzyme, the FFAs yield can be improved in many different ways, highlighting the disruption of the degradation pathway (Δ FadD or Δ FadE) and overexpressing the transcription factor FadR which represses the transcription of all genes that code for proteins of the β -oxidation cycle [Zhang et al., 2012].

Apart from increasing the yield of FFAs in *E. coli*, some researchers have focused their attention on changing the FFAs composition, mainly through the heterologous expression of plant thioesterases, which play a similar role than the 'TesA enzyme, but with different substrate specificity. These enzymes have been classified into two general families, termed FatA and FatB, with highest activity towards 18:1-ACP and saturated acyl-ACPs, respectively. When expressed in *E. coli*, they disturb the fatty acid metabolism causing a change in the fatty acid production and composition, depending on the acyl-ACP thioesterase used [Zhang et al., 2011; Lennen et al., 2012].

This part of the thesis focuses on the production of a desired fatty acid composition from the bacteria *E. coli* to have optimum properties once the fatty acids are converted into their correspondent methyl esters, main compounds of biodiesel. Three different enzymes have been overexpressed in *E. coli* (see Figure 20), native thioesterase 'TesA, the transcription factor FadR and the plant BnFatA from rapeseed (*Brassica napus*), election based on the efficient oil accumulation in its plants seeds and the high percentage of C18 fatty acids in its oil composition.

4.2 EXPERIMENTAL SECTION

4.2.1 STRAINS AND GROWTH CULTURES

The *E. coli* strains DH5 alpha and BL21 DE3 pLysS were used as plasmids construction and production of FFAs host, respectively. The bacteria were grown in LB media (1 wt % BactoTryptone, 0.5 wt % BactoYeast Extract, 1 wt % NaCl, pH 7) for cloning (see Figure 21) and M9 minimal media (M9 salts¹, 2 mM MgSO₄, 0.1 mM CaCl₂, 0.4 wt % glucose) for production essays. The liquid cultures were shaken at 200 rpm and 30 °C.

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¹ M9 salts (5X): Potassium phosphate (KH₂PO₄) (15.0 g/L), Sodium phosphate (Na₂HPO₄) (64.0 g/L), Sodium chloride (NaCl) (2.5 g/L), Ammonium chloride (NH₄Cl) (5.0 g/L).

For plasmid selection, 100 µg/mL of ampicillin, 50 µg/mL of kanamycin and 25 µg/mL of chloramphenicol were added as necessary.

Figure 20: Metabolic pathway of sustainable production of biofuel in *E. coli*. Enzymes overexpressed in this work are underlined

Figure 21: *E. coli* liquid cultures

4.2.2 PLANT MATERIAL, RNA EXTRACTION AND cDNA SYNTHESIS

Rapeseed (*B. napus*) plants were grown to flowering stage in a shaded greenhouse, 24°C day/20°C night. Approximately 2-3 g of developing seeds 26 days after flowering were ground in liquid nitrogen and transferred to a bead mill (Ventura Mix 2) for grinding and homogenization. RNA was extracted following a previously published protocol [Chang et al., 1993] and then was purified using the RNeasy Plus Mini Kit. Lastly, cDNA was obtained using the PrimeScript™ RT reagent Kit.

4.2.3 PLASMIDS CONSTRUCTION

Genes accession numbers, plasmids names and the sequence of all primers are shown in Tables 19, 20 and 21, respectively. Genes from *E. coli* were extracted from its genome obtained through a previously reported method [Syn et al., 2000]. Genes were assembled into pDEST14 plasmids, which contain the promoter T7, using Gateway cloning system [Katzen, 2007]. This system is based on the site-specific recombination properties of bacteriophage lambda [Landy et al., 1989], where the wild-type λ *att* recombination sites have been modified to improve efficiency and specificity in the two necessary and consecutive reactions: 1) The BP Reaction (lysogenic pathway), catalyzed by BP Clonase™ enzyme, recombines an *attB* substrate with an *attP* substrate (donor vector) to create an *attL*-containing entry clone; 2) The LR Reaction (lytic pathway), catalyzed by LR Clonase enzyme recombines an *attL* substrate (entry clone) with an *attR* substrate (destination vector) to create an *attB*-containing expression clone. Isolation and purification of DNA plasmids was done through QIAprep Spin Miniprep Kit. The Veriti thermocycler from Applied Biosystems was used to make the PCR amplifications using Taq DNA Polymerase from Invitrogen and DNA fragments were sent for sequencing to Stab Vida.

Table 19: Genes

Gene description	Host organism	Sequence database (^a EMBL)
TesA, acyl-CoA thioesterase 1	<i>E. coli</i>	U82664
FadR, fatty acid metabolism regulon transcriptional regulator	<i>E. coli</i>	AP009048
BnFatA, Oleoyl-acyl carrier protein thioesterase	<i>B. napus</i>	LK034251

^aEuropean Molecular Biology Laboratory

Table 20: Plasmids

Plasmids	Replication origin	Overexpressed genes	Resistance
^a pDONR221	pUC	-	kanamycin
^a pDEST14	pBR322	-	ampicillin
^a pDONR221p1p5r	pUC	-	kanamycin
^a pDONR221p5p2	pUC	-	kanamycin
pDEST14 ‘ <i>TesA</i>	pBR322	<i>‘TesA</i>	ampicillin
pDEST14 <i>FadR</i>	pBR322	<i>FadR</i>	ampicillin
pDEST14 <i>BnFatA</i>	pBR322	<i>BnFatA</i>	ampicillin
pDEST14 ‘ <i>TesA</i> + <i>FadR</i>	pBR322	<i>‘TesA</i> + <i>FadR</i>	ampicillin
pDEST14 <i>BnFatA</i> + <i>FadR</i>	pBR322	<i>BnFatA</i> + <i>FadR</i>	ampicillin

^aGateway plasmids

4.2.4 E. COLI LIPIDS ANALYSIS

10 mL-triplicates of *E. coli* BL21DE3 pLysS cells were grown in M9 minimal media, adding the correspondent antibiotic, with agitation of 200 rpm at 30 °C. When an OD600 of 0.6 was reached, the cells were induced with 0.2 mM of isopropyl-b-D-thiogalactopyranoside (IPTG) and grown an additional 40 h. FFAs were extracted from 500 µL culture by addition of 30 µL HCl and 500 µL of ethyl acetate, spiked with 20 mg L⁻¹ of methyl nonadecanoate as an internal standard. The culture tubes were vortexed for 10 s followed by shaking at 200 rpm for 20 min. The organic layer was separated and a second extraction was performed by the addition of another 500 µL of ethyl acetate to the culture tubes. The FFAs were then converted to methyl esters by the addition of 50 µL trimethylsilyl(TMS)-diazomethane, 5 µL HCl and 100 µL methanol. This reaction was allowed to proceed for 2 h and then was applied to the GC-MS Agilent HP-6890 Serie II, equipped with a 5973 Mass Selective Detector and a J&W 122-5532 UI, 30 m x 250 µm x 0.25 µm column. The GC program was as follows: split ratio 50:1, column constant flow rate (He) 1 mL/min, injector temperature 250 °C, initial temperature of the sample 40 °C, held by 5 min, ramped to 325 °C at 8 °C/min and held by 5 min. Final quantification analysis was performed with GC/MSD ChemStation software.

Table 21: Primers

Primer	Sequence 5' to 3'	Function
^a attB1 adapter	GGGGACAAGTTTGTACAAAAAAGCAG GCT	Preparation of sequence for BP reaction
^a attB2 adapter	GGGGACCACTTTGTACAAGAAAGCTG GGT	Preparation of sequence for BP reaction
EcTesAfw	AAAAAGCAGGCTTAGAAGGAGATATA CATATGGCGGACACGTTATTGA	Extraction of leaderless sequence of <i>TesA</i> (' <i>TesA</i> ') of <i>E. coli</i> genomic DNA
EcTesArv	AGAAAGCTGGGTTTATGAGTCATGA TTTACTAAAGG	
attB5rTesA	GGGGACAACCTTTTGTATACAAAGTTGT TTATGAGTCATGATTTACTAAAG	Preparation of gene ' <i>TesA</i> ' for 2- fragment recombination
EcFadRfw	AAAAAGCAGGCTTAGAAGGAGATATA CATATGGTCATTAAGGCGCAAAGC	Extraction of gene <i>FadR</i> of <i>E. coli</i> genomic DNA
EcFadRrv	AGAAAGCTGGGTATTATCGCCCCTGA ATGGC	
attB5FadR	GGGGACAACCTTTTGTATACAAAAGTTG CAGAAGGAGATATACATATGGTCATT AAGGCGCAAAGC	Preparation of gene <i>FadR</i> for 2- fragment recombination
BnFatAfw	AAAAAGCAGGCTTAGAAGGAGATATA CATATGTTGAAGCTTTCGTGT	Extraction of gene <i>BnFatA</i> from <i>B.</i> <i>napus</i> cDNA
BnFatArv	AGAAAGCTGGGTCTCATCGTGAGGAT TTCTTTCTCC	Extraction of gene <i>BnFatA</i> from <i>B.</i> <i>napus</i> cDNA
attB5rBnFatA	GGGGACAACCTTTTGTATACAAAGTTGT TCATCGTGAGGATTTCTTTCTCC	Preparation of cDNA BnFatA for 2- fragment recombination

^aGateway primers

4.3 RESULTS AND DISCUSSION

4.3.1 FATTY ACID ANALYSIS OF *E. COLI* CELLS EXPRESSING RECOMBINANT PROTEINS

Fatty acid profiles from IPTG induced cultures of *E. coli* strains BL21 were obtained to test the functionality and effect of recombinant proteins on the *E. coli* lipid biosynthetic pathway (Figure 22 and 23).

As reported previously [Liu et al., 2010] the strain BL21 DE3 has a very low fatty acid yield (28 mg L⁻¹), composed mainly of saturated fatty acids, with 77.2 % of palmitic acid

(C16:0). The overexpression of a leaderless version of the endogenous thioesterase ('TesA) hydrolyzes the acyl-CoAs to generate FFAs and consequently depletes long chain acyl-ACPs [Cho et al., 1993] and triplicates the yield obtained in the control. It is noteworthy to mention that the major presence of FFAs triggers the fatty acid degradation pathway [Cronan et al., 1998] so the disruption of that pathway can be a way to better improve the yield, which has been the aim of other studies [Steen et al., 2010]. In addition, it has been tested that the specific activity of 'TesA must be carefully regulated to reach the optimum [Liu et al., 2010], and the use of stronger promoter like T7 is useful to study the effect of various enzymes but not to reach a maximum yield.

The fatty acid profile changes as expected when the thioesterase BnFatA is expressed. The specificity of FatA thioesterases is $18:1 > 18:0 > 16:1$ [Salas et al., 2002] and this effect is reflected in Figure 22. However, despite a major affinity for C18:1, the highest change is of C16:1, from 3.9 to 27.9 %. The reason may reside in an increase of the rate of fatty acid biosynthesis that cannot compensate for the loss in C18:1 and leads to the accumulation of C16:1 [Serrano-Vega et al., 2005]. The yield is slightly lower than the 'TesA strain, maybe due to a toxicity effect, which has been reported by other authors working with FatA thioesterases from plants [Zhang et al., 2011; Cao et al., 2014], although this effect profoundly depends on the plant chosen and greatly improves using codon optimization [Zhang et al., 2011]. Double mutants express both thioesterases 'TesA and BnFatA along with the transcriptional factor FadR. The yield increases and, as reported previously [Zhang et al., 2012], there is a higher production of unsaturated fatty acids due that FadR activates the expression of *fabA* and *fabB* genes (see Figure 20), whose enzyme products catalyze the biosynthesis of unsaturated fatty acids.

4.3.2 PREDICTED PROPERTIES OF FATTY ACID METHYL ESTERS (FAMES)

FFAs were converted into FAMES with TMS-diazomethane, a lab-scale method for esterification and measurement in GC-MS, but FFAs can be converted into FAMES or FAEEs through an industrial process, using acid catalysis or high pressure reactions [Canakci et al., 2001; Kusdiana et al., 2001; Bolonio et al., 2018] to make biodiesel. The main biodiesel standards are the US specification ASTM D6751 and the slightly more restrictive European biodiesel specification EN 14214.

Figure 22: Free fatty acids composition of the different strains of *E. coli* (BL21 DE3 pLysS)

Figure 23: Free fatty acids yield of the different strains of *E. coli*

Table 22 shows the properties that can be predicted from the FAME profile of the correspondent biodiesel along with the limit specifications of EN 14214.

Table 22: Biodiesel standard EN 14214: specifications and prediction equations

Property	Specification	Prediction Equation	Reference
Density at 15°C (kg m ⁻³)	860-900	$\rho_{FAME} = 851.471 + \frac{250.718 db + 280.899}{1.214 + n}$ $\frac{1}{\rho} = \sum (x_{FAME} / \rho_{FAME})$	Lapuerta et al., 2010b
Kinematic viscosity at 40°C (mm ² s ⁻¹)	3.5-5.0	$\ln(\nu) = \sum x_{FAME} \times \ln(\nu_{FAME})$	Allen et al., 1999
Flash point (°C)	> 101	$T_f = 284.1 + 3.861N_c - 0.5154N_c^2 - \frac{37.02}{db} + \frac{2.372}{db^2}$	Samavi et al., 2016
Cetane number	> 51.0	$CN_{FAME} = -23.523 + (2.366 + 6.299e^{-0.411db}) \cdot ne^{-0.018n}$ $CN = \sum x_{FAME} \times CN_{FAME}$	Lapuerta et al., 2009
Oxidation stability 110°C (h)	> 8	$IT = 0.214P_{FAME} + 0.671$ $0 < P_{FAME} < 45$ $IT = -0.234U_{FAME} + 22.318$ $0 < U_{FAME} \leq 84$	Sarin et al., 2010b
Cold Filter Plugging Point (°C)	Location and season dependant	$CFPP = 18.019N_c - 0.804U_{FAME}$	Su et al., 2011
Lubricity (μm)	< 460 ^a	$WS1.4 = 381.54 - 8.17n + 49.05db$	Lapuerta et al., 2016

^aThe limit of lubricity is not specified in EN 14214, but in EN 590.

Properties of FAMES of the different *E. coli* strains were estimated using the equations of Table 22. Density is an important technical and economical parameter for fuels since they are usually measured by volume and sold by weight, and density relates both. Density has also influence, together with the heating value, on the fuel consumption, since the amount of fuel injected in the combustion chamber is delivered by volume. It has been reported previously [Mittelbach et al., 1983; Prankl et al., 1999; Knothe, 2005] that the density of FAME increases when (a) the chain length diminishes and (b) the number of double bonds increases. Density could be estimated for biodiesel with the equations reported in Table 22 (row 1) and the inverse additivity rule [Lapuerta et al., 2010b], where ρ_{FAME} is the density of each pure methyl ester, db is the number of double bonds, n is the number of carbon atoms of the fatty acid and x_{FAME} is the mass fraction of each FAME in the blend.

Fuel viscosity has influence on the injection and the combustion, especially at low engine operation temperatures. Kinematic viscosity of biodiesel increases with the chain length of both the fatty acids and the alcohols [Prankl et al., 1999]. This property is inversely related with the number of double bonds, higher saturation degree implying higher viscosities. The viscosity of a fuel is related to pumpability at the operating temperature and also with the ability of lubricate the pump. The kinematic viscosity at 40 °C of the FAME included in this study have been estimated using the equation included in Table 22 (row 2), which is a general equation to evaluate the kinematic viscosity of a blend, where ν is the kinematic viscosity of the blend and ν_{FAME} is the kinematic viscosity of each pure methyl ester [Allen et al., 1999; Llamas et al., 2012a; Llamas et al., 2012b]. This equation would correspond to the Grunberg – Nissan equation with a zero value of the interaction term [Grunberg et al., 1949; Allen et al., 1999].

The flash point is defined as the lowest temperature at which a liquid produces enough vapors to ignite in the presence of a source of ignition, and it is one of the most significant properties of flammable liquids in industrial processes when evaluating process safety [Lazzús, 2010]. This test gives an indication of the maximum temperature for fuel shipment, storage, and handling without serious fire hazard. Flash point could be estimated for biodiesel with the equations reported in Table 22 (row 3) and where T_f is the flash point of the biodiesel, db is the average number of double bonds and N_c is the weighted-average number of carbon atoms in the biodiesel.

Cetane number is one of the most important parameters for diesel fuels since it is a direct measurement of the quality of the combustion, high cetane number implying better ignition properties, good cold starting and lower smoke points. Biodiesel has usually higher cetane numbers than fossil diesel and cetane number notably depends on feedstock. Cetane numbers are higher for longer fatty esters and alcohol chains and for lower unsaturation degree. The cetane number for pure FAME could be estimated from the equations in Table 22 (row 4) and the additivity rule [Lapuerta et al., 2009], where CN_{FAME} is the cetane number of each pure methyl ester and CN is the cetane number of biodiesel.

The oxidative stability of biodiesel, which primarily affects the stability of biodiesel during extended storage, is measured by the Rancimat method (EN 14112) that reports the induction time (IT) in hours. Biodiesel with a high percent of unsaturated esters show usually shorter induction times than those with a lower amount of these unsaturated esters.

The FAME induction time has been estimated with correlations obtained from the literature and reported in Table 22 (row 5), where U_{FAME} is the total content of unsaturated FAMES (wt %) and P_{FAME} is the content of methyl palmitate (wt %).

The cold flow behavior of biodiesel is one of the main drawbacks for the generalized use of this biofuel, since it is worse than that of fossil diesel. A bad performance of the biofuel at low temperatures may cause problems of handling, storage, injection and cool starting of the engine [Foglia et al., 1997]. Table 22 (row 6) reports the equation used to estimate the value of the cold filter plugging point (CFPP, °C) using the fatty acid profiles, where U_{FAME} is the total content of unsaturated methyl esters (wt %) and N_c is the weighted-average number of carbon atoms in the biodiesel.

Lubricity is the measure of the reduction in friction or wear. This property is recently gaining attention because low blend levels of biodiesel can restore lubricity to (ultra-)low-sulfur petroleum-derived diesel (petrodiesel) fuels that are now the main diesel fuels available [Knothe et al., 2005]. The lubricity can be estimated using the equation included in Table 22 (row 7).

The estimated values are shown in Table 23. Density, kinematic viscosity, cetane number and lubricity results are all appropriate according to the standard, but some of the other properties are out of range. Flash point is below limit in the control and the strain overexpressing the enzyme ‘TesA, but improves when BnFatA or FadR are overexpressed. The two remaining properties, oxidation stability and cold flow performance, measured through the cold filter plugging point (CFPP), are usually the major drawbacks of biodiesel compared to petrodiesel [Atabani et al., 2012].

Table 23 shows how genetically unmodified *E. coli* has an unacceptable CFPP of 22 °C caused by the very high saturated FFAs composition. As a reference, although CFPP limits are different by country, the highest in Europe is 0 °C for some countries in summer (see Section 1.7). Overexpressing ‘TesA improves the CFPP value by a reduced weighted-average number of carbon but is still too high. As explained before, this poor cold flow performance goes accompanied by a good oxidation stability. Strains overexpressing BnFatA or FadR increase the unsaturated compounds and have much better CFPPs, reaching -25.2 °C in the case of both overexpressed enzymes. As a downside, the oxidation stability of these strains is worse, but still quite reasonable due to the lack of polyunsaturated compounds in *E. coli* composition (the value of 8 h of induction time is

a very strict limit and the ASTM standard has stated a 3 h limit for the same property). This allows to improve drastically the cold flow performance while maintaining an acceptable oxidation stability, which could be treated with antioxidants, a promising low-cost method for increasing its resistance to oxidation [Dunn, 2008].

Table 23: Estimated properties of FAMES from the control and recombinant strains of *E. coli*

	BL21 DE3 pLysS strains of <i>E. coli</i>				
Property	BL21 DE3 pLysS (control)	pDEST14 ‘TesA	pDEST14 BnFatA	pDEST14 ‘TesA+FadR	pDEST14 BnFatA+FadR
Density at 15 °C (kg m ⁻³)	867.6	869.3	872.8	874.4	877.4
Kinematic viscosity at 40 °C (mm ² s ⁻¹)	4.2	3.7	4.1	3.5	3.5
Flash point (°C)	58.9	75.3	129.6	143.4	154.2
Cetane number	77.4	73.2	69.7	65.9	62.7
Oxidation stability 110 °C (h)	19.4	18.2	9.7	6.8	5.3
Cold Filter Plugging Point (°C)	22.0	5.6	-1.6	-17.4	-25.2
Lubricity (µm)	249.0	257.2	264.0	271.5	278.5

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5. GREEN-FILAMENTOUS MACROALGAE *CHAETOMORPHA* CF. *GRACILIS* FROM CUBAN WETLANDS AS A FEEDSTOCK TO PRODUCE ALTERNATIVE FUEL: PHYSICOCHEMICAL CHARACTERIZATION

5.1 CONTEXT

Algae can become a suitable source of biomass for biofuels production. This chapter of the thesis is focused on macroalgae, which are multicellular organisms that grow in fresh or salt water [Sheehan et al., 1998]. They are classified into three broad groups according to the pigmentation: brown seaweed (*Phaeophyceae*), red seaweed (*Rhodophyceae*), and green seaweed (*Chlorophyceae*) [John et al., 2011; Kraan, 2013].

There are thousands of algae species. The more cultivated around the world are the brown algae *Laminaria japonica* and *Undaria pinnatifida*, the red algae *Porphyra*, *Eucheuma*, *Kappaphycus* and *Gracilaria*, and green algae *Monostroma* and *Enteromorpha* [Carlsson, 2007]. Among these algae species, green algae are cultivated easily since they can grow in all kinds of water environments.

Macroalgae are a promising source for biofuel production, they do not compete with the production of food [Singh et al., 2011] and unlike oil crops, do not require agricultural land for cultivation, pesticides or herbicides [Chisti et al., 2007]. They are normally cultured in freshwater ponds, brackish or seawater, even sewage, where they can consume nutrients from the wastewater to grow and develop. Moreover, macroalgae can fix CO₂ with a photosynthetic efficiency of 6-8 %, unlike terrestrial biomass which has an efficiency of 1.8 to 2.2 % [Chen et al., 2015].

Fourteen types of seaweed macroalgae collected individually from Galician coast were investigated by Maceiras [Maceiras et al., 2011] and Sánchez [Sánchez et al., 2012]. The algae oil extraction was carried out using Soxhlet method and n-hexane as solvent (300 mL of n-hexane for 30–80 g of dried algae depending on the algae type) according to EN ISO 734-1. They demonstrated that these algae do not have very high oil content, which could seem an inconvenient. However, only at south of Galicia approximately 100,000 tonnes/year of macroalgae are collected from beaches [Maceiras et al., 2011].

Sharif conducted a study [Hossain et al., 2008] about biofuel production from *Spirogyra* sp. and *Oedogonium* sp. macroalgae (also studied by Manikandan [Manikandan et al.,

2014]), which were collected from the Phycology Laboratory (Institute of Biological Science, Malaysia). The oil content for *Oedogonium sp.* (9.2 %) was higher than *Spirogyra sp.* (7.3 %), nevertheless, the biomass obtained after the oil extraction was higher in *Spirogyra* (43.3%) than *Oedogonium sp.* (33.6 %). In terms of sediments, (glycerin, water and pigments) similar results were obtained. A hexane-ether solution (20 and 20 mL) were mixed with the dried algae to extract the oil and the mixture was left settling for 24 h.

Tamilarasan [Suganya et al., 2013] developed an investigation of *Enteromorpha compressa* macroalgae due to its high content of free fatty acids (FFAs). This macroalga was collected from Gulf of Mannar, Mandapam (India). The oil extraction was conducted using Soxhlet method and mixed solvents (1 % diethyl ether and 10 % methylene chloride in n-hexane) for 6 h. The oil extraction yield was 11.14 %. The gas chromatography analysis showed more saturated fatty acids than unsaturated fatty acids. The density and kinematic viscosity of the oil were 922.15 kg/m³ and 35.51 mm²/s respectively.

Currently, an interesting investigation on *Cladophora glomerata* macroalga was reported by Yuvarani et al. [Yuvarani et al., 2017]. The oil was extracted using modified Soxhlet method obtaining maximum oil percentage of 18 wt % from methanol-chloroform solvent mixtures at 65 °C for 3.5 h. According to the fatty acid profile, the macroalga oil has the saturated fatty acids palmitic and pentadecanoic acid, as well as unsaturated fatty acids, oleic and linoleic acid.

In this context, the macroalgae oil for biofuels production in Cuba might be an interesting alternative due to its availability (e.g. coastline and wetlands) and Cuban climate (e.g. rich water in nutrients, abundant sun and natural environment). In addition, the Cuban new economic and social policies include the use of renewable energies, emphasizing in their applications and economic effects. However, the potential of macroalgae in Cuba has not been thoroughly studied (e.g. only one study about biogas production was found [Díaz, 2010]). For this reason, the aim of this chapter is to evaluate the green filamentous macroalgae *Chaetomorpha cf. gracilis* (see Figure 24), due to its high productivity rates (10 and 15 days) and availability. A complete description of the lipid extraction process and physicochemical characterization is developed and used to discuss the potential of this macroalga to become a biofuel.

Figure 24: *Chaetomorpha cf. gracilis*

5.2 MATERIALS AND METHODS

5.2.1 LIPID EXTRACTION FROM GREEN-FILAMENTOUS *MACROALGAE CHAETOMORPHA CF. GRACILIS*

2 kg of wet green-filamentous macroalgae *Chaetomorpha cf. gracilis* was collected from Cuban freshwater wetlands. The macroalga was previously washed with distilled water in order to remove all the residual stuck. After that, it was sun-dried for one day and then stored in a drying oven/incubator for 3 h at 100 °C until constant weight.

The dry macroalgae biomass was crushed using Retsch ZM-200 equipment as a prior step to oil extraction, obtaining a total of 1.652 kg. Then, the macroalgae oil was extracted through the Soxhlet method, which has been reported as an effective method for macroalgae oil extraction [Maceiras et al., 2011; Sánchez et al., 2012; Sreedhar, 2014; Salehzadeh et al., 2014].

The oil extraction was carried out using a 0.5 L round-bottomed glass flask coupled to a Soxhlet extractor and a condenser, as well as a proportion of 400 mL of n-hexane (purchased from Panreac) for each 40 g of dried biomass. The oil extraction time for each set was 3 h. The residual mix of n-hexane-oil was removed under vacuum at 335 mbar, 40 °C steam, 15 °C of cooling water and a thermostatic bath at 60 °C in a rotary evaporator. After each set of extraction, the solvent was recovered and reused for the next extraction batch.

5.2.2 CHARACTERIZATION OF THE COMPOSITION: FATTY ACIDS AND NATURAL PRODUCTS

The oil obtained from *Chaetomorpha cf. gracilis* was treated to convert all fatty acid components into the methyl esters for an easier determination. First, the oil acid value was measured following the method proposed in standard EN 14104. The acidity value was 22 mgKOH/g (>2 mgKOH/g). For the esterification step the oil was heated to 60 °C with stirring at 600 rpm, p-toluenesulfonic acid (mass ratio oil – p-toluenesulfonic acid, 200:1) solved in methanol (molar ratio of methanol–oil, 6:1) was added to the reactor, and the reaction mixture was kept at 60 °C with stirring at 600 rpm for 4 h. At the end of the reaction, freshly calcined calcium oxide (mass ratio oil–calcium oxide, 50:1) was added to the reactor at 60 °C with stirring, to neutralize the acid catalyst and to eliminate the water produced in the esterification, forming insoluble calcium hydroxide.

In the transesterification process, the mixture of oil and methanol from the esterification process was heated to 60 °C with stirring at 600 rpm. Sodium methoxide (mass ratio oil–sodium ethoxide, 100:2) dissolved in methanol (molar ratio of methanol–oil, 6:1) was added to the reaction mixture. After 3 h, the reaction mixture was cooled to room temperature in the reactor. It was decanted in the centrifuge tubes and centrifuged at 3000 rpm for 5 min, and the upper biodiesel phase was separated from the lower G-phase. The fatty esters were transferred to a rotary evaporator to eliminate the methanol excess, treated with 2 wt % Magnesol D-60 at 77 °C, with stirring at 800 rpm for 30 min, and filtered through a 0.45 µm filter.

The fatty acid profile of the sample was determined using a Hewlett-Packard 5890 series II gas chromatograph equipped with flame ionization detector (FID) and split/splitless injector. An HP-Wax column (30 m $\times$ 0.32 mm i.d. $\times$ 0.15 µm) of polyethylene glycol was used for separation. The following analytical conditions were followed: injector temperature, 210 °C; split ratio, 60:1; injection volume, 1 µL; column flow rate (He): 1 mL/min constant flow mode. FID temperature, 240 °C; H₂ flow rate: 40 mL/min; air flow rate: 400 mL/min; oven program: 210 °C hold 9 min, to 230 °C at 20 °C/min, hold 10 min; calibration standard, solution of methyl heptadecanoate in heptane (5 mg/mL); sample preparation, 250 mg of sample in 10 mL vial with 5 mL of methyl heptadecanoate solution. In addition, gas chromatography– mass spectrometry (GC–MS) was used to identify *natural products* present in the macroalgae. A gas chromatography (Hewlett-Packard 6890) coupled to a mass spectrometer (Hewlett-Packard 5973), equipped with

Agilent column DB-5MS (25 m × 0.25 mm i.d. × 0.20 μm) was used. Helium was used as the carrier gas. The injector temperature was 250 °C and the column temperature of each run was started at 50 °C for 3 min, then raised to 310 °C at 10 °C/min and maintained at 310 °C for 10 min.

5.2.3 DETERMINATION OF PHYSICOCHEMICAL PROPERTIES OF MACROALGAE OIL

Different properties such as kinematic viscosity, density, elemental composition (carbon, hydrogen, and nitrogen), lower and higher heating values of the macroalgae oil were tested according to the ASTM standards (see Table 24). In addition, the crystallization onset temperature (COT) of oil macroalgae obtained from differential scanning calorimetry (DSC) was analyzed using the DSC Q20 of TA Instruments to obtain the heating thermograms.

Table 24: Properties and standard procedures

Properties	Standard	Equipment
Kinematic viscosity at 40°C	ASTM D445	Viscosity meter HVM 472
Density at 15°C	ASTM D4052	Density meter DMA 48 de
Heating value	ASTM D240	6200 Calorimeter
Elemental composition	ASTM D5291	Elemental Analyzer Truspec CHN

For this purpose, an oil sample of 6.5 mg (within aluminum pans), an empty aluminum pan (as a reference) and a nitrogen flow rate of 40 mL/min were used. The melting thermograms at different heating rates were tested in order to analyze the effects on the COT. Each sample was heated to 70 °C, kept at this temperature for 5 min and then cooled from 70 to -80 °C, maintaining this temperature also for 5 min. The heating and cooling rate was 5 °C/min.

5.3 RESULTS AND DISCUSSION

5.3.1 EXPERIMENTAL RESULTS OF LIPID EXTRACTION AND METHYL ESTER FATTY ACID CHARACTERIZATION

The initial humidity content for *Chaetomorpha cf. gracilis* macroalgae was 66.7 %, and was reduced to 0.15 % after being dried in the oven. The oil content obtained using

Soxhlet extraction method was 4.2 mL/40 g, achieving a total oil content of 53 mL from the 1.652 kg of dry crushed biomass. The oil yield was 1.85 %.

The oil content obtained in this thesis is between the limits reported previously for macroalgae, 1.3 and 7.8 wt % [Hossain et al., 2008]. Some examples are *Cladophora*, 2.48 %; *Gracilaria*, 2.01 % and *Spirogyra*, 3.01 % as reported by Ahmed [Ahmed et al., 2013]. Other researchers as Vincecate [Vincecate, 2006] suggested that macroalgae contains around 5.5 % of oil. And one of the best oil contents was accomplished by Sharif [Hossain et al., 2008] (i.e. 7.3 % and 9.2 % for *Spirogyra* sp. and *Oedogonium* sp algae).

The fatty acid composition of the *Chaetomorpha cf. gracilis* oil, analyzed by GC-FID, is shown in Table 25. It showed higher unsaturated fatty acids (i.e. 56.14 %) compared to saturated fatty acids (i.e. 35.18 %), with the oleic acid content being 27.89 %. Same trend was achieved by Renita et al. [Sreedhar, 2014] with the *Sargassum myriocystum* FAME, as well as Xu et al. [Xu et al., 2014] but using a FAME of *Cryptococcus curvatus* yeast. However, opposite trends have been reported with other macroalgae species (see Table 25), where the fatty acid composition reported saturated fatty acids as most abundant.

The most common saturated fatty acid in algal cells is the palmitic acid (16:0) constituting 21–42 % of total fatty acids. However, red and brown algae, have lower levels of C₂₀ and C₂₂ polyunsaturated fatty acids, while the green algae contain high levels of C₁₆ and C₁₈ polyunsaturated fatty acids [Suutari et al., 2015]. The concentration of light fatty acids is high compared to most of vegetable oils, and especially that of palmitoleic acid. Also, the iodine value is not so high (102 g I₂/100 g oil) because the content in linoleic acid is much lower than that usually found in vegetable oils.

The analysis made with GC-MS permitted to identify other compounds than fatty acids, as shown in Figure 25. The chromatogram showed a high percentage of *natural products* (52 %). Mainly, 22,23-dihydrostigmasterol (18.6 %) and phytol (17.5 %). In addition, other products such as squalene, cholesterol and neophytadiene reported lower percentages. The sample has significant hydrocarbon content (21.6 %), between C₁₇ and C₂₉. These hydrocarbons with odd number of carbons are produced by decarboxylation of fatty acids caused by an enzyme recently found in algae [Sorigué et al., 2017]. This is important because hydrocarbons are a source of energy used worldwide, as components of petroleum and natural gas.

Table 25: Fatty acid composition of *Chaetomorpha cf. gracilis* oil and other macroalgae (%)

Fatty acid	<i>Chaetomorpha cf. gracilis</i> [This study]	<i>Sargassum myriocystum</i> [Sreedhar, 2014]	<i>Caulerpa peltata</i> [Tamilarasan et al., 2014]	<i>Cryptococcus curvatus</i> <i>yeast</i> [Xu et al., 2014]	<i>Enteromorpha compressa</i> [Suganya et al., 2013]	<i>Macro-Green algae</i> [Sonawane et al., 2015]	<i>Spirulina platensis</i> [Nautiyal et al., 2014] [Nautiyal et al., 2014]	<i>Nannochloropsis salina</i> [Reddy et al., 2014]
Caprylic	-	-	-	-	-	18.63	3.90	-
Lauric	8.40	-	7.28	-	-	1.01	1.14	-
Myristic	5.20	-	3.02	-	2.16	4.94	2.52	2.72
Palmitic	21.58	13.74	36.82	25.88	70.26	29.15	41.21	37.83
Palmitoleic	12.56	-	5.04	-	3.71	4.23	3.39	31.34
Stearic	-	14.77	4.58	12.83	2.95	4.04	1	5.63
Oleic	27.89	15.02	2.31	48.16	2.38	8.12	4.11	11.31
Linoleic	11.60	15.44	18.19	8.95	18.54	-	12.64	-
Linolenic	4.09	-	7.03	-	-	-	17.79	-
Eicosatrienoic	-	17.44	-	-	-	-	-	-
Eicosatetraenoic	-	19.46	-	-	-	-	-	-
Behenic	-	-	8.27	-	-	-	-	-
Others	8.68	4.13	7.46	4.18	0	29.88	12.3	11.17

Figure 25: Chromatogram GC-MS of FAME *Chaetomorpha cf. gracilis* oil

The presence of esters (content of fatty acids) was 4.8 %, lower compared to other macroalgae. For example, a yield of fatty acid methyl ester of 17.10 % for marine macroalgae was reported by Sanchez [Sánchez et al., 2012]. Alternatively, Manikandan [Manikandan et al., 2014] reported a yield of 26.3 % for the *Oedogonium sp* macroalgae.

5.3.2 PHYSICOCHEMICAL CHARACTERIZATION

The *Chaetomorpha cf. gracilis* macroalgae oil viscosity reported a lower value (see Table 26) compared to other algae and vegetable oils [Haik et al., 2011; Wahlen et al., 2012; Suganya et al., 2013; Tamilarasan et al., 2014]. However, similar values were obtained by Jesu, et al. [Martin et al., 2012] with orange oil (i.e. 0.95 mm²/s). In diesel engines, the fuel viscosity is a standardized property (e.g. ASTM D445), so the achieved results with the *Chaetomorpha* oil viscosity are even far from the standards. For this reason, the direct use of *Chaetomorpha* macroalgae oil as diesel engine fuel is not recommended. Nevertheless, this drawback might represent an advantage if *Chaetomorpha* oil is used as an additive to decrease the viscosity in the blend with other oils.

The assessment of density shows a similar result as the viscosity (see Table 26). Lower value compared to other vegetable/algae oil and biodiesel fuels [Haik et al., 2011; Wahlen et al., 2012; Tamilarasan et al., 2014; Bhuiya et al., 2016] was reported. This result might be linked to the presence of *natural compounds*, as previously reported (see Section 5.3.1). Based on this fact, Silitonga et al. [Silitonga et al., 2013] pointed out that it is possible to increase the density adding unsaturated esters with more than two double bonds.

According to the heating value analysis shown in Table 26 the *Chaetomorpha* oil shows comparable value to diesel fuel (40-45 MJ/kg). This value is also higher than other macroalgae oils [Demirbaş, 2008; Haik et al., 2011; Wahlen et al., 2012; Milledge et al., 2015; Chen et al., 2015], as well as palm oil (39.11 MJ/kg) and *Simmondsia chinensis* oil (47.38 MJ/kg) [Bhuiya et al., 2016]. A higher heating value of *Chaetomorpha* oil represents a plus because it might lead to improvement if it is used as an additive of other oils. Likewise, the lower heating value has a similar value as the high heating value regarding other algae oils [Porphy et al., 2012].

Regarding the elemental composition (carbon, hydrogen and nitrogen), the results obtained (see Table 26) are not similar to other macroalgae species (e.g. *L. digitata*, *L. hyperborea*, *F. vesiculosa*, *F. serratus* and *C. filum*) [Ross et al., 2008; Chen et al., 2015; Milledge et al., 2015]. The carbon percentage of these species is generally between 31 and 39. The hydrogen content of *Chaetomorpha cf. gracilis* oil is seven times higher than five species of Brown seaweed studied by Ross and Milledge [Ross et al., 2008; Milledge et al., 2015]. Nevertheless, nitrogen content for *L. digitata* macroalgae is similar to

Chaetomorpha cf. gracilis. These results might be related to the environmental conditions during its growth cycle, biochemical composition changes or the presence of *natural compounds*.

Table 26: Characterization of *Chaetomorpha cf. gracilis* macroalgae oil

Property	Value
Kinematic viscosity at 40 °C (mm ² /s)	0.826
Density at 15 °C (kg/m ³)	699.6
Higher heating value (MJ/kg)	48.50
Lower heating value (MJ/kg)	45.87
Carbon (% m/m)	58.67
Hydrogen (% m/m)	12.8
Nitrogen (% m/m)	0.053

As previously mentioned, the COT was analyzed using a melting thermogram obtained by DSC (see Figure 26). In this chart, the COT was defined as the temperature when the first exothermic peak begins.

The COT from a melting thermogram was 11.74 °C. Several studies have correlated the COT with cold filter plugging point, in the case of diesel fuel [Claudy et al., 1986] or biodiesel [Dunn et al., 1999]. In our case, it is not possible to estimate the CFPP but it is clear that the COT is too high and the oil should be used as a mixture with branched chain alkyl esters as well as diesel fuel.

5.3.3 FINAL CONSIDERATIONS

According to the characterization in this study, the *Chaetomorpha cf. gracilis* macroalgae might be a promising source for the production of liquid biofuel. This macroalgae has not been used before in Cuba and might cause damages in the ecosystem due to its fast growing in freshwater ponds.

From the energy point of view, the oil extraction of *Chaetomorpha cf. gracilis* macroalgae could be a key factor for this proposal. The oil extraction procedure is quite energy intensive (collection of algae, pre-drying, drying, oil-extraction and separation), so an analysis based on the ratio energy-out/energy-in for oil production is indeed necessary and recommended.

Figure 26: Melting thermogram from the DSC of *Chaetomorpha cf. gracilis* oil

For this reason, an analysis focused on the net energy ratio (NER) was developed from the energy contained in the final product, the output energy, divided by the sum of cumulative energy demand energy for the oil extraction.

For the NER analysis, two scenarios were defined in order to identify the influence of the energy consumed in the oil extraction process. The analyzed scenarios were: oil extraction from a conventional method (oven/incubator, crushed, heating mantle, rotary evaporator, thermostatic bath), as well as a second scenario that integrates renewable energy (solar dryer, crushed, heating mantle, rotary evaporator, thermostatic bath). The assessment of NER analysis is shown in Figure 27.

Figure 27: Energy balance of the oil extraction process

Both scenarios are feasible because the energy consumed is lower than the energy available in the final product (see Table 26). However, it is valid to point out that the integration of the solar dryer in the oil extraction process represents an attractive alternative because it demands less energy for the oil extraction process.

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6. BIODIESEL FROM DESERT PLANTS

6.1 FATTY ACID METHYL AND ETHYL ESTERS (FAME AND FAEE) OBTAINED FROM RARE SEEDS FROM TUNISIA: *AMMI VISNAGA*, *CITRULLUS COLOCYNTHIS*, *DATURA STRAMONIUM*, *ECBALLIUM ELATERIUM* AND *SILYBUM MARIANUM*

6.1.1 CONTEXT

One of the most important issues, related to the production of biofuel is its competitive relationship with conventional agriculture. The rapidly increasing use of sugarcane and maize grains for production of ethanol, or the use of soybean, rapeseed and palm oil for production of biodiesel has been met with mounting criticism and concern [UN Energy, 2007] [Foley et al., 2005; Hill et al., 2006] based on the fact that the resource allocation for biofuel production may compete with production of food [Koonin, 2006; Ragauskas et al., 2006; Kennedy, 2007]. Even perennial high biomass producing plants like switchgrass (*Panicum virgatum*) or elephant grass (*Miscanthus giganteus*) compete with "green fuel crops" for the same scarce resources i.e., arable land and fresh water [Tilman et al., 2006].

Arid and semi-arid lands are often seen as a potential place where biofuel production can attain environmental sustainability [Chávez-Guerrero et al., 2010], as it is assumed that (1) these lands have a limited contribution to food production and thus a small potential for indirect land use change; and (2) the ecosystems of arid and semi-arid lands generally deliver fewer ecosystem services than those in more humid climates (e.g. in terms of carbon stock and biodiversity) [Costanza et al., 1997].

A big variety of crops have been studied and proposed as alternatives of conventional sources of biodiesel. Among them, jatropha is one of the most promising options. It has been promoted as a sustainable biodiesel crop for arid and semi-arid lands resulting in large investments and land conversions [Achten, 2010]. However, despite the fact that jatropha grows abundantly in the wild, it has never really been domesticated. Its yield is not predictable; the conditions that best suit its growth are not well defined and the potential environmental impacts of large-scale cultivation are not understood at all [Fairless, 2007]. This has often caused yields below expectations [Sanderson, 2009] and the search for other alternatives.

Some plant species highly adapted to currently unused desert arid lands capable of assimilating CO₂ and producing significant amounts of triacylglycerides may be good candidates for use as feedstock in the biodiesel industry. For example, *Balanites aegyptiaca* L. Delile (popularly known as the Desert date, belonging to the family Zygophyllaceae) is highly adapted to the drier parts of Africa and south Asia, it is distributed in most adverse arid desert environments [Hall et al., 1991] so it could be an alternative [Chapagain et al., 2009]. *Agave*, a genus of some 200–300 species within the family Agavaceae, has been reported to have a potential to produce biofuel [Davis et al., 2011]. The natural distribution of the *Agave* genus is limited to the Americas, with the greatest diversity of species in Mexico, although many species have been distributed across the Mediterranean area. It often grows on rocky soils of poor quality in regions with extreme drought and elevated temperatures. [Kirby, 1963]. Other studies [Eshel, 2010] have focused on halophytic plant species (grow in waters of high salinity) that can cope with conditions considered stressful for food crops, but can be introduced into fuel production. Water is a limiting factor for plant production. However, in many desert areas large quantities of saline water can be found. Such water is too saline for conventional agriculture or for human consumption and cannot be used for such purposes.

The seeds studied in this chapter of the thesis were all recollected from wild grown plants in Tunisia, North of Africa. Tunisia's climate is Mediterranean on the northern coast, with mild, rainy winters and hot, sunny summers, while it is semi-desert or desert in inland areas. None of the plants, except *Citrullus* [Giwa et al., 2010], has been studied previously with the aim of producing biofuels. Moreover, most of them produce non edible oils, which add to their advantages the absence of food competing problems.

Two different field campaigns were done, collecting small amounts of seeds of plants. For the sake of clarity, Section 6.1 will describe plants of the first campaign and Section 6.2 will describe plants of the second one.

In this part, seeds were collected from the bishop's weed (*Ammi visnaga* Lam), the bitter apple (*Citrullus colocynthis* Shard), the thorn apple or stramonium (*Datura stramonium*), the squirting cucumber (*Ecballium elaterium*) and the milk thistle (*Silybum marianum*). Later, oils were extracted and transformed into fatty acid methyl (FAME) and ethyl (FAEE) esters. Some of the biofuel properties were measured following the standard methods, and later, the estimated and measured properties were compared to check the

accuracy of the predictions. The final objective of this work was to assess the potential use of these five plants as biofuel sources.

***Ammi visnaga* (L.) Lam**

The bishop's weed, *Ammi visnaga* (L.) Lam. (see Figure 28) is a phanerogam herbaceous annual plant of the Apiaceae family. This plant is native from the South of Europe, Asia Minor and the North of Africa, although it has been also introduced in Central Europe, Mexico, Chile, Argentina and the East of India. It grows in ditches, road edges, fallows, stubble and altered soils with poor permeability up to 1500 m of altitude. The plant can reach from 80 to 150 cm height, lacks any hairs and presents a smooth and straight stem with small lines of different tones that grow over it. The flowering season starts in June and the recollection of the ripe fruits takes place in the summer. The fruit is a small ellipsoid of 2.0 to 2.5 cm.

Figure 28: *Ammi visnaga*

***Citrullus colocynthis* (L.) Shard**

The bitter apple (*Citrullus colocynthis* (L.) Shard, see Figure 29) is a climbing plant of the Cucurbitaceae family, native from the North of Africa, Nubia and Egypt but it has extended to all the Mediterranean area. The seeds are very nutritious but if the whole fruit is ingested, a resinous substance called colocynthine is released and it produces intestinal pain and inflammation. In Tunisia, the plant is found in Tozeus, Gafsa, Sfax, Kairouan, Degach, Metlaoui, Zeramdine, Médenine, Hamman-Lif, Sidi Makhoulouf and Souassi [Althawadi et al., 1986; Sen et al., 1974; Giwa et al., 2010].

Figure 29: *Citrullus colocynthis*

***Datura stramonium* (L.)**

The thorn apple or stramonium (*Datura stramonium* L, see Figure 30) is a poisonous Angiospermae plant from the Solanaceae family, which grows in all temperate parts of the world, and is able to adapt to all types of soils but growing better in humid soils with abundant nitrates. It is native from the deserts of Northwest of America, but also from South and Central America, Europe, Asia and Africa. It is an annual plant, which can reach 50-220 cm height. The flowering season ranges from May to November. The fruit is an oval capsule with more than 35 thorns that opens by four valves.

Figure 30: *Datura stramonium*

***Ecballium elaterium* (L.)**

The squirting cucumber (*Ecballium elaterium* L., see Figure 31) is a perennial herbaceous plant of the Cucurbitaceae family. This plant grows in the Mediterranean area, mainly in the South of Europe and Middle East. Specifically, it grows in the North of Tunisia, around Tunis, Zembra, Korba, Soliman, Djérissa and Zaghouan. Its main raising demands are mild temperatures, clay basic soils and abundance of nitrogen, and thus, it grows near

people and cattle. This plant is not especially tall and its height does not surpass 30 cm. The plant is covered by small hairs that allow it to survive in dry climates. The flowers are yellow and their flowering period range from May to September. The most distinguishing character is the fruit that during the ripening period swells up until the maximum hydrostatic pressure, when a light touch or the wind action explodes it expelling the dark seeds from the inside and reaching long distances (up to 3 m) thus favoring the colonization of new lands by the plant.

Figure 31: *Ecballium elaterium*

Silybum marianum

The milk thistle, *Silybum marianum* (see Figure 32), a wild annual plant of Compositae family is widely cultivated in China, Hungary, Argentina, Venezuela and Ecuador and naturally grows at mild climatic regions of different parts of Northern Africa, Southern Europe and Asia, particularly, Iraq. The medicinal and pharmaceutical aspects of the plant have been researched over the years [Wu et al., 2009]. The extract from these seeds for instance is used traditionally for treatment of hepatotoxicity and acute and chronic liver diseases [Wu et al., 2009; Doehmer et al., 2011]. Morazzoni and Bombardelli [Morazzoni et al., 1995] indicated that the pharmacologically active component of the extract (silymarin) is made of isomeric mixture of flavonolignans, silychristin, silydianin, diastereoisomers silybin and isosilybin. Current studies reveal that the seeds of the plant contain a lot of oil [Ghavami et al., 2008; Li et al., 2012]. While Ghavami and Ramin [Ghavami et al., 2008] reported the oil content of the seed to be more than 28 %, Li et al. [Li et al., 2012] even indicated it to be more than 45 %. Moreover, in silymarin industrial oil production, the oil is considered a byproduct.

Figure 32: *Silybum marianum*

6.1.2 EXPERIMENTAL SECTION

FAME and FAEE Production

Soxhlet extraction was carried out with medium boiling point petroleum ether as solvent during a period of 24 h [Llamas et al., 2012b]. Oils refining processes, like degumming and esterification of free fatty acids, and transesterification of triacylglycerides were carried out following previously published methods [Canoira et al., 2008]. The fatty acid profiles obtained have been determined by GC-FID after the standard method EN 14103 and by GC-MS when it was necessary to identify unknown compounds.

Measurement of selected properties

The density at 15 °C was measured in an automatic densimeter Digital DM48 (ASTM D1298; accuracy: $\pm 0.3 \text{ kg/m}^3$). The kinematic viscosity at 40 °C was determined in a viscosimeter Proton, using tubes Turbiscan model 9506 of Polyscience, immersed in a bath Tamson model TV 2000 (ASTM D445; accuracy: $\pm 0.0013 \cdot \text{mean, mm}^2/\text{s}$). The CHN content was determined in a Leco CHN-932 elemental analyzer (ASTM D5291; accuracy: $\pm 0.71 \text{ wt \%}$ for C, $\pm 0.18 \text{ wt \%}$ for H). The higher heating value (*HHV*) was determined in a Leco AC-300 bomb calorimeter, using benzoic acid tablets as the external standard (ASTM D240; accuracy: $\pm 0.25 \text{ MJ/kg}$). The lower heating value (*LHV*) was calculated from the *HHV* following the ASTM D240 modified for oxygenated fuels. The acidity index of the biodiesel was analyzed in an apparatus Methrom model 702 Titrino (EN ISO 14104; accuracy: $\pm 0.02 \text{ mg KOH/g}$). The lubricity was measured with a high frequency reciprocating rig (HFRR_PCS Instruments) following the procedure EN ISO 12156-1 (accuracy $\pm 70 \text{ }\mu\text{m}$).

The Crystallization Onset Temperature (COT) was determined as the temperature at which the heat release from crystallization starts, using a Q20 TA Differential Scanning Calorimeter (DSC) Instrument and following a previously reported method (Bolonio et al., 2015).

6.1.3 RESULTS AND DISCUSSION

The yields of the seeds extraction to produce oil were 7.2 wt % for *Ammi*, 25.3 wt % for *Citrullus*, 23.0 wt % for *Datura*, 37.7 wt % for *Ecballium* and 27.8 wt % for *Silybum*. Although these yields are low when compared with other non-edible biodiesel feedstock like *Jatropha*, where the extraction yield could reach around 45 wt % based on seeds, it should be noted that these are wild plants that grow in arid lands without much need of water and the huge availability of this type of land could compensate the low oil content of the seeds. Moreover, good agricultural practices could improve very much the oil content of the seeds.

Measured and estimated properties

The fatty acid profiles and the COTs, both used to estimate the biofuel properties, are shown in Tables 27 and 28 respectively. DSC is a thermo-analytical technique that has been proved very useful to study the cold flow behavior of biodiesel [Lee et al., 1995; Dunn, 1999], especially because it needs a very small sample (less than 10 mg) which is a great advantage when handling reduced amounts as it is the case in this study. Figure 33 shows the thermograms of FAME and FAEE from *Datura stramonium* and Table 28 shows the COTs obtained from the DSC analysis.

Table 29 reports the equations used to estimate the values of cloud point (CP), pour point (PP) and cold filter plugging point (CFPP) where U_{FAME} and U_{FAEE} are the total content of unsaturated acids (wt %), N_c is the weighted-average number of carbon atoms of each biodiesel and P_{FAME} is the wt % of palmitic.

Table 28 reports the estimated values of CP, PP and CFPP using this set of equations. The CFPP estimated value of -2.9 °C for *Silybum* FAEE matches very well the experimental value of -3 °C [Takase et al., 2014]. The FAME and FAEE induction time (IT) has been estimated with correlations obtained from the literature and reported in Table 29.

Table 27: Fatty acid profiles *Ammi visnaga*, *Citrullus colocynthis*, *Datura stramonium*, *Ecballium elaterium* and *Silybum marianum*^a

<i>Fatty acid</i>	<i>Ammi visnaga</i>	<i>Citrullus colocynthis</i>	<i>Datura stramonium</i>	<i>Ecballium elaterium</i>	<i>Silybum marianum</i>
<i>C</i> _{8:0}	0.21			0.02	0.05
<i>C</i> _{14:0}				0.12	0.08
<i>C</i> _{15:0}				0.02	0.02
<i>C</i> _{16:0}	4.58	8.84	13.77	8.45	9.44
<i>C</i> _{16:1}	0.21		0.46	0.06	0.07
<i>C</i> _{17:0}				0.07	0.11
<i>C</i> _{17:1}					0.06
<i>C</i> _{18:0}		8.60	2.44	5.75	7.11
<i>C</i> _{18:1}	76.10	12.31	25.67	25.57	25.48
<i>C</i> _{18:2}	15.60	70.25	57.30	48.85	49.26
<i>C</i> _{18:3}	0.34		0.37	0.11	0.19
<i>C</i> _{20:0}				0.18	3.95
<i>C</i> _{20:1}	0.13			0.17	0.92
<i>C</i> _{20:2}					0.04
<i>C</i> _{20:3}	1.36				0.03
<i>C</i> _{20:4}					0.45
<i>C</i> _{22:0}				4.84	2.66
<i>C</i> _{22:1}	0.15			4.99	0.03
<i>C</i> _{22:6}	1.31				
<i>C</i> _{24:1}					0.04
<i>C</i> _{18:1} (OH) ^b				0.80	
Fatty acid ester content (wt %)	79.32	98.32	99.7	88.61	97.63

^aAll fatty acid profiles have been normalized to 100 wt % ^bRicinoleic acid

Table 28: Crystallization onset temperature (COT) obtained from the thermograms, and estimated values of cloud point (CP), pour point (PP) and cold filter plugging point (CFPP) based on COT or ester profiles (first campaign)

			<i>Properties estimated from COT</i>						<i>Properties estimated from the ester profile</i>					
<i>Seed</i>	<i>COT (°C)</i>		<i>CP (°C)</i>		<i>PP (°C)</i>		<i>CFPP (°C)</i>		<i>CP (°C)</i>		<i>PP (°C)</i>		<i>CFPP (°C)</i>	
<i>Biodiesel</i>	<i>FAME</i>	<i>FAEE</i>	<i>FAME</i>	<i>FAEE</i>	<i>FAME</i>	<i>FAEE</i>	<i>FAME</i>	<i>FAEE</i>	<i>FAME</i>	<i>FAEE</i>	<i>FAME</i>	<i>FAEE</i>	<i>FAME</i>	<i>FAEE</i>
<i>Ammi visnaga</i>	-33.1	-	-30.9	-	-37.6	-	-26.8	-	-2.6	-7.7	-9.6	-12.8	-5.5	-14.4
<i>Citrullus colocynthis</i>	-0.7	-3.7	4.9	-1.5	3.0	-5.8	1.2	-6.1	-0.3	-2.5	-6.1	-5.7	-3.3	-7.6
<i>Datura stramonium</i>	-5.1	-7.3	-0.1	-4.9	-2.6	-10.8	-2.6	-10.3	-0.2	-3.2	-7.1	-6.7	-3.2	-8.5
<i>Ecballium elaterium</i>	-5.4	-5.1	-0.3	-2.8	-2.9	-7.7	-2.8	-7.7	-0.6	-2.1	-4.3	-5.2	-3.5	-7.0
<i>Silybum marianum</i>	10.8	8.6	17.5	10.1	17.3	11.4	11.1	8.6	-0.03	1.3	-2.3	-0.6	-3.0	-2.9

Table 29: Equations used for the estimation of properties of FAME and FAEE

Property	Equation		Reference	
	FAME	FAEE	FAME	FAEE
Cloud Point, °C	$CP = 5.5997 + 1.1014 \times COT$ $CP = 0.526 \times P_{FAME} - 4.992$ $0 < P_{FAME} < 45$ $CP = -0.576 \times U_{FAME} + 48.255$ $0 < U_{FAME} \leq 84$	$CP = 1.96 + 0.94 \times COT$ $CP = -81.62 - 0.45U_{FAEE} + 5.87N_c$	Dunn et al., 1999 Sarin et al., 2009	Bolonio et al., 2015
Pour Point, °C	$PP = 3.8151 + 1.2489 \times COT$ $PP = 0.571 \times P_{FAME} - 12.24;$ $0 < P_{FAME} < 45$ $PP = -0.626 \times U_{FAME} + 45.594;$ $0 < U_{FAME} \leq 84$	$PP = -0.62 + 1.40COT$ $PP = -125.04 - 0.62U_{FAEE} + 8.61N_c$	Dunn et al., 1999 Sarin et al., 2009	Bolonio et al., 2015
Cold Filter Plugging Point, CFPP, °C	$CFPP = 1.7914 + 0.8629 \times COT$ $CFPP = 0.511 \times P_{FAME} - 7.823;$ $0 < P_{FAME} < 45$ $CFPP = -0.561 \times U_{FAME} + 43.967$ $0 < U_{FAME} \leq 84$	$CFPP = -1.7 + 1.19 \times COT$ $CFPP = -103.47 - 0.59U_{FAEE} + 7.30N_c$	Dunn et al., 1999 Sarin et al., 2009	Bolonio et al., 2015
Oxidation stability, h	$IT = 0.214 \times P_{FAME} + 0.671;$ $0 < P_{FAME} < 45$ $IT = -0.234 \times U_{FAME} + 22.318;$ $0 < U_{FAME} \leq 84$	$IT = 9.50 - 0.03MU_{FAEE} - 0.14BAPE$ $IT = 4.45 - 0.04 \times BAPE$	Sarin et al., 2010b	Bolonio et al., 2015

Cetane number	$CN_{FAME} = -23.523 + (2.366 + 6.299e^{-0.411db}) \cdot ne^{-0.018n}$ $CN = \sum x_{FAME} \times CN_{FAME}$	$CN_{FAEE} = -25.192 + (-3.565 + 14.818e^{-0.185db}) \cdot ne^{-0.032n}$ $CN = \sum x_{FAEE} \cdot CN_{FAEE}$	Lapuerta et al., 2009
^a Density, kg/m ³	$\rho_{FAE} = 851.471 + \frac{250.718 \cdot db + 280.899 - 92.182(m-1)}{1.214+n}$, with $m=1$ for FAME and $m=2$ for FAEE $\frac{1}{\rho} = \sum (x_{FAE} / \rho_{FAE})$		Lapuerta et al., 2010b
Kinematic viscosity at 40 °C, mm ² /s	$lnv_{FAME} = -12.503 + 2.496 \cdot lnMW_{FAME} - 0.178 \cdot db$ $lnv = \sum x_{FAME} \times lnv_{FAME}$	$\eta_{FAEE} = (1.16 \times 10^{-4} MW_{FAEE}^2 - 0.026 MW_{FAEE} + 2.28) \frac{0.147db^2 - 1.09db + 4.82}{4.82}$ $v_{FAEE} = (\eta_{FAEE} \times 10^3) / \rho_{FAEE}$ $lnv = \sum x_{FAEE} \times lnv_{FAEE}$	Ramírez-Verduzco et al., 2012
HHV, MJ/kg	$HHV = \frac{\Delta H_{f(g)} - m \times \Delta H_{fCO_2(g)} - p/2 \times \Delta H_{fH_2O(g)}}{W} - \Delta H_{vap}$		Lapuerta et al., 2010a
LHV, MJ/kg	$LHV = \frac{HHV - 213.65 \times H - 0.77 \times O}{1000}$		Llamas et al., 2013
OESI	$OESI = a \frac{N+m/4-p/2}{SP} + b$ for a molecular formula C _N H _m O _p		Barrientos et al., 2013
Lubricity, wear scar, μm	$WS1.4 = 381.54 - 8.17n + 49.05db - 66.79(m - 1)$		Lapuerta et al., 2016

^aFAE: Fatty acid ester

Figure 33: Thermograms for FAEE and FAME from *Datura stramonium*, obtained with DSC

Table 30 reports the estimated values of the IT based on those equations. The reported IT for *Silybum* FAEE is 2.1 h [Takase et al., 2014], in excellent agreement with this work estimation of 1.8 h. In general, the esters used in this work are expected to have poor IT and would need some amount of additives. The cetane number for pure FAME and FAEE could be estimated from the equations in Table 29 and the additivity rule [Lapuerta et al., 2009], where CN_{FAME} and CN_{FAEE} are the cetane number of each pure methyl or ethyl ester, db is the number of double bonds, n is the number of carbon atoms of the fatty acid, x_{FAME} and x_{FAEE} are the mass fractions of each methyl and ethyl ester and CN is the cetane number of biodiesel. Table 30 reports the estimated CN values, all higher than 51.0, thus fulfilling the EN 14214 standard. The reported CN for *Silybum* FAEE is 52 [Takase et al., 2014], somewhat apart from the estimated value of 58.4. Density could be estimated with the equations reported in Table 29 and the inverse additivity rule [Lapuerta et al., 2010b], where ρ_i is the density of each pure methyl or ethyl ester, n is the number of carbon atoms of the fatty acid and m is the number of the carbon atoms of the alcohol used for the transesterification.

Table 30 reports the density results which fullfill the EN 14214 standard with the exception of *Datura* FAME, 900.4 kg/m³. The kinematic viscosity at 40 °C have been estimated using the equations included in Table 29 (row 7), where ν is the kinematic viscosity of the blend, ν_{FAME} and ν_{FAEE} the kinematic viscosity of the pure FAME or FAEE respectively, MW_{FAME} and MW_{FAEE} are the molecular weight of pure FAME or FAEE respectively and η is the dynamic viscosity.

Table 30: Estimation of other properties based on the ester profiles (first campaign)

<i>Seed Biodiesel</i>	<i>Induction time (h)</i>		<i>Cetane number</i>		<i>Kinematic viscosity at 40 °C (mm²/s)</i>		<i>Density at 15°C (kg/m³)</i>		<i>LHV (MJ/kg)</i>		<i>OESI</i>		<i>Lubricity (μm)</i>	
	<i>FAME</i>	<i>FAEE</i>	<i>FAME</i>	<i>FAEE</i>	<i>FAME</i>	<i>FAEE</i>	<i>FAME</i>	<i>FAEE</i>	<i>FAME</i>	<i>FAEE</i>	<i>FAME</i>	<i>FAEE</i>	<i>FAME</i>	<i>FAEE</i>
<i>Ammi visnaga</i>	1.7	4.9	58.6	59.3	4.30	4.80	881.6	876.8	37.39	37.63	8.02	9.29	285.3	210.3
<i>Citrullus colocynthis</i>	3.0	1.6	52.8	52.5	3.96	4.36	886.1	881.2	37.30	37.55	8.33	9.59	302.3	227.4
<i>Datura stramonium</i>	3.6	0.6	54.3	54.4	3.99	4.40	884.7	879.9	37.30	37.54	8.14	9.41	297.6	222.6
<i>Ecballium elaterium</i>	2.5	1.9	58.6	58.2	4.38	4.73	883.1	878.3	37.42	37.66	8.25	9.52	289.0	214.0
<i>Silybum marianum</i>	2.7	1.8	58.8	58.4	4.27	4.71	882.6	877.8	37.39	37.63	8.14	9.41	289.2	214.3

All the biodiesel viscosities would meet the EN 14214 specification as reported in Table 30, with the exception of *Ecballium* FAME with 5.24 mm²/s. The reported kinematic viscosity at 40 °C for *Silybum* FAEE is 4.73 mm²/s [Takase et al., 2014], in excellent agreement with the estimated value of 4.71 mm²/s.

The *HHV* and *LHV* have been estimated according to equations included in Table 29 (rows 8 and 9) where $\Delta H_{f(g)}$ and ΔH_{vap} are respectively the enthalpies of formation and vaporisation of the fuel which could be estimated by the groups contribution method, and *H* and *O* are the wt % of hydrogen and oxygen respectively from the elemental analysis of the biodiesel. The values are reported in Table 30, showing for all the biodiesels very similar figures around 37 MJ/kg. The Oxygen Extended Sooting Index (*OESI*) was recently proposed as a prediction tool for soot formation. The values of *OESI* for these FAME and FAEE are estimated and summarized in Table 30, showing all very close values around 8 for FAMES and around 9 for FAEEs, meaning that all of them have substantial potential for soot formation suppression with respect to diesel fuel, which has a value around 52 [Barrientos et al., 2013]. The lubricity values are estimated using the equation proposed in Table 29 (row 11). All of them (Table 30) are well below the limit (460 µm) so the FAMES or FAEEs studied could potentially be used to improve the lubricity of ultra-low sulfur diesel fuels.

Comparison of measured and estimated properties

Table 31 shows the results of some selected measured properties of the FAME studied in this work, and their comparison with the corresponding estimated values. The relative errors (% Δ) is calculated as the difference between the estimated and measured property values, divided by the measured value.

For the density at 15 °C, the relative error ranges from -0.01 % to -1.74 %, that is, the estimation could be considered as excellent. The *HHV*, *LHV* and the elemental composition also show very low relative errors. The error is, as expected, always lower for % C (from 0.47 % for *Ecballium* to 2.21 % for *Datura*) and higher for % H (from 6.81 % for *Ecballium* to 8.72 % for *Datura*), since the accuracy of the experimental % H measurement is lower. This good prediction of the elemental composition from the ester profile is an important support of this work, since it means that the ester profiles that are at the basis of most of the estimated properties, have been very accurately determined.

Table 31: Comparison of selected measured and estimated properties of FAME

	<i>Citrullus Colocynthis</i>			<i>Datura stramonium</i>			<i>Ecballium elaterium</i>		
	<i>Measured</i>	<i>Estimated</i>	<i>Δ(%)</i>	<i>Measured</i>	<i>Estimated</i>	<i>Δ(%)</i>	<i>Measured</i>	<i>Estimated</i>	<i>Δ(%)</i>
Density at 15°C (kg/m³)	886.2	886.1	-0.01	900.4	884.7	-1.74	891.7	883.1	-0.96
Kinematic viscosity at 40 °C (mm²/s)	4.13	3.96	-4.12	4.68	3.99	-14.74	5.24	4.38	-16.41
HHV (MJ/kg)	39.66	39.85	+0.47	39.35	39.86	+1.30	39.81	40.00	+0.48
LHV (MJ/kg)	37.40	37.30	-0.27	37.08	37.30	+0.59	37.42	37.41	-0.03
TAN (mg KOH/g)	0.32	-	-	0.48	-	-	0.34	-	-
C wt %	76.37	77.16	+1.03	75.39	77.06	+2.21	76.79	77.15	+0.47
H wt %	11.00	11.9	+8.20	11.00	11.96	+8.72	11.30	12.07	+6.81

The most significant deviations are found in the kinematic viscosity. The explanation lies in the effect that small amounts of non-FAME or FAEE polar compounds have on this property [Knothe et al., 2007]. *Ecballium* great deviation could arise from the presence in the biodiesel of small amounts of asperentin (3.14 wt %), squalene (0.30 wt %), 2-dodecen-1-yl succinic anhydride (0.38 wt %) and non-typical FAME like 9-oxo-nonanoic acid methyl ester (0.10 wt %) and ricinoleic acid methyl ester (0.71 wt %) that have been detected by GC-MS. due to the fact that the polar non-fatty compounds always increase the viscosity [Knothe et al., 2007]. The results for *Ammi* have not been included in Table 31 because in this case the low total ester content of 79.32 wt % and the presence of small amounts of non-fatty ester compounds detected by GC-MS affect deeply the measured properties: seseline (0.36 wt %), visnagin (1.39 wt %), β -mircene (0.62 wt %), δ -3-carene (5.11 wt %), khellin (1.03 wt %), 7-methylbenz[c]acridine (0.69 wt %), 8-methyl-2-oxo-2H, 8H-benzo[1,2-b:3,4-b']dipyrro-9,10-diyl-10-acetate-9-(2-methylbutyrate) (1.65 wt %) and 6-acetyl-3,5-dimethoxy-7-methylnaphthoquinone (1.72 wt %). Table 32 shows the results of some selected properties of FAME and FAEE of *Silybum* that have been reported in the literature [Takase et al., 2014] and their comparison with the properties estimated in this work. *Silybum* is the only seed that has been extensively studied. In general terms, the agreement between the measured values and their estimation is good.

The FAEE kinematic viscosity shows a relative error of only -0.42 % (for FAME is also acceptable with -4.26 % error) probably because *Silybum* has very high total ester content of 97.63 wt % and thus, the effect of non-fatty ester compounds is negligible.

Table 32: Comparison of selected measured and estimated properties of FAME and FAEE of *Silybum marianum*

	<i>FAME</i>				<i>FAEE</i>			
	<i>Measured</i>	<i>Estimated</i>	Δ	$\Delta(\%)$	<i>Measured</i>	<i>Estimated</i>	Δ	$\Delta(\%)$
<i>Cetane number</i>	51	58.8	+7.8	+15.3	52	58.4	+6.4	+12.3
<i>Kinematic viscosity at 40 °C (mm²/s)</i>	4.46	4.27	-0.19	-4.26	4.73	4.71	-0.02	-0.42
<i>Induction time (h)</i>	2.1	2.7	+0.6	+28.6	2.1	1.8	-0.30	-14.3
<i>Cloud Point, °C</i>	-1	-0.03	+0.97	-	-2	1.34	+3.3	-
<i>Pour point, °C</i>	-1	-2.3	-1.3	-	-3	-0.6	+2.4	-
<i>CFPP, °C</i>	-2	-3.0	-1	-	-3	-2.9	+0.1	-

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6.2 FATTY ACID METHYL ESTERS (FAME) FROM OLEAGINOUS SEEDS GROWN IN ARID LANDS: *IBICELLA LUTEA*, *ONOPORDUM NERVOSUM*, *PEGANUM HARMALA*, *SMYRNIUM OLUSATRUM* AND *SOLANUM ELAEAGNIFOLIUM*

6.2.1 CONTEXT

This part of the thesis studies the properties of biodiesel of fatty acid methyl esters (FAMEs) obtained from seed oils of plants grew in arid and non-arable lands in Tunisia, North of Africa. Most of the oils are non-edible, diminishing the option of food competing problems. The methodology followed has been reported in the previous Section and by Houachri et al. [Houachri et al., 2018].

Ibicella lutea

The devil horn *Ibicella lutea* (see Figure 34) is named after the form of its seeds pod, with a double horn, which adheres to the skin of animals that rub the plant, acting as a mean of biological dispersion. The plant belongs to the family of the Martyniaceae and it is native from South America, although it has expanded to other arid regions of the world, since it grows in dry and desert climatic conditions.

Figure 34: *Ibicella lutea*

Onopordum nervosum arabicum

The cotton thistle *Onopordum nervosum arabicum* (see Figure 35) is a plant from the Asteraceae family, endemic from the Iberian Peninsula, although it has expanded to other Mediterranean regions and it has also been introduced in the South of England. The plant grows at roadsides and lands left to fallow, in basic soils more or less nitrified and

eventually siliceous, at heights between sea level and 1500 m. The seeds have a very peculiar morphology.

Figure 35: *Onoropordum nervosum*

Peganum harmala

The Sirian rue *Peganum harmala* (see Figure 36) is a plant that belongs to the Nitraceae family, native from the Mediterranean area and South East Asia. The plant grows in brackish (salty) zones and gypsum marls (loams), reaching only 0.3-0.8 m height, but its roots can penetrate 6 m deep if the soil is extremely dry. The flowering season is from May to June and the fruit is a globe divided into three cameras, which holds more than 50 seeds.

Figure 36: *Peganum harmala*

Smyrniium olusatrum

The horse celery *Smyrniium olusatrum* (see Figure 37) is a plant that belongs to Apiaceae family. Although it is native from the Mediterranean area, it has expanded to Northern regions. The plant can reach 1.20-1.50 m height, and it grows in pathways around cliffs, being the first yearly coastal vegetation. The flowers are yellow-green and the fruits are black.

Figure 37: *Smyrniium olusatrum*

Solanum elaeagnifolium

The silverleaf nightshade or *Solanum elaeagnifolium* (see Figure 38) is a plant from the Solanaceae family, native from South and Central America, although it has extended all over the world. It grows in habitats altered by man, like roadsides, fallow lands and urban areas. The plant is perennial, reaching 0.2-0.5 m height and it is extremely toxic to cattle and humans. The seeds are planar discs with a brownish tone.

Figure 38: *Solanum elaeagnifolium*

6.2.2 EXPERIMENTAL SECTION

FAMES production

Seed oils were obtained following the Soxhlet method, extracting the dry seeds with medium boiling point petroleum ether as solvent during a period of 24 h [Llamas et al., 2012a]. Extracted oils were refined and transformed into esters following a previously published method [Canoira et al., 2008]: First, oils were degummed to remove the phosphoglycerides or gums, then free fatty acids were esterified and finally triacylglycerides were transesterified into fatty acid esters. The progress of the transesterification reaction was followed by thin layer chromatography (TLC) on silica-gel plates using a mobile phase composed of light petroleum ether (90 vol %), diethyl ether (9 vol %) and acetic acid (1 vol %). All reagents and solvents were of synthesis grade and were used without previous purification.

Determination of ester profiles and crystallization onset temperatures (COTs)

The quantification of the percentage of the different fatty acids in the oils was carried out by a HP-5890 series II gas chromatograph equipped with flame ionization detector (FID) and split/splitless injector. The samples were run through an HP-Wax column (30 m $\times$ 0.32 mm i.d. $\times$ 0.15 μ m) of crosslinked polyethylene glycol following these analytical conditions: injector temperature, 210 $^{\circ}$ C; FID temperature, 240 $^{\circ}$ C; split ratio, 70:1; injection volume, 1 μ L; constant flow rate mode (He) 1 mL/min; H₂ flow rate: 40 mL/min; air flow rate: 400 mL/min; oven program, start 9 min at 200 $^{\circ}$ C, heat at 20 $^{\circ}$ C/min up to 230 $^{\circ}$ C and hold 10 min. Each sample was prepared weighting 250 mg in a 10 mL vial with 5 mL of methyl heptadecanoate solution (5 mg of methyl heptadecanoate per milliliter of n-heptane). All chromatograms were repeated at least three times to test their repeatability and the ester profiles shown in the tables represent the mean of the measurements.

The identification of the different fatty acids in the samples was carried out in an Agilent 6890 series II gas chromatograph equipped with a mass selective detector (MSD5973) and splits/splitless injector. The samples were run through a J&W 122-5532 column (30 m $\times$ 0.25 mm i.d. $\times$ 0.25 μ m) following these analytical conditions: injector temperature, 250 $^{\circ}$ C; split ratio: 50:1; injection volume, 1 μ L; constant flow rate mode (He) 1 mL/min; oven program: start 9 min at 50 $^{\circ}$ C, heat at 10 $^{\circ}$ C/min up to 310 $^{\circ}$ C and hold 10 min. Each sample was prepared weighting 250 mg of sample in a 10 mL vial with 5 mL of methyl

nonadecanoate solution (5 mg of methyl nonadecanoate per milliliter of toluene). Conditions of the mass detector were: electronic impact: 70 eV; m/z scan from 50 to 800 Da; resolution 1000. The chromatograms and mass spectrum of compounds were analysed by the MSD ChemStation Data Analysis Application software of Agilent and the spectral library Wiley 275.

The COT was determined as the temperature at which the heat release from crystallization starts, using a Q20 TA differential scanning calorimeter (DSC) instrument. The procedure consisted of adding a small amount of each of the studied esters (around 8 mg) to a 40 μ L sealed vial under a nitrogen flow rate of 40 mL/min. Then the samples were carried through a heating-cooling cycle: heat to 70 °C, hold 5 min, cool from 70 to -80 °C other 5 min, heat again to 70 °C, reaching the starting point. The rate of temperature was chosen as 5 °C/min following previous studies in FAMEs which reported a good combination of resolution characteristics and timeliness [Dunn, 1999; Lee et al., 1995. Giraldo et al., 2013]. The process was repeated twice to test an appropriate repeatability.

6.2.3 RESULTS AND DISCUSSION

Oil extraction

The oil yields of the seeds obtained by Soxhlet extraction were 24.4 wt % for *Ibicella*, 30.1 wt % for *Onopordum*, 10.5 wt % for *Peganum*, 23.4 wt % for *Smyrnum* and 8.7 wt % for *Solanum*. When comparing the oil yields, it should be taken into account that the wild plants studied in this work grow in arid lands with the need of minimum amount of water. Moreover, domestication of these plants and good agricultural practices could substantially improve the oil content of the seeds and the overall production. As an example, the world average soybean yield has been increasing by about 60 % in 30 years from 1980 (1.6 t ha⁻¹) to 2010 (2.6 t ha⁻¹) [Ohyama et al., 2013].

Fatty acid profiles

Table 33 shows the fatty acid ester profiles obtained in this work after the identification and quantification of the different compounds determined by GC-MS and GC-FID respectively. It is noteworthy the low total ester content of the oils of *Peganum* and *Smyrnum* (only 63.13 and 60.63 wt % respectively) which means that a great part of the seed extract is not triacylglycerides; also the high oleic acid content of the oils of *Ibicella* and *Smyrnum* (52.36 and 74.14 wt % respectively) with lesser amount but still important

of linoleic acid (35.88 wt %) for *Ibicella*. High total ester contents and very similar esters profiles can be observed for *Onopordum* and *Solanum*, with a very high content of linoleic acid (more than 60 wt %) and lower amount of oleic acid (around 20 wt %) and saturated acids (palmitic, around 9 wt % and stearic, around 4 wt %). *Peganum* and *Solanum* present some not negligible amounts of linolenic acid (2.44 and 1.07 wt % respectively) that could affect the oxidation stability properties. The saturated fraction shows some differences: 1) Palmitic acid contents are similar for three oils, *Ibicella*, *Onopordum* and *Solanum* (around 9 wt %) which would affect deeply the cold flow behavior of their FAMES, with lesser amounts for *Peganum* and *Smyrniun*; 2) Stearic acid contents are low in general.

Table 33: Fatty acid profiles of *Ibicella lutea*, *Onopordum nervosum*, *Peganum harmala*, *Smyrniun olusatrum* and *Solanum elaeagnifolium*¹

<i>Fatty acid</i>	<i>Ibicella lutea</i>	<i>Onopordum nervosum</i>	<i>Peganum harmala</i>	<i>Smyrniun olusatrum</i>	<i>Solanum elaeagnifolium</i>
<i>C</i> _{10:0}				2.36	
<i>C</i> _{14:0}				0.16	
<i>C</i> _{15:0}				0.07	
<i>C</i> _{16:0}	9.10	9.08	4.02	5.26	9.86
<i>C</i> _{16:1}	0.33			0.26	0.59
<i>C</i> _{18:0}	2.33	3.56	2.57	1.07	4.24
<i>C</i> _{18:1}	52.36	27.02	26.93	74.14	20.92
<i>C</i> _{18:2}	35.88	60.34	53.62	14.1	63.32
<i>C</i> _{18:3}			2.44	0.48	1.07
<i>C</i> _{20:0}			1.27	0.25	
<i>C</i> _{20:1}				0.08	
<i>C</i> _{20:2}				0.26	
<i>C</i> _{20:3}				0.08	
<i>C</i> _{20:4}				0.05	
<i>C</i> _{22:0}				0.08	
<i>C</i> _{22:1}				1.07	
<i>C</i> _{22:6}			9.15	0.26	
Fatty acid ester content (wt %)	97.9	99.6	63.13	60.63	97.9

¹ All fatty acid profiles have been normalized to 100 wt %

Thermograms

Figure 39 shows the thermogram obtained with the analysis of the FAME of *Onopordum* where COT is 8.39 °C. The already explained exothermic peak is clearly visible in the

upper part of the curve, with an almost symmetric endothermic peak in the heating cycle corresponding to the melting point. This peak is always placed at a higher temperature than the COT (in the case of *Onopordum* is 14.1 °C), that is, there is always a hysteresis where the sample tends to remain in the solid state during the heating process.

Figure 39: Thermogram for FAME from *Onopordum nervosum*, obtained with DSC

Table 34 shows the COT values obtained from the thermograms for the FAMEs studied in this work. In the thermogram for *Smyrniium*, the exothermic peak is not visible above -26.9 °C, since a totally flat line appears between 70 °C and -26.9 °C, where the first exothermic peak appears. However, the -26.9 °C could not be considered as the COT but as the freezing point of the oleate C18:1, which *Smyrniium* has in a big amount of 74.14 wt %. This thermogram could be due to the low total ester content of *Smyrniium* and that it has the lowest amount of stearate C18:0, only 1.07 wt %. From the above explanation, the COT values reported in Table 34 are significant for all FAMEs except for *Smyrniium*.

Estimation of FAME properties

The cold flow points (CP, PP and CFPP) can be estimated from the composition or fatty acid profile, in this work determined by GC-FID and/or GC-MS, and from the COT obtained from the DSC thermograms. This measurement consists of a direct test of the samples cooling cycle and gives a most representative value of the cold flow performance.

Table 34: Crystallization onset temperature (COT) obtained from the thermograms, and estimated values of cloud point (CP), pour point (PP) and cold filter plugging point (CFPP) based on COT and ester profiles (second campaign)

FAME	COT (°C)	Properties estimated from COT			Properties estimated from the ester profile		
		CP (°C)	PP (°C)	CFPP (°C)	CP (°C)	PP (°C)	CFPP (°C)
<i>Ibicella lutea</i>	-0.5	5.0	3.2	1.3	-0.2	-7.0	-3.2
<i>Onopordum nervosum</i>	8.4	14.8	14.3	9.0	-0.2	-7.1	-3.2
<i>Peganum harmala</i>	3.2	9.1	7.8	4.5	-2.9	-9.9	-5.8
<i>Smyrniun olusatrum</i>	-26.9	-24.0	-29.7	-21.8	-2.2	-9.2	-5.1
<i>Solanum elaeagnifolium</i>	-5.5	-0.5	-3.1	-3.0	0.2	-6.6	-2.9

The equations used to estimate the values of CP, PP and CFPP using the COT or the fatty acid profiles have been reported previously [Houachri et al., 2018]. These equations were obtained from the literature, where authors correlate the ester profile [Sarin et al., 2009] and the COT [Dunn, 1999] with the CP, PP and CFPP. Table 34 reports the estimated values of CP, PP and CFPP using this set of equations. In general, the cold flow behavior of these FAMES is poor, since none of them fulfill the CFPP specification for temperate climates in winter and only *Solanum* would fulfil the summer specification, according to the estimation from COT values.

The induction time (IT), which represents the oxidation stability, has been estimated with correlations obtained from the literature [Sarin et al., 2010b] and reported previously [Houachri et al., 2018]. Table 35 reports the estimated values of the IT. In general, the esters used in this work are expected to have poor IT because of their high content in linoleic fatty acid and would therefore need some additivation. In the case of *Peganum harmala*, the IT estimated differs significantly with the IT reported by Chang et al. [Chang

et al., 2017] of 20.9 h. This could be due to the content of antioxidant compounds, which are not considered in the estimated equations. In fact, as it will be described later, *Peganum* oil has 3.20 wt % of δ -tocopherol, a natural antioxidant which has been reported to improve the oxidation stability of biodiesel [Dunn, 2005].

The cetane number (CN) has been estimated using the equations previously reported [Houachri et al., 2018], which first estimate the CN of pure FAMES and then the CN of the samples through the additivity rule [Lapuerta et al., 2009]. Table 35 reports the estimated CN values for all the biodiesels studied and all except *Peganum* are higher than 51.0, thus fulfilling the EN 14214 standard.

The density has been estimated with the equations reported previously [Houachri et al., 2018] and the inverse additivity rule [Lapuerta et al., 2010b]. In this case, all the biodiesels included in this study fulfill the EN 14214 standard since the densities fall within the range of 860 and 900 kg m⁻³ as it is shown in Table 35.

The kinematic viscosity at 40 °C has been estimated using the equations reported previously [Ramírez-Verduzco et al., 2012; Houachri et al., 2018]. These equations can evaluate the kinematic viscosity of any blend [Allen et al., 1999; Llamas et al., 2012a; Llamas et al., 2012b]. All the biodiesels kinematic viscosities, reported in Table 35, meet the EN 14214 specification (3.5-5.0 mm² s⁻¹).

The higher and lower heating values (HHV and LHV) have been estimated according to equations reported previously [Lapuerta et al., 2010a; Llamas et al., 2013; Houachri et al., 2018]. The enthalpy values can in turn be estimated from the ester profile and the Benson group contribution method [Cohen et al., 1993]. Results are reported in Table 35, showing very similar LHV figures in all the biodiesels, around 37 MJ/kg.

Oxygen Extended Sooting Index (*OESI*), which was recently proposed as a soot formation predictor [Barrientos et al., 2013], has been estimated using the previous reference and the results are summarized in Table 35, showing that all samples show very close values around 8, meaning that they have substantial potential for soot formation suppression with respect to diesel fuel, which has a value around 52.

Table 35: Estimation of properties based on the ester profiles (second campaign)

<i>FAME</i>	<i>Induction time (h)</i>	<i>Cetane number</i>	<i>Kinematic viscosity at 40 °C (mm²/s)</i>	<i>Density at 15°C (kg/m³)</i>	<i>HHV¹ (MJ/kg)</i>	<i>LHV² (MJ/kg)</i>	<i>OESI³</i>	<i>Lubricity (μm)</i>
<i>Ibicella lutea</i>	2.6	57.4	4.19	882.4	39.94	37.35	8.01	288.6
<i>Onopordum nervosum</i>	2.6	53.3	4.00	885.4	39.87	37.31	8.26	299.9
<i>Peganum harmala</i>	1.5	49.1	3.85	890.1	39.89	37.38	8.98	317.9
<i>Smyrniium olusatrum</i>	1.8	60.0	4.29	880.3	39.94	37.33	7.72	281.1
<i>Solanum elaeagnifolium</i>	2.8	52.7	3.95	885.9	39.85	37.30	8.29	301.8

¹HHV: Higher heating value

²LHV: Lower heating value

³OESI: Oxygen extended sooting index

The lubricity has been estimated using the equation reported previously [Lapuerta et al., 2016; Houachri et al., 2018] and the values of the wear scar are shown Table 35. All the values are below the limit so the FAMEs studied could be used as lubricity enhancers.

The accuracy of this properties estimation has been checked previously by the authors comparing the properties estimated and measured for FAME of *Silybum marianum*, also obtained from seeds of this plant grown wild in Tunisia [Houachri et al., 2018].

Analysis of not-fatty compounds by GC-MS

The low total ester content of *Peganum* (63.13 wt %) due to the presence of some amounts of non-fatty ester compounds detected by GC-MS could affect deeply the quality of the properties estimation: 6-methoxy-1-methyl- β -carboline (0.20 wt %), 9,17-octadecadienal (34.30 wt %), 9,12-octadecadien-1-ol (2.15 wt %) and δ -tocopherol (3.20 wt %) have been unambiguously identified and quantified by GC-MS. In the case of *Smyrniun*, the quality of its properties estimation could also be negatively affected by the presence of some amounts of non-fatty compounds that have been detected and quantified by GC-MS: sabinene (0.05 wt %), germacrene D (1.32 wt %), 3,6-dimethyl-5-(1-methylethenyl)-6-ethenyl-4,7-dihydrobenzofuran (5.18 wt %), germacrene B (0.93 wt %), 11-methylanthra[2,1-b]furan (2.09 wt %), 7,14-dimethylene-7,14-dihydro-syn-1,6:8,13-bismethano[14]annulene (14.1 wt %), alexandrofuran (6.16 wt %), 17,18-dehydro-D-noreburnamonine (0.45 wt %), methyl 8-methoxybenzyl(1,2-b:5.4-b)difuran-2-carboxylate (3.34 wt %) and 2-formyl-4-phenyl-6-aminoquinoline (4.87 wt %).

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7. BIODIESEL FROM WASTE FEEDSTOCKS

7.1 FATTY ACID ETHYL ESTERS (FAEES) OBTAINED FROM GRAPE SEED OIL

7.1.1 CONTEXT

Although biofuels cannot currently compete with fossil fuels in terms of prices and availability, the rising levels of carbon in the Earth atmosphere [Hogue, 2015] are enough worrying to pursue research into new feedstocks for biofuels. Trying to avoid competence also with food crops and to use fertile lands, the search of residual feedstocks for producing biofuels remains a challenging objective.

The grape seeds from the plant *Vitis vinifera* of the *Vitaceae* family are a by-product of the wine industry. Spain amounts for 1.2 Mha of vineyards, the biggest extension of this type of cultivation in the world, with around half of this land (48.8 %) in the central region of Castilla-La Mancha. However, the oil extraction yields from the grape seeds are generally low and depend a lot on the grape variety, since there are almost 600 different types of vines [Pardo et al., 2009]. This oil has a very limited use in the cosmetic and food sectors. Such a limited demand of grape seed oil together with the huge wine production, especially in Spain, has as a consequence an enormous surplus of this residual product. On the other hand, the average Spanish wine production during the period 2013-2015 was 44.52 MhL/year, which exceeds the consumption and results in a 3.5 vol % wine excess that is derived to the production of bioethanol by distillation.

With the aim to use both residual feedstocks from the wine industry (grape seed oil and ethanol surplus), the production of fatty acid ethyl esters (FAEE) and not methyl esters (FAME) has been studied in this work. Moreover, other by-products of the wine industry like the grape skins (7 wt %) and the stalks (5 wt %) are also used to produce bioethanol by distillation. The production of FAEEs from grape seed oil and bioethanol from the wine surplus / skins / stalks distillation would thus result in a totally renewable biofuel with possible industrial outputs in Spain and other wine production countries. The production and characterization of FAME from grape seed oil has been studied previously [Fernández et al., 2010] but, to the best of our knowledge, the production and characterization of grape seeds FAEE has been reported in the literature only marginally [Ceriani et al., 2008; Freitas et al., 2008; Mohammadi et al., 2015].

Figure 40 shows a scheme of the wine production process integrated with the biodiesel production process proposed in this work. The grape seeds oil studied in this work has also nutraceutical components like resveratrol and melatonin [Meng et al., 2017] that would thus fulfil the biorefinery concept, that is, a refinery that produces not only biofuels but also other great added value compounds like monomers and nutraceuticals.

In this work, the most relevant properties of this grape seed FAEE have been measured and estimated, since properties estimation methods based on the ester profiles have become a very useful tool in recent years to predict biodiesel performance without the need of producing great biodiesel amounts [Sajjadi et al., 2016].

Figure 40: Production of grape seed FAEE integrated with wine production

7.1.2 EXPERIMENTAL SECTION

Materials

Grape seeds of the red wine variety *Tempranillo* were obtained from the Madrideojos (Toledo, Spain) cooperative wine cellar and were stored wet until extraction. Absolute ethanol 200 proof was purchased from Scharlau. Sodium metal, citric acid, sodium chloride and 4 Å molecular sieve were of commercial grade and were also bought from Scharlau. Medium boiling point petroleum ether was purchased also from Scharlau. Thin layer chromatography (TLC) plates of silica gel 60 were bought from Macherey-Nagel GmbH, Düren, Germany.

Seeds extraction

The grape seeds were dried in air at room temperature for three days just before extraction. Afterwards, the seeds (2 kg) were grounded in a mortar and introduced in a Soxhlet extractor with medium boiling point petroleum ether for 8 h. Later the solvent was evaporated at reduced pressure in a rotavapor to yield 128 g (6.41 %) of a yellowish clear oil.

Pretreatments on oil

Grape seed oil (128 g) was degummed by treatment with 60 wt % orthophosphoric acid (2 wt % of the oil), heating the mixture at 95 °C under stirring at 500 rpm for 30 min. Afterwards, the gums were separated by centrifugation at 3000 rpm to yield 121 g (94.6 %) of grape seed degummed oil.

The free fatty acids (FFA) content of the grape seed oil was 1.5 mg KOH /g making unnecessary an esterification process previous to the transesterification [Canoira et al., 2008].

Transesterification

The grape seed oil (120 g) was transesterified in a 2 L stirred tank reactor with absolute ethanol, using a molar ratio 6:1 of ethanol to oil and 2 wt % of sodium ethoxide as catalyst (freshly prepared from metallic sodium and absolute ethanol). The reaction mixture was stirred at 600 rpm and 70 °C during 3 h. The evolution of the transesterification was controlled by thin layer chromatography (TLC) on silica gel 60 plates using medium boiling point petroleum ether (90 vol %), diethyl ether (9 vol %) and glacial acetic acid (1 vol %) as the mobile phase. After these 3 h of reaction, the G-phase (composed of glycerol, ethanol, soaps and salts) was left to settle in the lower part of the reactor tank and it was subsequently separated and discarded. The ethanol remaining in the upper biodiesel phase was removed at reduced pressure in a rotavapor. The biodiesel was neutralized once with a 1 wt % aqueous solution of citric acid (25 vol % of the biodiesel) and later it was washed once with a 3 wt % aqueous solution of sodium chloride (100 vol % of the biodiesel). Finally, the biodiesel was washed three times with 100 vol % of distilled water. The biodiesel and the aqueous phases of these purification processes were separated in all these steps by centrifugation at 1500 rpm. Finally, the biodiesel was dried over 4 Å molecular sieve (8 wt % of the biodiesel) previously activated overnight at

200 °C and it was vacuum filtered twice through 0.45 µm acrylamide filters. The biodiesel yield in the transesterification described above was 108.4 g (90.3 %).

Equipment for the determination of the fatty acid profile

A Hewlett-Packard 5890 series II gas chromatograph equipped with FID detector and split/splitless injector was used for the analysis. An HP-Wax column (30 m × 0.32 mm i.d. × 0.15 µm) of crosslinked polyethylene glycol was used for separation. The following analytical conditions were used for the analysis: injector temperature, 210 °C; split ratio, 70:1; injection volume, 1 µL; column flow rate (He), 1 mL/min constant flow rate mode; FID temperature, 240 °C; H₂ flow rate, 40 mL/min; air flow rate, 400 mL/min; oven program, 200 °C, hold 9 min, to 230 °C at 20 °C/min, hold 10 min; calibration standard, solution of methyl heptadecanoate in n-heptane (5 mg/mL); sample preparation, 250 mg of sample in 10 mL vial with 5 mL of methyl heptadecanoate solution. The sample was injected in the chromatograph three times. The ester profile is the average of the three measurements and the relative standard deviation of the percentages shown in Table 36 is lower than 1.6 %.

Table 36: Fatty acid profile¹ and total ester content of grape seed FAEE

<i>Fatty acid</i>	<i>wt %</i>
<i>C_{16:0}</i>	7.61
<i>C_{18:0}</i>	7.81
<i>C_{18:1}</i>	30.63
<i>C_{18:2}</i>	53.62
<i>C_{20:0}</i>	0.33
Fatty acid ester content (wt %)	98.33

¹Fatty acid profile has been normalized to 100 wt %

Equipment for the measurement of thermochemical and physical properties

The density at 15 °C was measured in an automatic densimeter Anton Paar DMA48 (procedure ASTM D4052; accuracy: ± 0.3 kg/m³). The cetane number (CN) was measured in the Cetane ID 510 instrument (procedure ASTM D7668 or EN 16715; repeatability 0.0198 (CN – 21)). The kinematic viscosity at 40 °C was determined in a viscosimeter Proton, using tubes Turbiscan model 9506 of Polyscience, immersed in a bath Tamson model TV 2000 (procedure ASTM D445; accuracy: ± 0.0013 · mean, mm²/s). The CHN content was determined in a Leco CHN-932 elemental analyzer

(procedure ASTM D5291; accuracy: ± 0.71 wt % for C, ± 0.18 wt % for H). The higher heating value (HHV) was determined in a Leco AC-300 bomb calorimeter, using benzoic acid tablets as the external standard (procedure ASTM D240; accuracy: ± 0.25 MJ/kg). The lower heating value (LHV) was calculated from the HHV following the procedure ASTM D240 modified for oxygenated fuels. The acidity index of the biodiesel was analyzed in an apparatus Methrom model 702 Titrino (procedure ASTM D664; accuracy: ± 0.02 mg KOH/g). The oxidative stability was measured in an apparatus Rancimat Methrom 743 (procedure EN 14112; accuracy $0.09 \cdot \text{mean} + 0.16$ h). The cloud point (CP) and the pour point (PP) were determined in an PAC CPP 5Gs apparatus (procedure ASTM D2500; accuracy ± 0.1 °C for CP and 1 °C for PP). The cold filter plugging point (CFPP) was measured in an apparatus PAC FPP 5Gs (procedure EN ISO 116; accuracy ± 1 °C). The lubricity was measured with a high frequency reciprocating rig (HFRR_PCS Instruments, London, U.K.) (procedure EN ISO 12156-1, accuracy ± 70 μm).

The Crystallization Onset Temperature (COT) was determined as the temperature at which the heat release from crystallization starts, using a Differential Scanning Calorimeter (DSC) Q20 from TA-Instrument. A 6 mg amount of grape seed FAEE was added to a 40 μL sealed vial under a nitrogen flow rate of 40 mL/min. DSC study comprises two heating-cooling cycles. First, the sample was heated to 30 °C and kept at this temperature during a period of 5 min. Second, the sample was cooled from 30 to -80 °C. Once this temperature was reached, the sample was kept at -80 °C for 5 min. The process was repeated twice to verify repeatability. The rate of temperature was chosen as 5 °C/min following previous studies in FAMES which reported a good combination of resolution characteristics and timeliness. Figure 41 shows the thermogram obtained when the FAEE of grape seed oil was analyzed.

7.1.3 RESULTS AND DISCUSSION

Production yields

The Soxhlet extraction of the grape seeds has a low yield of 6.41 % (standard deviation 0.22 %) which is similar to the yield reported in the literature of 7 % [Pardo et al., 2009; Freitas et al., 2008]. However, the huge production of wine in Spain (44.52 MhL/year) could account for around 15.22 kt / year of grape seed oil, supposing a yield of 75 % of wine on grapes and a 4 wt % of seeds in the grapes and the above extraction yield [Franco-Mora et al., 2015].

Figure 41: Thermogram for FAEE from grape seed oil, obtained with DSC

This amount of oil feedstock is not great but it is not negligible at the same time, and as the yields of degumming and transesterification are practically quantitative, the amount of FAEE that could be obtained from this feedstock would be 16.02 kt/year, which could reach the 1.8 % of the total biodiesel consumption in Spain, which was 876.8 kt/year the past few years (2013-2015) [EurObserv'ER].

The biodiesel “wet wash” purification method was chosen in this case because the “dry wash” method was unsuccessful with this FAEE of grape seed oil [Canoira et al., 2008]. For instance, the solubility of glycerol in a FAEE-glycerol-ethanol mixture was approximately five times higher than that in a FAEE-glycerol mixture system thus making the elimination of glycerol by the “dry wash” method unsuccessful. [Follegatti-Romero et al., 2012; Rostami et al., 2012].

Ester profile and total ester content

The ester profile of major components and the total ester content of the grape seed FAEE are summarized in Table 36. The total ester content of 98.33 wt % is higher than 96.5 wt % which is the lower accepted limit in the EN 14214 standard for FAME (there is not yet a standard for FAEE). This is a pretty unsaturated biodiesel (with iodine value of 119 gI₂/100g, as shown in Table 37), with more than 88 wt % of C18:1 plus C18:2 and this would result in a good cold flow behaviour as reported below but would probably require antioxidant additives.

Measured and estimated properties

Some properties of the grape seed FAEE were measured following the standard procedures indicated in the experimental part. Results are shown in Table 37, left column.

Table 37: Comparison of selected measured and estimated properties of grape seed FAEE

Property	Measured	Estimated	$\Delta(\%)$
Density at 15°C (kg/m ³)	882.4	879.3	-0.35
Kinematic viscosity at 40 °C (mm ² /s)	4.32	4.50	+4.10
HHV ¹ (MJ/kg)	39.95	40.17	+0.55
LHV ² (MJ/kg)	37.63	37.58	-0.13
Induction time (h)	2.01	1.07	-
Induction time (h) (biodiesel additivated with BHT)	5.49	1.07	-
Cetane number	52.8	55.6	+5.30
Cloud point, °C	-6.2	-6.5	-
Pour point, °C	-8.0	-6.4	-
CFPP ³ , °C	-10.5	-8.3	-
TAN ⁴ (mg KOH/g)	0.40	n/a	-
C wt %	76.90	77.49	-0.77
H wt %	11.30	12.11	+7.17
Lubricity, wear scar, μm	163.47	214.26	+31.0
Iodine value, gI ₂ /100g	-	119.0	-

¹HHV: Higher heating value; ²LHV: Lower heating value; ³CFPP: Cold filter plugging point

⁴TAN: Total acid number

These same properties, and other that were not measured, were estimated following empirical methods reported in the literature. The estimated values and the relative errors, obtained as the difference between the estimated and measured property value, divided by the measured value and expressed in percentage, are also shown in this Table 37. Table 38 shows the equations used for the estimation of properties, with the method references reported in the right column of this table.

Density can be estimated for biodiesel with the equations reported in Table 38 (row 6) and the inverse additivity rule [Lapuerta et al., 2010b], where ρ_{FAEE} is the density of each pure ethyl ester, db is the number of double bonds and n is the number of carbon atoms of the fatty acid. The density at 15 °C shows a good value of 882.4 kg/m³ that is in the

range of the EN 14214 standard, and the estimation shows also an excellent agreement with the measured value, the error being only -0.35 % [Ceriani et al., 2008].

The dynamic viscosities η at 40 °C of the pure FAEs can be estimated according to the equation included in Table 38 (row 7), assuming that the effect of unsaturation described by Allen [Allen et al., 1999] can be applied to all unsaturated esters, where MW is the molecular weight. The kinematic viscosities can then be calculated from the dynamic viscosities (see row 7 of Table 38), where ν is the kinematic viscosity at 40 °C in mm²/s and ρ is the density at 40 °C in kg/m³. The kinematic viscosity at 40 °C shows a good value, 4.32 mm²/s also in the range of the standard. In this property, the estimation is somewhat worse due to the fact that small amounts of non-fatty polar compounds present in the biodiesel increase always the kinematic viscosity. For an identical carbon chain, the influence of the functional groups on the kinematic viscosity of an organic compound follows the order COOH > C-OH > COOR > C=O > C-O-C [Knothe et al., 2007].

The lubricity was estimated using the equation included in Table 38 (row 10). The estimated value 214.3 μm is higher than the measured one, 163.5 μm . Although this difference stays in the range of repeatability of the measurement ($\pm 70 \mu\text{m}$), it could be due to small contents in polar compounds like free fatty acids (see TAN in Table 37), which tend to reduce the wear scar significantly [Knothe et al., 2005]. This low scar diameter represents a very good lubricity, far from the limits stated by international standards as the EN 590 Europe, which sets the limit in 460 μm and the ASTM D975 limit, 520 μm . Lubricity is an important parameter in fuels and particularly in the case of biodiesel, which can be used to lower the lubricity of ultra-low sulfur diesel. Sulfur compounds provide a protective layer that reduces adhesion and limit friction or wear but the increasing restriction of sulfur content in fuels is causing low lubricity and as a consequence the need of additives, where biodiesel can have an important role [Barbour et al., 2000].

The HHV and LHV of these biodiesels have been estimated according to equations included in Table 38 (rows 8 and 9 respectively) [Lapuerta et al., 2010a] where $\Delta H_{f(g)}$ and ΔH_{vap} are respectively the enthalpies of formation and vaporisation of the fuel which could be estimated by the Benson group contribution method [Cohen et al., 1993], and H and O are the wt % of hydrogen and oxygen respectively from the elemental analysis of the biodiesel.

Table 38: Equations used for the estimation of properties of grape seed FAEE

Property	Equation	Reference
Cloud Point, °C	$CP = 1.96 + 0.94 \times COT$	Bolonio et al., 2015
Pour point, °C	$PP = -125.04 - 0.62U_{FAEE} + 8.61N_c$	Bolonio et al., 2015
CFPP ¹ , °C	$CFPP = -103.47 - 0.59U_{FAEE} + 7.30N_c$	Bolonio et al., 2015
Oxidation stability, h	$IT = 9.50 - 0.03MU_{FAEE} - 0.14BAPE$	Bolonio et al., 2015
Cetane number	$CN_{FAEE} = -25.192 + (-3.565 + 14.818e^{-0.185db}) \cdot ne^{-0.032n}$ $CN = \sum x_{FAEE} \cdot CN_{FAEE}$	Lapuerta et al., 2009
Density, kg/m ³	$\rho_{FAEE} = 851.471 + \frac{250.718 db + 280.899 - 92.182}{1.214 + n}$ $\frac{1}{\rho} = \sum (x_{FAEE} / \rho_{FAEE})$	Lapuerta et al., 2010b
Kinematic viscosity at 40 °C, mm ² /s	$\eta_{FAEE} = (1.16 \times 10^{-4} MW_{FAEE}^2 - 0.026 MW_{FAEE} + 2.28) \frac{0.147 db^2 - 1.09 db + 4.82}{4.82}$ $\nu_{FAEE} = (\eta_{FAEE} \times 10^3) / \rho_{FAEE}$ $ln \nu = \sum x_{FAEE} \times ln \nu_{FAEE}$	Houachri et al., 2018
HHV ² , MJ/kg	$HHV = \frac{\Delta H_{f(g)} - m \times \Delta H_{fCO_2(g)} - p/2 \times \Delta H_{fH_2O(g)}}{MW} - \Delta H_{vap}$	Lapuerta et al., 2010a
LHV ³ , MJ/kg	$LHV = \frac{HHV - 213.65 \times H - 0.77 \times O}{1000}$	Llamas et al., 2013
Lubricity, wear scar, µm	$WS1.4 = 381.54 - 8.17n + 49.05db - 66.79$	Lapuerta et al., 2016

¹CFPP: Cold filter plugging point; ²HHV: Higher heating value; ³LHV: Lower heating value (*H* and *O* are the hydrogen and oxygen weight percent).

The heating values HHV and LHV are lower than those of fossil diesel, but in the range of other biodiesel fuels. In these cases, the agreement between the estimated and measured values is very good, showing as a good tool for prediction.

The FAEE induction time (IT) has been measured by the Rancimat test (EN 14112). The measured IT of this FAEE was 2.01 h. However, this IT is still much lower than the minimum 8 h value required in the standard for FAME, and thus, the grape seed FAEE, has been additivated with 1000 mg/kg of 1,6-di-*tert*-butyl-4-methylphenol (BHT) [Schober et al., 2004]. The additivated FAEE showed an improved IT of 5.49 h. This result shows that an additional additivation would be necessary to reach the 8 h.

The CN for FAEE can be estimated from the equations in Table 38 (row 5) and the additivity rule [Lapuerta et al., 2009], where CN_{FAEE} is the cetane number of each pure ethyl ester, db is the number of double bonds, n is the number of carbon atoms of the fatty acid, x_{FAEE} is the wt % of each ethyl ester and CN is the cetane number of biodiesel. The cetane number shows a good value of 52.8 points in accordance with other biodiesels, and the estimation is also good [Mohammadi et al., 2015].

The good agreement between the calculated and measured elemental analysis, better for carbon since the experimental error is lower than for H, shows that the measured ester profile is accurate, and thus all the properties estimated upon this profile should also be accurate as far as the estimation equations used are sound.

The cold flow behaviour of grape seed FAEE has been measured by cloud point (CP), pour point (PP) and cold filter plugging point (CFPP), and DSC. DSC has proven to be a very useful technique because it needs a very small amount of sample (only around 10 mg) and when crystallization starts, a clearly visible exothermic peak appears in the thermogram. The temperature at which this peak appears has been defined as the crystallization onset temperature (COT) [Lee et al., 1995; Giraldo et al., 2013]. Figure 41 shows the thermogram for grape seed FAEE where a COT of -8.9 °C could be inferred. The cooling-heating cycle described in the experimental part has been repeated twice for the sake of repeatability, and the DSC analysis has been run for two independent grape seed FAEE samples with identical results for both of them. The heating process (lower line in Figure 41) shows the melting point of grape seed FAEE, always higher than the COT, that is, the biodiesel sample tends to remain in the solid state once the COT has been surpassed. This hysteresis has been observed with many other feedstocks [Llamas

et al., 2013]. Table 38 reports the estimated values of CP, PP and CFPP using this set of equations. The measurement and estimation of cold flow properties show good cold flow behaviour for this grape seed FAEE. This was expected from the esters profile, since the amount of unsaturated esters surpasses 83 wt %.

From a comparison based on the degree of unsaturation, the milk thistle FAEE (*Silybum marianum*) with an iodine value between 118-125 gI₂/100g has a set of properties very similar to this grape seed FAEE: IT, 2.1 h; kinematic viscosity, 4.73 mm²/s; cetane number, 52; CP, -2.0 °C; PP, -3 °C; CFPP, -3 °C [Houacri *et al*, 2018]. In addition, the sesame FAEE (*Sesamum indicum*) with an iodine value of 100-120 gI₂/100g has a very similar set of properties: IT, 2.33 h; CP, 0 °C; PP, -3 °C; CFPP, -6 °C [Bolonio *et al*, 2015].

From an economic point of view, FAEE from grape seed turns out to be very attractive. It is well known that a huge percentage of the biodiesel production price is due to feedstock prices (85 % according to Hass et al. [Haas et al., 2006]). Grape seed oil, considered a waste product from the wine industry, can be currently produced at a low price and has some advantages over other well-known biodiesel waste sources. On one hand, the collection infrastructure and logistics management to produce biodiesel from waste frying oil and other feedstocks must be optimized to lower the cost [Araujo et al., 2010], while grape seed production is located in wineries and would be easy to collect. On the other hand, low cost oils and fats often contain large amounts of free fatty acids that increase the cost of production [Canacki & Van Gerpen, 2001], while grape seed oil has been reported to have medium and low free fatty acids content in this work and previous studies [Fernández et al., 2010].

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7.2 FATTY ACID ETHYL ESTERS FROM ANIMAL FAT USING SUPERCRITICAL ETHANOL PROCESS

7.2.1 CONTEXT

Animal fat is an attractive feedstock for the production of biofuel, specifically biodiesel. It is obtained through a process of rendering, which converts animal by-products into stable and value-added materials. These animal by-products arise from slaughterhouse, meat and dairy processing industry as well as from food retail trade, restaurants and industrial kitchens.

Some of the advantages of using animal fat for the production of biodiesel are: 1) Much of the animal fat produced is not considered edible and, although used for some industrial purposes, its market is very limited, what makes it a byproduct with lower costs than vegetable oils; 2) It is produced in large amounts by food processing and service facilities spread all over the world; 3) A life cycle analysis (LCA) of the production of biodiesel from animal fat conducted by the Institute for Product Development at the Technical University of Denmark [Hedegaard et al., 2007] resulted in a net overall reduction in GHG emissions and came out as the most preferable transport biofuel in comparison to rapeseed biodiesel and maize kernels bioethanol. Concerning the properties of biodiesel obtained from animal fat, several studies [Wyatt et al., 2005; Canoira et al., 2008] have reported higher cetane numbers (CNs) than vegetable oil biodiesel fuels (values over 60 are common while for example soybean oil based biodiesel usually has a CN of about 48-52). In addition, the saturated fatty acids in animal fats contribute to better oxidative stability in contrast with vegetable oils which contain higher amounts of polyunsaturated fatty acids, such as linoleic and linolenic acid, that makes them prone to rancidity. The major drawback resides in the higher cloud (CP) and pour points (PP) which would make necessary the implementation of techniques to improve the cold flow performance such as PP depressants [Bhale et al., 2009] or winterization [Pérez et al., 2010].

Animal fats include tallow (beef tallow from domestic cattle and mutton tallow from sheep), pork lard (rendered pork fat) and chicken fat. All of them are high in free fatty acids (FFAs) content and therefore need a more expensive processing method for its conversion to biodiesel. This is the main drawback for the generalized use of biodiesel from animal fat and can be overcome following different strategies. Bhatti et al. [Bhatti et al., 2008] studied the acid catalyzed transesterification of waste tallow reporting the

optimal processing conditions for 5 g of mutton tallow: 2.5 g of concentrated H_2SO_4 and molar rate oil/methanol 1:30 at 60 °C, yielding 93.21 % of biodiesel after 24 h. Meanwhile, Dias et al. [Dias et al., 2009] pretreated acid waste lard with an acid value up to 14.57 mg KOH g^{-1} and reported that the pre-treatment was effective, enabling acid waste lard to serve as a single raw material for biodiesel production with acceptable quality, but a low yield (65 wt %). As an alternative, Lu et al. [Lu et al., 2007] applied the use of *Candida sp.* 99–125 to enzymatically transesterified lard obtaining as optimal conditions for 1 g of lard: 8 mL n-hexane as solvent, 0.2 g immobilized lipase, 20 % of water at a temperature of 40 °C and a three-step addition of methanol (30 hours); final yield of fatty acid methyl esters (FAMES) was 87.4%. In addition, Alptekin et al. [Alptekin et al., 2010] found as optimum conditions to transform chicken fat with 13.45 % of FFAs the following: 20 % sulfuric acid and 40:1 methanol to oil molar ratio for 80 min at 60 °C; the methyl ester yield was 87.4 %. Best results are obtained by enzymatic and acid-catalyzed transesterification but both reactions are slower and have lower yields than the alkaline-catalyzed transesterification. Moreover, enzymatic transesterification has still important costs while acid-catalyzed transesterification need higher molar ratio of alcohol to oil, is strongly inhibited by the presence of water and causes corrosion problems and environmental threat [Canakci et al., 1999].

As an alternative to the methods described above, the supercritical fluids transesterification has been considered recently as a non-catalyst method with several notable advantages over the conventional processes [Leung et al., 2010]. For reactions in supercritical methanol and ethanol, no catalyst is required and nearly complete conversions can be achieved in a very short time, primarily because supercritical alcohol and oil coexist in a single phase. The process becomes especially interesting when dealing with high FFAs feedstocks as waste cooking oil [Demirbas, 2009] or algae [Chen et al., 2012], saving the cost of the required acid pre-esterification reaction. There are two distinguished supercritical processes: 1) A single step transesterification, which happens at drastic reaction conditions, 350 °C and 20–50 MPa [Da Silva et al., 2010; Olivares-Carrillo et al., 2011; Gonzalez et al., 2013]; 2) A two-step process consisting in a hydrolysis of triacylglycerides to FFAs in subcritical water and subsequent methyl or ethyl esterification of FFAs to fatty acid methyl (FAME) or ethyl (FAEE) esters without any catalyst [Kusdiana et al., 2004b; Minami et al., 2006]. The two-step process happens at more moderate reaction conditions (270 °C/7–20 MPa) than those of the one-step

process. Furthermore, reaction of glycerol with final esters can be suppressed because glycerol is removed away prior to esterification, improving the quality of the biodiesel produced.

A worrying problem of supercritical reactions for biodiesel production is the degradation of unsaturated compounds [Imahara et al., 2008; Vieitez et al., 2011], which can produce undesirable products and a reduction of yield. It has been reported that side reactions of unsaturated FAMES occur when the reaction temperature is over 300 °C and can lead to a significant loss of material [He et al., 2007].

Animal fat seems an ideal feedstock to apply the described techniques, because of its high FFA and the low polyunsaturated composition, which prevents lost by degradation. This work studies the production of biodiesel from animal fat (tallow) with supercritical ethanol, comparing the direct transesterification and the two-step process of hydrolysis and esterification. Yields and degradation of polyunsaturated compounds have been assessed with changes of the temperature, ethanol:animal fat molar ratio and reaction time.

7.2.2 MATERIALS AND METHODS

Characterization of the animal fat

Tallow feedstock was provided by Argent Energy (UK) Limited, whose properties are shown in Table 39. Density and viscosity were measured following the standard analytical procedures EN ISO 3675 and EN ISO 3104 respectively, using the Anton Paar (AUT) SVM 3000 Stabinger Viscometer. Water content was determined through a coulometric Karl Fischer titration according to the EN ISO 12937. FFAs were measured according to the standard EN 14104. The analysis of the total fatty acid content was analyzed according to DGF-C-III 2. The sulfur content was investigated by ultraviolet fluorescence spectrometry using a Mitsubishi TS-100 total sulfur analyzer equipped with a sulfur detector SD-100 according to EN ISO 20846. Sodium (Na), potassium (K), calcium (Ca), magnesium (Mg) and phosphorus (P) were determined by inductively coupled plasma optical emission spectrometry (Spectro Genesis ICP-OES) based on EN 14538 and EN 14107.

Table 39: Animal fat properties

Properties	Animal fat	Standard deviation
Density ($\text{g}\cdot\text{cm}^{-3}$)	0.897	± 0.0002
Dynamic viscosity ($\text{mPa}\cdot\text{s}$)	39.1	± 0.04
Kinematic viscosity ($\text{mm}^2\cdot\text{s}^{-1}$)	43.6	± 0.04
Water content (ppm)	314.6	± 5.89
FFAs content ($\text{mg KOH}\cdot\text{g}^{-1}$)	20.8	± 0.02
Total fatty acids (wt %)	92.9	-
S (ppm)	25	± 1.93
Na (ppm)	102	± 2.23
K (ppm)	41	± 1.01
Ca (ppm)	171	± 4.20
Mg (ppm)	22.7	± 0.79
P (ppm)	182	± 0.60

Catalyzed biodiesel production

FAEEs produced by non-supercritical ethanol were used as a control method and prepared following an adaption of a previously reported protocol [Canoira et al., 2008]. The animal fat was heated to 95 °C with stirring at 500 rpm and 85 wt % orthophosphoric acid (0.2 vol % of the fat) was added to the reactor, holding the reaction for 30 min. Then, the reaction mixture was centrifuged at 3000 rpm for 15 min, and the bottom part in the centrifuge tubes (phosphoglycerides or gums) was removed. The esterification reaction with absolute ethanol in excess (molar ratio of ethanol:animal fat, 6:1) was catalyzed by p-toluenesulfonic acid (mass ratio animal fat:p-toluenesulfonic acid, 200:1). The reaction was heated to 70 °C with stirring at 600 rpm for 4 h. At the end of the reaction, acid catalyst was neutralized and water removed by freshly calcined calcium oxide, produced from calcium carbonate heated at 850 °C overnight (mass ratio animal fat:calcium oxide, 50:1), added to the reactor at 70 °C with stirring. Both calcium hydroxide and p-toluenesulfonate were removed by centrifugation. The resultant mixture of FAEEs, animal fat and ethanol was used for the transesterification step without any further treatment, only with adjustment of the amount of ethanol. The mixture was heated to 70 °C with stirring at 600 rpm. Sodium ethoxide (mass ratio animal fat:sodium ethoxide, 100:2) dissolved in ethanol (molar ratio of ethanol:animal fat, 6:1) was added to the

reaction mixture. After 3 h the reaction mixture was cooled to room temperature and centrifuged at 3000 rpm for 5 min, separating the upper biodiesel phase from the lower glycerol phase. The biodiesel was transferred to a rotary evaporator to eliminate the excess ethanol. For purification, the catalyst was neutralized with 25 vol % of a 1 wt % solution of citric acid in a decantation funnel, and separated from the aqueous layer by centrifugation at 3000 rpm for 5 min. The biodiesel was washed with 100 vol % of a 3 wt % solution of sodium chloride, and later with three batches of 100 vol % of deionized water and separated by centrifugation at 3000 rpm for 5 min. Finally, it was dried by stirring with 8 wt % of 4 Å molecular sieve, previously dried at 200 °C for 2 h, and filtered through a 0.45 µm filter. Final composition was assessed by gas chromatography – mass spectrometry (GC-MS) to identify the different compounds and gas chromatography – flame ionization detector (GC-FID) to quantify the percentage of esters in the mixture (shown in Figure 42).

Figure 42: FAEE profile (wt %) of the animal fat.
Total fatty acid ester content 99.7 wt %

Supercritical methods

One-step process

Animal fat and ethanol were mixed at different ratios to react without any catalyst at temperatures and periods of time specified in the results section. All the reactions happened with 300 mL initial volume of reactants in a 0.5 L high pressure vessel (Parr

instrument company Moline, IL. USA). Temperature was precisely controlled and pressure measured by the 4848B Expanded Reactor Controller. Afterwards, the reaction mixture was cooled to room temperature with water cooling system and centrifuged at 3000 rpm for 5 min, separating the biodiesel from the lower glycerol phase. The biodiesel was transferred to a rotary evaporator to eliminate the excess ethanol and the efficiency of the reaction was assessed through gel permeation chromatography (GPC) (to assess the percentage of non-reacted triacylglycerides) and titration with 0.1 M potassium hydroxide (to assess the percentage of FFAs) (EN 14104). The remaining FFAs were esterified at 78 °C for 4 h using a commercial acidic polymer-based resin (Lewatit® GF 101) and ethanol in a molar ratio ethanol-FFAs, 6:1. Finally, the reaction mixture was filtered through a 0.45 µm filter and the ethanol was removed by a rotary evaporator. Final composition was assessed by GC-MS and GC-FID.

Two-step process

Animal fat and water were reacted without any catalyst for 1 h at temperatures specified in the results section and in the same high pressure reactor described previously, using a molar ratio water:animal fat 2:1 and a total initial volume of 300 mL of reactants. Afterwards, the reaction mixture was cooled in a decantation funnel, where 100 vol % of hexane was added to dissolve the FFAs and improve their separation with the glycerol and water. The FFAs solution was transferred to a rotary evaporator to eliminate the hexane and a sample was taken to measure the efficiency of the hydrolysis through GPC.

Esterification of FFAs was carried out in the high pressure reactor, at a molar ratio ethanol:FFAs 7:1, temperature and periods of time specified in the results section. The biodiesel was transferred to a rotary evaporator to eliminate the excess ethanol and the efficiency of the reaction was determined through GPC and titration with 0.1 M potassium hydroxide. After removal of the excess ethanol, residual FFAs were turned into esters using Lewatit® GF 101 beads as described previously. Finally, the reaction mixture was filtered through a 0.45 µm filter and the ethanol was removed by a rotary evaporator. Final composition was assessed by GC-MS and GC-FID.

Gel permeation chromatography (GPC)

GPC has already been proved to be a useful technique to study transesterification reactions [Darnoko et al., 2000; Kucek et al., 2007]. A Hewlett-Packard (HP) 1100 Series GPC analysis system coupled to the refractive index (RI) detector Knauer K-2301 was

used for the analysis. Two consecutive Phenomenex Phenogel™ 5 μm size exclusion columns composed of highly cross-linked polystyrene-divinylbenzene (PSDVB) were used for separation (pore size 100 and 500 Å). Diameter and length of the tubes were 7.8 and 300 mm respectively. The following analytical conditions were used: injection volume: 50 μL ; temperature: 40 °C; solvent: tetrahydrofuran (THF); flow rate: 1 $\text{mL}\cdot\text{min}^{-1}$. The amounts of tri-, di- and monoacylglycerides, separated from the FFAs and ethyl esters were analyzed by the HP interface 35900.

Gas chromatography

Gas chromatography – mass spectrometry (GC-MS)

An HP 6890 series II gas chromatograph equipped with a 5973 mass selective detector and a split/splitless injector was used for the analysis. A J&W 122-5532UI column (30 m $\times$ 0.25 mm i.d. $\times$ 0.25 μm) was used for separation. The following analytical conditions were used: injector model, Agilent 6805 Series model; injection volume: 1 μL ; injection temperature: 250 °C; split ratio: 50:1; column flow rate (He): 1 $\text{mL}\cdot\text{min}^{-1}$ constant flow rate mode; oven program: 50°C, held for 9 min, to 310 °C at 10 °C $\cdot\text{min}^{-1}$, held for 10 min. Mass spectra of electron ionization: 70 eV; m/z scan from 50 to 800 Da by quadrupole detector set at 150 °C; resolution 1000; source temperature: 230 °C. Chemical compounds were identified using the software MSD ChemStation E.02.02.1431 Data Analysis Application of Agilent and the spectral library Wiley 275.

Gas chromatography – flame ionization detector (GC-FID)

An Agilent 7890 GC System equipped with a FID detector and split/splitless injector was used for the analysis. An HP-InnoWax column (30 m $\times$ 0.32 mm i.d. $\times$ 0.25 μm) of crosslinked polyethylene glycol was used for the separation. The following analytical conditions, based on the EN 14103:2011 protocol were used for the analysis: injector temperature: 210 °C; split ratio: 80:1; injection volume: 1 μL ; column flow rate (He): 2 $\text{mL}\cdot\text{min}^{-1}$ constant flow rate mode; FID temperature: 250 °C; H₂ flow: 30 $\text{mL}\cdot\text{min}^{-1}$; air flow rate: 350 $\text{mL}\cdot\text{min}^{-1}$; oven program: 60 °C held for 2 min, to 200 °C at 10 °C $\cdot\text{min}^{-1}$, to 240 °C at 5 °C $\cdot\text{min}^{-1}$, held for 7 min; sample preparation: 250 mg of sample in a 10 mL vial with 5 mL solution of ethyl nonadecanoate in hexane (10 $\text{mg}\cdot\text{mL}^{-1}$).

7.2.3 RESULTS AND DISCUSSION

Reactions yield

One-step process

The conditions and results of the one-step process reactions (OSR) are shown in Table 40. Yield of FAEEs and percentage of remaining FFAs depend on the ethanol concentration, temperature, time and ethanol:animal fat molar ratio of the experiments.

Table 40: Yields of one-step process experiments with different ethanol concentration, temperature, time duration or ethanol: animal fat molar ratio

	^a OSR1	OSR2	OSR3	OSR4	OSR5	OSR6	OSR7	OSR8	OSR9	OSR10
Type of ethanol	96 %	96 %	96 %	96 %	96 %	Absolute	Absolute	Absolute	Absolute	Absolute
^b Temperature (°C)	300	300	350	350	350	300	350	350	350	350
Time (min)	40	120	40	40	120	40	40	40	120	120
Molar ratio (ethanol:animal fat)	40:1	40:1	20:1	40:1	20:1	40:1	20:1	40:1	20:1	40:1
^c % of FAEEs	63.7	69.4	87.7	89.6	86.8	77.3	91.6	98.4	93.4	96.5
^c % of FFAs	14.0	9.5	7.9	7.3	8.8	4.8	4.0	1.6	5.5	3.5

^aOSR: One-Step process Reaction.

^bTemperature standard deviation: ± 0.2 °C.

^cRelative Standard Deviation (RSD) for percentages of FAEEs or FFAs was equal or less than 2 %.

Effect of ethanol concentration

Production of anhydrous or absolute ethanol in large scale is commercially made by extractive distillation using conventional solvents, which consumes 50–80 % of the energy used in a typical fermentation ethanol manufacturing [Lee et al., 1985]. The use of 96 % ethanol in the production of FAEEs could reduce the overall cost of the process. The comparison of the pairs of experiments OSR3-OSR7, OSR4-OSR8 and OSR5-OSR9 shows how the use of absolute ethanol increases the yield of esters up to 98 % and reduces the FFAs to less than 2 %. Previous experiments have shown that the presence of water did not have a significant effect on the yield studies [Kusdiana et al., 2004a], but these experiments were carried out in a 5 mL reaction vessel for 1 to 4 min. These very short reaction times mean that the samples have been heated and cooled almost instantly, not allowing the chemical equilibrium between FAEEs, FFAs and water to be reached. The batch-system of this work, with a 300 mL reactor, represents better real operation conditions, where the water affects the reaction yields. A possible solution to this problem is the use of a continuous process where the reaction temperature could be easily reached and the residence time reduced [Silva et al., 2007; Vieitez et al., 2011].

Effect of the temperature

Temperature is the most important factor for the non-catalyzed transesterification reaction. The pairs of experiments OSR1-OSR4 and OSR6-OSR8 show the yield of esters is reduced by more than 20 % when the temperature goes from 350 °C to 300 °C. This is consistent with other studies that show how only temperatures higher than 300 °C are useful for this process [Kusdiana et al., 2001].

Effect of the residence time

The dependence of the transesterification reaction on the residence time can be assessed comparing the pairs of experiments OSR1-OSR2, OSR3-OSR5, OSR7-OSR9 and OSR8-OSR10. When the residence time increases, the yield of FAEEs follow two different behaviors. On one hand, with lower temperature (300 °C for OSR1-OSR2), the yield of the reaction is low, and an increase in the residence time slightly improves it. On the other hand, when the temperature is 350 °C and the FAEE yield is high (near 90 % or more), an increase in time diminishes the yield and increases the final percentage of FFAs. It seems that the presence of water produces an equilibrium between FAEEs and FFAs, which can increase the percentage of FFAs with time. There is also the possibility of side reactions of unsaturated FAEEs which can reduce their yield as it has been proposed by other studies [Imahara et al., 2008] and will be discussed in the last section of the results.

Effect of the ethanol:animal fat molar ratio

The comparison of the pairs of reactions OSR3-OSR4, OSR7-OSR8 and OSR9-OSR10 shows that the yield of FAEEs increases significantly and the final FFAs decreases when the ethanol:animal fat ratio changes from 20:1 to 40:1. These results are consistent with other studies that point out the need of high methanol or ethanol ratios to optimize the transesterification yield [Demirbaş, 2002; Gui et al., 2009].

Two-step process

The results of the hydrolysis reactions are summarized in Figure 43 and are consistent with other studies [Kusdiana et al., 2004b; Levine et al., 2010]. Yield of FFAs reaches 90 % or higher only when temperatures are 250 °C or higher. Moreover, another test was done to reduce the water from the volume ratio 2:1 to 1:1 (at 300 °C and 1 h of residence time), and the yield was reduced from 95.6 to 92.8 %. This confirms the need of big amounts of water to reach the optimum yield of the reaction [Kusdiana et al., 2004a].

Figure 43: Yield of FFAs after hydrolysis of the animal fat with water (volume ratio water:animal fat, 2:1; residence time: 60 min). RSD $\leq$ 2 %

The results of the esterification reactions are summarized in Figure 44. In the same way as hydrolysis process, temperatures below 250 °C do not produce an optimum yield. It is worth noting that as other studies have shown [Abdala et al., 2014b], optimum results are reached with much lower ethanol:FFAs molar ratio (7:1) compared to the one used during the one-step process (40:1).

Figure 44: Yield of FAEEs after esterification of FFAs (molar ratio ethanol:FFAs, 7:1; residence time: 60 min). RSD $\leq$ 2 %

Based on these results, temperatures between 250 and 300 °C would reach a good yield both in hydrolysis and esterification reactions, while in the case of one-step process reactions, the optimal temperature is 350 °C. This difference of almost 100 °C has important consequences, economically because of the energy saving in heating but especially in the reduction of the polyunsaturated compounds degradation.

Degradation of mono- and polyunsaturated compounds

One-step process

Experiments with FAEs yields higher than 90 % and performed with absolute ethanol (low FFAs) were used for this study (see Figures 45 and 46). The percentages of oleic acid ethyl ester (C18:1) and linoleic acid ethyl ester (C18:2) obtained after the catalyzed method were used as control. Figures 45 and 46 show how the degradation increases with higher residence time or molar ratios. The increase in residence time causes a greater probability for side reactions to happen, while the increase in molar ratio causes an increase in pressure and accelerates the degradation.

Figure 45: Percentage of oleic acid ethyl ester after one-step process reactions
*MR: molar ratio

Figure 46: Percentage of linoleic acid ethyl ester after one-step process reactions

The percentage of degradation of C18:1 and C18:2 ethyl esters was calculated according to Equations 16 and 17, where the subscript “model” refers to the concentrations obtained in the catalyzed method:

$$\% \text{ degradation of C18:1} = \frac{[C18:1]_{\text{model}} - [C18:1]_{\text{experiment}}}{[C18:1]_{\text{model}}} \times 100 \quad \text{Eq. 16}$$

$$\% \text{ degradation of C18:2} = \frac{[C18:2]_{\text{model}} - [C18:2]_{\text{experiment}}}{[C18:2]_{\text{model}}} \times 100 \quad \text{Eq. 17}$$

In the worst case (350 °C, 120 min and molar ratio ethanol:animal fat 40:1), the degradation of C18:1 ethyl ester reaches 4.7 % (from 38.5 % to 36.7 %) and of C18:2 ethyl ester reaches 38.7 % (from 3.1 % to 1.9 %). Meanwhile, using the following reaction conditions: 350 °C, 40 min and molar ratio ethanol:animal fat 20:1, there is no degradation of C18:1 ethyl ester and the degradation of C18:2 ethyl ester is reduced to 22.6 % (from 3.1 % to 2.4 %). In this case, the yield would reach 91.6 % (see Table 39).

These results prove the importance of using raw materials with low polyunsaturated compounds to produce biodiesel via the one-step supercritical method. A reduction of linoleic acid ethyl ester from 3.1 % to 2.4 % could be acceptable, but the same percentage reduction in oils with high C18:2 fatty acid content, such as rapeseed or soybean oil, would cause a major loss of material, and the formation of gaseous products or polymers which could affect the final biodiesel properties [Imahara et al., 2008]. It is worth mentioning that the presence of water can reduce the degradation [Abdala et al., 2014a; Silva et al., 2014] but as described before, this could affect the yield of FAEEs in batch production and only could be recommended in a continuous process.

Two-step process

The degradation of oleic and linoleic acid ethyl esters is shown in Figures 47 and 48. All the experiments with acceptable yield (more than 90 %) were considered, and to compare with the one-step process reactions, the percentage of ester was measured at the end of the process, consisting of 1 h of hydrolysis and 1 h of esterification. The volume ratio water:animal fat was always 2:1 and the molar ratio ethanol:FFAs was 7:1 for the second step.

Figure 47: Percentage of oleic acid ethyl ester after two-step process reactions

Figure 48: Percentage of linoleic acid ethyl ester after two-step process reactions

In the worst case (350 °C), degradation is even higher than the one produced during the one-step process. The C18:1 ethyl ester is reduced by 7.8 % (from 38.5 % to 35.5 %) while the C18:2 ethyl ester is reduced by 51.6 % (from 3.1 to 1.5 %). This bigger degradation during the two-step process, when the whole process is at 350 °C and for 2 h, could be caused by a lower stability of FFAs compared to the triacylglycerides.

Nevertheless, the most important result obtained in Figures 47 and 48 is that when hydrolysis and esterification reactions are performed at 250 °C, there is no perceptible degradation of neither C18:1 ethyl ester nor C18:2 ethyl ester. The yield of FAEEs would be 90 % (see Figure 44). This result shows a great advantage of the two-step process over the one-step process, especially when dealing with oils with high content of polyunsaturated compounds. Other studies have also reported degradation of polyunsaturated compounds during high pressure and temperature reactions [Vieitez et al., 2011; Imahara et al., 2008] and come to the conclusion that supercritical processes should always be performed at temperatures lower than 300 °C. Moreover, the higher reaction times used in this work for the two-step process compared to the one-step process can be reduced with the use of a continuous process or cosolvents as shown in previous works [Mello et al., 2017a; Mello et al., 2017b].

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8. CONCLUSIONS

Estimation of key biodiesel properties:

Cold flow performance and oxidation stability

Rapeseed, camelina, soybean, palm kernel, coconut, linseed, sesame, sea-buckthorn, waste frying oils and animal fat have been transesterified with ethanol to produce biodiesel of ethyl esters. The composition of biodiesel fuels was measured using gas chromatography (GC-FID). The cold properties, cloud point (CP), pour point (PP) and cold filter plugging point (CFPP), were measured following the corresponding ASTM and EN standards. The oxidation stability was assessed through the induction time (IT) measured with the Rancimat test. A multiple linear regression model was applied to find the correlation between total percentage of unsaturated fatty acid ethyl esters (FAEEs) and length of the average carbon chain with the CP, PP and CFPP. Considering the difficulty in the prediction of these properties, using only the composition, the results were successful ($R^2=0.89$ in the CP-composition regression). The crystallization onset temperature (COT) was used to predict the cold properties through a simple linear regression resulting in a strong correlation ($R^2=0.97$ in both properties CP and PP). The prediction of the oxidation stability was done using as parameters the percentage of monounsaturated compounds ($MU_{FAEE} = C16:1 \text{ (wt \%)} + C18:1 \text{ (wt \%)}$) and the bis-allylic position equivalents index, which contains the information of the polyunsaturated compounds ($BAPE = C18:2 \text{ (wt \%)} + 2 \cdot C18:3 \text{ (wt \%)}$). A multiple linear regression was necessary for values of C18:3 lower than 10 % using MU_{FAEE} and BAPE as variables. On the other hand, for values of C18:3 higher than 10 % the parameter MU_{FAEE} was not significant and a simple linear regression was done. Both regressions showed good results with $R^2=0.97$ and $R^2=0.94$ respectively.

An inexpensive and rapid way to predict the cold flow properties and the oxidation stability of the FAEE biodiesel using a minimum amount of substance is proposed. Researchers of biodiesel that work with microorganisms can use these equations to know these two crucial properties using milligrams of the sample. As an application of the equations an amount of *E. coli* BL21 (DE3) was produced following the methods above described and the compositions were used to predict the properties of *E. coli* biodiesel using the equations proposed. Biodiesel from unmodified *E. coli* presents a good oxidation stability and moderate cold flow behavior because of the presence of a high

percentage of monounsaturated compounds (C16:1 and C18:1) and lack of polyunsaturated compounds.

Lubricity

A high frequency reciprocating rig (HFRR) test has been used to study the influence of composition and ambient humidity of the individual components of biodiesel (methyl and ethyl esters) and also of actual biodiesel fuels from different oils on their lubricity. A correlation has been proposed to calculate the normalized wear scar of a biodiesel fuel as a function of the average number of carbons of the acid chain, the average number of double bonds (as a measure of the unsaturation) and the alcohol used for transesterification. The influence of humidity on the lubricity of methyl and ethyl esters and biodiesel fuels has also been studied through the humidity correction factor (HCF). This factor is approximated by the lubricity standard to 60 $\mu\text{m/kPa}$ and all the measurements obtained in this chapter of the thesis are below this value. Consequently, the lubricity of biodiesel fuels is underestimated or overestimated (depending on the ambient vapor pressure) when the factor provided by the standard is used. An additional correlation has been proposed to calculate this factor. From both correlations, it can be concluded that when the number of carbon atoms in the acid chain or in the alcohol used for transesterification increase, both the normalized wear scar and the humidity correction factor decrease. The effect of the unsaturation on the wear scar is not so clear because it was opposite for pure esters than for biodiesel fuels. These correlations are useful to predict the lubricity of a biodiesel fuel, as well as the effect of humidity on the lubricity, from its fatty acid profile, with good accuracy, as far as the content of impurities in the biodiesel is small enough. Finally, it has been proved that the change in lubricity at different humidities (HCF) is derived from the different chemical composition of the fuel, and also that it is the water content in the fuel what mainly leads to an increase in the wear scar. This implies that the benefits in lubricity of biodiesel fuels could be counteracted by their high hygroscopy when they are exposed to open air for long time.

Genetic change of fatty acids composition of *E. coli* to obtain optimum biodiesel properties

Production of biofuels from microorganisms is a promising alternative to increase the sources of sustainable energy. *E. coli* can be used as an ideal model for the synthesis of fatty acids, a key step in the production of biodiesel. This thesis studies the genetic

modification of *E. coli* to yield an adequate fatty acid composition aiming appropriate properties on the methyl or ethyl esters or biodiesel obtained from the fatty acids. Unmodified *E. coli* BL21 has a very high saturated fatty acid composition, which must be reduced to improve the final cold flow performance of the methyl esters produced. This goal has been achieved following two different strategies: 1) Overexpression of the transcription factor FadR, which inhibits the fatty acid degradation pathway and increases the percentage of unsaturated compounds; 2) Heterologous expression of a FatA thioesterase from *B. napus*, which has a higher specificity for oleic and palmitoleic acids (C18:1, C16:1 respectively), and therefore increases the percentage of unsaturation. Results show how the initial cold flow performance, measured through the cold filter plugging point, is drastically improved, from 22.0 °C of the control to -25.2 °C in the strain that overexpresses both BnFatA and FadR enzymes. Oxidation stability is reduced but in a small amount due to the lack of polyunsaturated compounds in *E. coli*, important difference with the majority of the oil plants, where the common presence of linoleic acid (C18:2) causes a general low oxidation stability, a main drawback of biodiesel performance. Strains overexpressing BnFatA and 'TesA+FadR enzymes show as very promising strains for trying the pilot plant scale production since the biodiesel obtained would meet all the specifications of the standards.

A physicochemical characterization of green-filamentous macroalgae *Chaetomorpha cf. gracilis*

This thesis has analyzed the *Chaetomorpha cf. gracilis* macroalgae, which has an oil yield of 1.85 %. A gas chromatography analysis showed a higher percentage of natural products and alkanes. The analysis of the oil showed a lower kinematic viscosity and density compared to other macroalgae oils. Conversely, the heating values and crystallization temperature values were higher. Concerning the elemental composition, carbon and hydrogen content was also different to other algae. The analysis conducted on the net energy ratio for the oil extraction process showed that the *Chaetomorpha cf. gracilis* macroalgae oil might represent an attractive alternative for liquid biofuel production. In addition, *Chaetomorpha* oil might be also recommended as an additive to prepare alternative fuels.

Fatty acid methyl and ethyl esters obtained from rare seeds from Tunisia

Small amounts of oleaginous seeds from plants grown in arid lands in Tunisia have been collected in two field campaigns: 1) *Ammi visnaga*, *Citrullus colocynthis*, *Datura Stramonium*, *Ecballium elaterium* and *Silybum marianum*; 2) *Ibicella lutea*, *Onopordum nervosum*, *Peganum harmala*, *Smyrniolum olusatrum* and *Solanum elaeagnifolium*. The seeds have been extracted in Soxhlet apparatus with petroleum ether, the oils have been refined and transesterified to FAMES (all of them) and FAEEs (only the ones collected in the first campaign). Their fatty acid profiles have been determined by GC-FID and GC-MS and their crystallization onset temperatures have been deduced from the thermograms obtained by DSC. Based on these measurements, several properties to test their potential use as biofuels have been predicted: cold flow behavior (CP, PP and CFPP), oxidative stability, cetane number, density, kinematic viscosity, heating value, oxygen extended sooting index and lubricity. Moreover, some selected properties of several esters studied in this part of the thesis have been measured and compared with the corresponding estimated values. As a general conclusion, all the oils studied in this section of the thesis have adequate properties for their use as biofuel, similar to other well-studied oils such as palm or soybean, but with the advantage of growing in arid lands, so need small amounts of water and do not compete with food plants. Oxidation stability and cold flow behavior remain as usual as two of the more controversial properties, requiring the need of additives or treatments to improve them before blending with diesel fuel.

Biodiesel from waste feedstocks

Fatty acid ethyl esters (FAEEs) obtained from grape seed oil

Grape seed represents a biodiesel feedstock of waste origin with some potential future in Spain and other wine producing countries due to the huge wine production. Moreover, in this thesis, FAEE production is proposed, making a totally renewable biodiesel from a waste feedstock. The grape seed FAEE presents very good properties like higher and lower heating values, density, kinematic viscosity, lubricity and cold flow behaviour, all inside the range of the EN 14214 standard. Only the oxidation stability presents low values of induction time that could be amended with the addition of antioxidants like BHT.

Biodiesel production using supercritical animal fat and ethanol

One of the main problems of using waste oils and fats for the production of biodiesel is their high free fatty acids content, which makes the traditional catalytic process more expensive, adding an acid catalyzed esterification before the transesterification. An alternative method is to use high pressure and temperatures without catalyst, overcoming the problem of acidity. This thesis has studied the production of biodiesel (FAEEs) from animal fat, using ethanol and two different supercritical methods. The generalized use of ethanol would make biodiesel a complete green energy, contrary to the use of methanol, which currently has a fossil origin. Briefly, the two processes considered have been: 1) The one-step process, where FAEEs are obtained through one single reaction of the animal fat with ethanol and 2) The two-step process, where first, a hydrolysis reaction turns the raw animal fat into free fatty acids and second, free fatty acids react with ethanol to produce FAEEs. On one hand, the two-step process seems especially convenient when the oil used for the production of FAEEs is rich in unsaturated compounds, because an acceptable yield is reached at 250 °C with no degradation of mono- and polyunsaturated compounds. On the other hand, the one-step process is simpler and can be an interesting alternative for animal fat or oil sources with low polyunsaturated compounds.

ABBREVIATURES

ACP: Acyl carrier protein

ASTM: American Society for Testing and Materials

ATP: Adenosine triphosphate

BAPE: Bis-allylic position equivalent

cDNA: Complementary DNA

CoA: Coenzyme A

COT: Crystallization onset temperature

CFPP: Cold filter plugging point

CN: Cetane number

CP: Cloud point

db: Weighted-average number of double bonds

DNA: Deoxyribonucleic acid

DSC: Differential scanning calorimetry

EN: European standard

Fab: Fatty acid biosynthesis

Fad: Fatty acid degradation

FAEE: Fatty acid ethyl ester

FAME: Fatty acid methyl ester

Fat: Fatty acid thioesterase

FFA: Free fatty acid

FAE: Fatty acid ester

GC-FID: Gas chromatography – Flame ionization detector

GC-MS: Gas chromatography – Mass spectrometry

GPC: Gel permeation chromatography

HCF: Humidity correction factor

HFRR: High frequency reciprocating rig

HHV: Higher heating value

Hy: Hygroscopy

ISO: International Organization for Standardization

IT: Induction time

LB: Lysogeny broth

LHV: Lower heating value

m: Number of carbon atoms of the alcohol used for the transesterification

MU_{F_{AEE}}: Weight percentage of monounsaturated esters

MW: Molecular weight

MWSD: Mean wear scar diameter

n: Weighted-average number of carbon atoms of the fatty acid

N_c: Weighted-average number of carbon atoms of the fatty acid ester

NER: Net energy ratio

OESI: Oxygen extended soothing index

P_{FAME}: Weight percentage of methyl palmitate

PP: Pour point

RNA: Ribonucleic acid

RSD: Relative standard deviation

SP: Smoke point

T_f: Flash point temperature

TGA: Triacylglyceride

TLC: Thin layer chromatography

U: Weight percentage of total unsaturated fatty acid esters

UCO: Used cooking oil

ULSD: Ultra-low sulfur diesel

WACF: Water-air correction factor

WFCF: Water-fuel correction factor

WS1.4: Normalized wear scar

x: Mass fraction

$Y_{\text{H}_2\text{O}/\text{air}}$: Mass fraction of water vapor in the humid air

$Y_{\text{H}_2\text{O}/\text{fuel}}$: Mass fraction of water in the fuel

$\Delta H_{f(g)}$: Enthalpy of formation of the fuel

ΔH_{vap} : Enthalpy of vaporization of the fuel

η : Dynamic viscosity

ν : Kinematic viscosity

ρ : Density

GLOSSARY

Bacto Tryptone: peptide mixture resulting from the trypsinolytic digestion of milk casein; it is commonly used to prepare bacterial culture media.

Bacto Yeast Extract: extract of autolyzed yeast cells (water-soluble fraction) used for bacterial culture media.

Cytosol: aqueous component of the cell cytoplasm, within which the nucleus and organelles are suspended.

***Escherichia coli* BL21 DE3 pLysS:** *E. coli* strain for highly-efficient protein expression under the control of T7 promoters. DE3 indicates that a lysogenic λ -DE3 phage was used to introduce a genomic copy of the gene for T7 RNA polymerase, under the control of the *lac UV5* promoter. The pLysS plasmid carries a gene for T7 lysozyme, which inhibits the T7 RNA polymerase. Its presence lowers background expression of recombinant proteins.

***Escherichia coli* DH5 alpha:** *E. coli* strain commonly used for DNA cloning. This strain carries several mutations to maximize stability of recombinant DNA, such as *recA1* and *endA1*.

Lysogenic cycle: is one of the two alternative life cycles of a virus inside a host cell (the other being the lytic cycle), whereby the virus DNA inserts itself into the host genomic DNA and replicates when the host cell divides.

Lytic cycle: is one of the two alternative life cycles of a virus inside a host cell (the other being the lysogenic cycle), whereby the virus that has entered a cell takes over the cell's replication mechanism, makes viral DNA and viral proteins, and then kills (breaks open) the cell, allowing the newly produced viruses to leave to infect other cells.

Periplasm: space between the inner and outer membrane in Gram-negative bacteria. In Gram-positive bacteria, the periplasmic space is found between the inner membrane and the peptidoglycan layer. This term is also used for the intermembrane spaces of fungi and organelles.

Plasmid: structure containing genetic information and that can replicate independently of the genomic DNA; it typically consists of a small circular DNA molecule found in the

cytoplasm of bacteria and protozoa. Engineered plasmids are commonly used as **vectors** to carry genetic information between organisms.

Primer: short nucleic acid sequence that provides a starting point for DNA synthesis.

Promoter: region of DNA where transcription of a gene initiates and its expression is controlled.

Ribosome binding site (RBS): RNA sequence found in messenger RNA (mRNA) to which ribosomes must bind in order to initiate protein biosynthesis (translation).

Thioesterase: enzyme of the esterase family that exhibit activity towards thiol (-SH) groups.

Transcription factors: proteins that bind to DNA-regulatory sequences (enhancers and silencers), mainly localized in the 5'-upstream region of genes (promoter). Such binding modulates the rate of gene transcription into mRNA.

Vector: DNA molecule used as a vehicle to transfer genetic material between different organisms (see **Plasmid**).

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While rapeseed and palm are still the dominant sources in the EU (accounting for 47 and 18 %, respectively), both oils have been criticized by numerous studies which point to the extensive occupation of land and the consequences of deforestation [Gilbert, 2012a; Gilbert, 2012b].

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In Figures 4, 5 and 6, the continuous lines represent the confidence intervals while the dash lines represent the prediction intervals.

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In Table 22, the formulas for kinematic viscosity (ν) are:

$$\ln(\eta) = \sum x_{FAME} \times \ln(\eta_{FAME})$$
$$\nu = (\eta \times 10^3) / \rho$$

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In Table 22, the formula for lubricity is:

$$WS1.4 = 381.54 - 8.17N_c + 49.05db$$

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The kinematic viscosities at 40 °C of the FAMEs included in this study have been estimated using the equation included in Table 22 (row 2), which is a general equation to evaluate the dynamic viscosity of a blend, where η is the kinematic viscosity of the blend and η_{FAME} is the dynamic viscosity of each pure methyl ester [Allen et al., 1999; Llamas et al., 2012a; Llamas et al., 2012b].

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In Table 29, the formulas for viscosity are:

For FAMEs:

$$\ln \nu_{FAME} = -12.503 + 2.496 \cdot \ln MW_{FAME} - 0.178 \cdot db$$

$$\eta_{FAME} = (v_{FAME} \times \rho_{FAME})/10^3$$

$$\ln(\eta) = \sum x_{FAME} \times \ln(\eta_{FAME})$$

For FAEs:

$$\eta_{FAEE} = (1.16 \times 10^{-4} MW_{FAEE}^2 - 0.026 MW_{FAEE} + 2.28) \frac{0.147 db^2 - 1.09 db + 4.82}{4.82}$$

$$\ln(\eta) = \sum x_{FAME} \times \ln(\eta_{FAME})$$

$$v = (\eta \times 10^3)/\rho$$

In Table 29, the formula for lubricity is:

$$WS1.4 = 381.54 - 8.17N_c + 49.05db - 66.79(m - 1)$$

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In Table 37, the estimated iodine value is:

113.0

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In Table 38, the formula for density is:

$$\rho_{FAEE} = 851.471 + \frac{250.718 db + 188.717}{1.214 + n}$$

In Table 38, the formulas for kinematic viscosity are:

$$\eta_{FAEE} = (1.16 \times 10^{-4} MW_{FAEE}^2 - 0.026 MW_{FAEE} + 2.28) \frac{0.147 db^2 - 1.09 db + 4.82}{4.82}$$

$$\ln(\eta) = \sum x_{FAME} \times \ln(\eta_{FAME})$$

$$v = (\eta \times 10^3)/\rho$$

In Table 38, the formula for lubricity is:

$$WS1.4 = 314.75 - 8.17N_c + 49.05db - 66.79(m - 1)$$