University of São Paulo
"Luiz de Queiroz" College of Agriculture

Prospection and identification of antioxidant compounds in the products of the soybean oil ethanolic extraction

Larissa Braga Bueno Borges

Thesis presented to obtain the degree of Doctor in Science. Area: Food Science and Technology

Piracicaba
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Larissa Braga Bueno Borges
Pharmacist

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Advisor:
Prof. Dr. MARISA APARECIDA BISMARA REGITANO D'ARCE DE BAPTISTA
Co-advisor:
Prof. Dr. SEVERINO MATIAS DE ALENCAR

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2019

Dados Internacionais de Catalogação na Publicação
DIVISÃO DE BIBLIOTECA – DIBD/ESALQ/USP

Borges, Larissa Braga Bueno

Prospection and identification of antioxidant compounds in the products of the soybean oil ethanolic extraction / Larissa Braga Bueno Borges. - - Piracicaba, 2019.

85 p.

Tese (Doutorado) - - USP / Escola Superior de Agricultura “Luiz de Queiroz”.

1. Extração com etanol 2. Isoflavonas 3. Antioxidantes 4. Estabilidade oxidativa 5. Soja 6. Biodiesel 7. Refino de óleo I. Título

This work is dedicated to all students.

ACKNOWLEDGMENTS

I would like to thank the support provided by ESALQ and the Agri-food Industry, Food and Nutrition Department throughout the years and to the São Paulo Research Foundation - FAPESP, project numbers 2013/03650-6 and 2017/02819-8. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

My heartfelt thanks to those that have somehow contributed to the conclusion of this work.

CONTENTS

RESUMO

Prospecção e identificação de compostos de atividade antioxidante em miscelas etanólicas de óleo de soja

O óleo de soja é obtido comercialmente através de um processo de extração a quente utilizando a hexana, também conhecida como hexano comercial. Esse solvente tóxico, derivado de petróleo, deve ser posteriormente evaporado para que o óleo passe por etapas de refino antes de seguir para o armazenamento e transporte ou diretamente para a síntese de biodiesel. A extração de óleo de soja com o etanol, um solvente renovável e de baixa toxicidade, resulta na produção de uma miscela rica em óleo que pode ser diretamente esterificada para produzir ésteres etílicos (biodiesel), sem a necessidade de etapas de refino de óleo e evaporação do solvente. Essa extração gera também uma miscela pobre em óleo que pode ser reutilizada em extrações posteriores como solvente. As bases teóricas da extração etanólica de óleo de soja foram estabelecidas pelos trabalhos de muitos autores, visando maiores rendimentos de extração. No entanto, o perfil antioxidante e a estabilidade oxidativa dos produtos da extração etanólica de óleos vegetais não foram abordados até o momento. Este estudo visou ampliar os conhecimentos sobre a composição antioxidante das miscelas ricas e pobres, oriundas da extração de óleo de soja, utilizando o etanol como solvente. Os resultados evidenciaram que embora a miscela rica apresente quantidades inferiores de tocoferóis em relação a óleos extraídos com hexano, a mesma é acrescida de compostos antioxidantes de natureza polar, devido a afinidade com etanol. Entre esses compostos foram identificadas isoflavonas, que normalmente não são encontradas nos óleos de soja obtidos pela extração com hexano. A reutilização da miscela pobre promove o aumento da estabilidade oxidativa das miscelas ricas, através da adição de compostos antioxidantes de maior afinidade pelo etanol. Esse efeito foi também observado no uso da miscela pobre em lavagens do óleo de fritura, resultando em aumento no período de indução. No entanto, a reutilização de miscelas pobres em extrações consecutivas demonstrou atingir um limite no aumento do período de indução das miscelas ricas. Esse fenômeno pode ser um indício de efeito pró-oxidante decorrente do aumento das concentrações de compostos presentes na miscela rica. Por essa razão, o estudo das interações entre diferentes classes de compostos antioxidantes nas miscelas oriundas da extração de óleo de soja pode ser uma excelente via para a compreensão dos mecanismos envolvidos na estabilidade oxidativa de sistemas lipídicos complexos.

Palavras-chave: Extração com etanol; Isoflavonas; Antioxidantes; Estabilidade oxidativa; Soja; Biodiesel; Refino de óleo

ABSTRACT

Prospection and identification of bioactive compounds in the products of the soybean oil ethanolic extraction

Soybean oil is commercially obtained through an extraction process using hot commercial hexane. This toxic solvent, derived from petroleum, must be subsequently evaporated before the oil can be submitted to refining steps prior to storage and transportation or directly moving to biodiesel synthesis. Extraction of soybean oil with ethanol, a renewable, low-toxicity solvent, results in the production of an oil-rich miscella that can be directly esterified to produce ethyl esters (biodiesel) without the need for oil-refining steps and evaporation of the solvent. This extraction also generates an oil-poor miscella that has been successfully reinserted in further extractions as the solvent. The theoretical bases of the ethanolic extraction of soybean oil have been established by the works of many authors, focusing on the insurance of high oil yields. However, the effects of the ethanolic extraction of vegetable oils on the antioxidant profile and oxidative stability of the extraction products have not yet been addressed. This study aimed to increase the knowledge on the antioxidant composition of the rich and poor miscellae derived from the extraction of soybean oil using ethanol as solvent. Results showed that although the rich miscella presents lower amounts of tocopherols in comparison to oils extracted with hexane, it contains antioxidant compounds of polar character with high affinity to ethanol, which would not be found in soybean oils extracted with hexane. Among these compounds isoflavones have been identified and quantified. The reuse of the poor miscella in subsequent extractions promotes an increase in the oxidative stability of the rich miscella, possibly, by adding antioxidant compounds with high affinity to ethanol. This effect was also observed in the use of the oil-poor miscella in liquid-liquid washings with waste cooking oil, resulting increase of the induction period. However, consecutive extractions of soybean oil have shown that the increase in the induction period of the oil-rich miscella reaches a limit. This phenomenon may be an indication of pro-oxidant effects, resulting from the increase in the content of the compounds present in the rich-miscella. For this reason, the study of the interactions between different classes of antioxidant compounds present in the soybean ethanolic extraction products may be an excellent way to understand the mechanisms involved in the oxidative stability of complex lipid systems.

Keywords: Ethanol extraction; Isoflavone; Antioxidants; Oxidative stability; Soybean; Biodiesel; Oil refining

1. INTRODUCTION

In Brazil, soybeans are the main crop for vegetable oil production. Industrial extraction of soybean oil is accomplished with the use of commercial-grade hexane. Hexane is a solvent derived from petroleum with apolar characteristics and, consequently, high affinity for lipid compounds. It is highly toxic, explosive, flammable, and derives from non-renewable sources.

Since the 80s, the Laboratory of Oils and Fats of ESALQ/USP has been developing projects on the extraction of vegetable oils which use ethanol as a substitute solvent for hexane. The ethanolic extraction presents challenges on matching the same efficiency achieved with hexane extraction. However, by using ethanol, the quality of products and byproducts is improved, whilst reducing health and environmental risks. The main product obtained from extraction with ethanol is an alcohol-in-oil emulsion, hereafter named oil-rich miscella (plural, oil-rich miscellae). The extraction also renders a byproduct consisting of 90% ethanol and small amounts of oil, namely oil-poor miscella. Over the years, some of the unusual antioxidative-related characteristics of these products were observed, urging further investigations.

In this study, we aimed to investigate the characteristics related to the antioxidant composition of the two main products obtained in the ethanolic extraction of soybean oil: the oil-rich and oil-poor miscella. Chapter 2 comprehends a detailed literature review on the relevant topics discussed here. Chapter 3 encompasses the first published scientific paper, produced by our research group, that approached the antioxidant composition and singular oxidative stability of the oil-rich miscella. The characteristics of this product and its derived biodiesel were compared to soybean oils extracted industrially with hexane at different levels of oil-refining. Chapter 4 proposes a simple application for the soybean oil-poor miscella as a washing antioxidant-adding vehicle for improving waste cooking oil quality. Here, a sample of waste cooking oil was treated with the oil-poor miscella derived from soybean oil extraction and a few quality parameters were assessed. Chapter 5 addresses the consecutive ethanolic extraction of soybean oil by reusing the oil-poor miscella as solvent for the next extraction. Fifty extractions were performed through solvent reuse and the antioxidant profile and oil stability of extraction products were investigated.

2. BIBLIOGRAPHIC REVIEW

2.1. Pre-treatment of soybeans (Glycine max) before solvent extraction

Originally from the North and East regions of Asia, today soybean (Glycine max (L.) Merr.) has a worldwide distribution (Kim et al., 2006). In Brazil, soybean is a major food commodity and its nutritional characteristics are fully explored by the industrial sector. Soybeans are valued for their amino acid range, being the protein fraction the main product obtained from seed processing. For this reason, there's an oil surplus in the soybean industry, which makes it the most consumed oil by Brazilians due to the accessible price range and availability. They present protein and oil contents of, approximately, 40% and 20%, respectively (Clemente & Cahoon, 2009), but besides these two main products, soybeans are a good source of a number of compounds such as: sugars, isoflavones, saponins, phytic acid and peptides (Medic et al., 2014).

Although frequently referred to as an oilseed, soybeans are legumes (Leguminosae family). With an oil-content of 18-20%, these compact seeds must be conditioned with the purpose of facilitating oil removal. Industrial processes are constantly optimized for maximum yield, therefore conditioning of the seeds is of great importance. It prepares the oil-bearing material for the upcoming solvent extraction.

A pre-cleaning is performed to remove most of the impurities and contaminants from the grains initially. Cotyledons are separated from the peels, which can either be burned to feed the boilers or ground to be re-added to the meals and "dilute" its final protein content for feed purposes. After cracking, hot steam is applied to softened pieces of cotyledons to efficiently mold them into very thin flakes through rolling operations (Othmer & Jaatinen, 1956). The heat is also responsible for the inactivation of enzymes involved in lipid-degradation reactions (Mandarino et al., 2001). Flaking increases the seed's contact surface and disrupts cell walls, improving solvent extraction efficiency. During extraction, solvent must percolate a complex capillary system formed by the crushed seed matrix (Othmer & Jaatinen, 1956). Soybean flakes will present 0.2-0.4 mm thickness and 1-2 cm^2 surface. Soybean flaking is a step of major importance for oil retrieval and it has been used as pretreatment for other types of oil extraction, including aqueous enzyme-assisted (Lamsal et al., 2006).

2.2. Conventional soybean oil extraction

Classical knowledge on oil extraction postulates several traits desired for oil extraction solvents. No single compound possesses all features, so the optimal solvent of choice will vary

depending on the local circumstances and process priorities. Those features are: high solubility in oil at lower temperatures, low boiling point, high selectivity to triacylglycerols, low chemical reactivity, hydrophobicity, low flammability and explosiveness, low viscosity and surface tension (Birch, 2000). Hexane is compliant with many of these characteristics, so the oil extraction industry has developed technologically and logistically to the standards of this solvent (Li et al., 2004).

Although generally known as hexane, the most industrially used solvent worldwide is in fact a mixture of isomers, less costly and with greater extractability than pure n-hexane. This mix is commonly referred to as “commercial hexane” or “extraction hexane” (Anderson, 2018). Ultimately, hexane is the almost exclusive choice of solvent in the current oil-extraction process because it is the most energy efficient approach (Cheng et al., 2018).

Inside an oil-extraction plant hexane is used to remove the oil from the seed matrix, being later recovered and reused. This is repeated continuously, consolidated by what is known as countercurrent flow process. The most commonly used extractor for oilseeds such as sunflower, canola and soybean, is the percolator. In this process, stacked oilseed flakes are placed on a moving porous screen. This stack of seed flakes is referred to as “bed” (Becker, 1978). A heated mixture of solvent and oil, named miscella, is sprinkled onto the bed, percolating the seed flakes, draining the enriched miscella by gravity. Once collected, the now more concentrated miscella will be sprinkled onto a fresher, more concentrated in oil, portion of seed flakes. This miscella is capable of extracting oil from the flakes because of the concentration gradient. The system is structured to enable maximum utilization of the solvent power, still existing in concentrated miscellae until a certain oil limit is reached (Anderson, 2018). As the pure solvent or miscella is poured onto the oil-bearing material, it removes some amount of oil from the seed flakes, increasing the concentration of the miscella, which is then drained, collected and poured countercurrently onto flakes containing greater amounts of oil than the previous. At the final steps of the extraction run, seed flakes with little remaining oil will get the pure solvent instead of the miscella. After this process, flakes will ideally contain roughly 0.5% of oil (Regitano-d’Arce, 2006) and hexane present in the defatted seeds (meal) will be recovered in a steam toaster (Becker, 1978). In this process, the use of fresh solvent is kept to a minimum because of the high price, making up for the most efficiency of the countercurrent system (Anderson, 2018). The collected oil-concentrated miscella will leave the extractor to be further concentrated during solvent recovery distillation steps. Small amounts of solvent present in the crude oil will be further removed during oil refining.

After oil extraction with solvent, if there is enough water absorption from the environment to the oil, a deposit can be formed at the bottom of the oil tank, referred to as gums. Gums are mainly composed of phosphatides (phospholipids), but also sugars, fines (meal particles) and oil. Such deposits are fermentable and hygroscopic and must be removed (Djikstra, 2018). Phospholipids are

composed of fatty acids and a phosphate group esterified to a glycerol molecule. In phospholipids, the phosphate “head” is always attached to a compound that can be an amino acid, inositol, choline or other simple amines (Medic et al., 2014; Regitano-d’Arce, 2006). The phosphate moiety is strongly hydrophilic, contrasting with the non-polar fatty acid portion, which confers surface-active properties to phosphatides. Soy lecithin is a valued natural emulsifier, produced after purification of gums obtained during the degumming stage, and is composed of three main phospholipids: phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol (Van Nieuwenhuyzen & Tomás, 2008). The degumming step in industrial oil refining consists of the addition of water to the heated crude oil to induce the hydration and precipitation of phospholipids and other substances, facilitating its removal through centrifugation (Mandarino et al., 2001). However, a class of phosphatides, namely non-hydratable phosphatides demand acidification with phosphoric or citric acid in order to be removed (Leung et al., 2010).

The risks involved in the exposure to hexane in the oilseed processing plant are concentrated in the solvent extraction and desolventization areas, although risks still exist during solvent transfer from trucks to tanks, storage and potential piping leaks (EPA, 1978). These represent a great source of hazardous air pollutant emissions into the atmosphere (EPA, 2001). The hazard profile of hexane comprehends two main categories: fire and toxicity risks (Cheng et al., 2018). Thermal risks involve its high flammability and known likelihood to explode when the lower limit in air is reached (1.2% v/v) (Zabetakis, 1965). In fact, small amounts of hexane leaking into the air represent a more dangerous scenario than having larger amounts of the solvent and air mixing. For that reason, hexane’s fire-related hazards are considered major disadvantages of working with this solvent, having been the cause of many accidents involving extraction plants in the last century (Kingsbaker, 1983). As to other risks, hexane is linked to moderate acute toxicity to humans and other organisms and is strongly related to chronic toxicity, which is the development of physical adverse effects in the long-term exposure (Cheng et al., 2018).

To minimize the use of hexane or even improve its efficiency, many techniques have been studied in the past decades. The use of ultrasound was investigated to aid in hexane, isopropanol and hexane/isopropanol extraction of soybean oil (Li et al., 2004).

2.3. Chemical oil refining

Refining comprises three major steps: alkali neutralization, bleaching and deodorization. Refining, performed after oil extraction and degumming, has the purpose of removing compounds that might negatively impact the oil’s shelf-life and visual aspects in terms of commercial acceptance in

order to meet legal standards (de Souza et al., 2008). Alternatives to the chemical processing of oils following extraction have been demonstrated with the use of membranes (Manjula & Subramanian, 2007). Subsequent to degumming, neutralization with alkali will take place in order to remove free fatty acids. This is achieved by mixing the oil with aqueous NaOH to generate soap and water. Firstly, the oil is heated to constant temperature. The amount of caustic soda necessary to neutralize free fatty acid and the remaining phosphoric acid is calculated based on a simple titration (Zeldenrust, 2018). The oil is thoroughly mixed to the alkali and later separated from the soapstock. Washings are performed to remove residual alkalis in the oil. Once vacuum dried, oil is ready for bleaching, when it will be mixed with adsorbent and porous materials for removal of metals, soaps, pigments and oxidation products. Bleaching clays or "earths" are adsorbents with high surface area, capable of complex reactions (Corma, 1997; Brooks et al., 2018). Finally, during deodorization, the filtered bleached oil will be poured down a column with almost absolute vacuum and dry steam application from bottom to top, carrying away by distillation the acidity compounds still present, oxidation-related volatiles as well as characteristic odors (Rodrigues et al., 2014). Tocopherols are largely removed at this stage, remaining in the recovered distillate, namely soybean oil deodorizer distillate (SODD). Removal and loss of naturally occurring antioxidants is one of the major drawbacks of industrial oil refining, along with the massive energy and water expenditure as well as generation of untreated effluents (de Souza et al., 2008).

2.4. Biodiesel production

Biodiesel is the popular designation to a renewable fuel composed of fatty acid monoalkyl esters (Mittelbach & Gangl, 2001). This fuel is not chemically derived from the diesel fuel or any petroleum-based products. In the year of 1890, Dr. Rudolph Diesel invented the internal combustion engine to mechanize the local farming equipment by using vegetable oils as fuel (Demirbas, 2008). Many years after the invention of the diesel engine there was a need for lower viscosity fuels. This low-viscosity mixture of alkyl esters became known as biodiesel because it was intended to feed the diesel engine.

Industrial biodiesel production is carried out by transesterification reaction (Figure 2.1), in which a triacylglycerol molecule reacts stoichiometrically with three molecules of a short chain alcohol, typically methanol or ethanol (Li et al., 2013). This reaction replaces the alcoholic portion of the triacylglycerol ester (in this case, glycerol) with an ethyl or methyl group (if methanol or ethanol are used), thus generating three methyl or ethyl ester molecules and one glycerol molecule (Regitano-d'Arce et al., 2017).

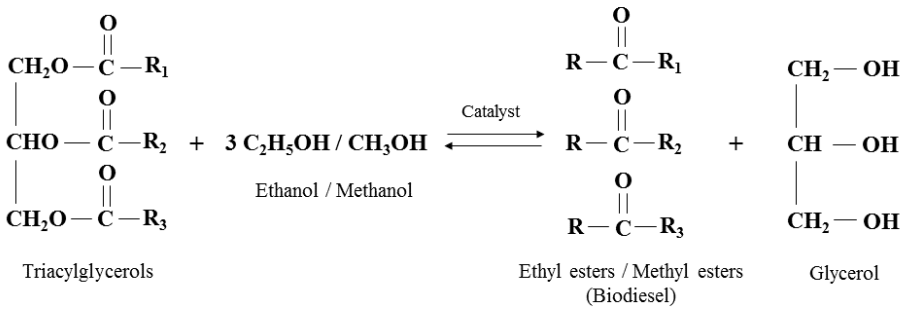


Figure 2.1. Transesterification reaction (Regitano-d'Arce et al., 2017)

One of the most important considerations in the industrial synthesis of biodiesel is to achieve maximum yield during transesterification. Several factors affect this yield, because it is a reversible chemical equilibrium. That is why the ethanol-to-oil molar ratio is kept high, as a way to overcome equilibrium shifts and ensure reaction completion (Atadashi et al., 2010). High contents of free fatty acids, phosphatides and water are undesirable to the feedstock, because, through several mechanisms, they interfere with the reaction kinetics, generate oil and biodiesel loss and can hinder phase separation (Savaliya et al., 2015). This is why the quality of the oil is of paramount importance (Pereyra-Irujo et al., 2009).

In order to lower the energy requirements of the transesterification, a catalyst must be employed. Non-catalytic transesterification, however, has been achieved by means of supercritical fluid technology (Zabeti et al., 2009). In this process, methanol or ethanol are pressurized and heated to their supercritical state where hydrophobicity, diffusion and solubility between oil and alcohol are improved, enabling high rate conversion to alkyl esters (Kulkarni & Dalai, 2006). Although high ester yields can be obtained with supercritical fluids, inconsistencies, high costs and complexity of this technology demand further studies (Li et al., 2013).

Catalyzed transesterification can occur via acid or alkaline. Many different catalysts have been tried and used along the years experimentally, each one with specific advantages and drawbacks. Heterogeneous catalysts do not readily dissolve in the oil/alcohol medium as the reaction occurs between chemical groups on the surface of theses solids (Guan et al. 2009). Heterogeneous catalysis (acid, alkaline, enzymatic) offer the possibility of being reused, provided some level of chemical recovery or activation is performed afterwards, since there is loss of catalyst activity over time (Knote & Razon, 2017). In homogeneous catalysis, the catalyst is solubilized in the alcohol, facilitating diffusion and mass transfer interactions with the oil phase (Vieitez et al., 2011). Homogeneous catalysis via alkaline transesterification is, by far, the most used route for industrial biodiesel production. Reliable and rapid conversion yields, low catalyst price and complexity of methodology are some of its

innumerous advantages. Alkalis such as NaOH and KOH are used to generate alkoxide ions which rapidly initiate the substitution of glycerol ester bond (Zabala et al., 2014). Alkaline transesterification works well under the recommendation limit of 1% on free fatty acid (FFA) levels in the feedstock and ideally anhydrous conditions (Samios et al., 2009). For that reason, highly acidic oils such as palm, jatropha and used frying oils have low performances with homogeneous alkalis (Tiwari et al., 2007; Enweremadu & Mbarawa, 2009). The homogenous route also demands extensive washing and purification steps, which greatly consumes water and energy resources, also generating large amounts of chemically untreated effluents (Refaat, 2010).

2.5. Ethanol as the alternative solvent to hexane

Oil extraction using heated anhydrous ethanol was first used in the 1930s by the South Railway Manchuria company in Dairen, Manchuria (current China), with the development of a pioneering soybean oil extraction plant (Okatomo, 1937). Advances in the field of extraction have brought awareness to the study of solvents and techniques less harmful than hexane (Sicaire et al., 2016; Chanioti et al., 2017). As part of the scientific mobilization in search for environmentally cleaner processes and lower risk for the analyst, the extraction of oil using ethanol has been studied as an alternative to commercial extraction of oil with hexane (Johnson & Lusas, 1983; Capellini et al., 2017).

At 78 °C this extraction can reach the same efficiency than hexane in conventional processes (Beckel et al., 1946). The solubility of ethanol in oil has been shown to be a directly dependent function of temperature and indirectly dependent on solvent hydration (Johnson & Lusas, 1983). As the hot mixture of oil and solvent collected from the extractor begins to cool, two phases appear. The lower (denser) phase has 6-8% ethanol and roughly 90% oil, namely oil-rich miscella or, simply, rich miscella (Beckel & Smith, 1944). The upper phase is mostly composed of ethanol (85-95%), containing also small amounts of oil (1-3%), hereafter named oil-poor miscella, or just poor miscella. Due to ethanol's high polarity, poor miscella concentrates primarily water, phospholipids and free fatty acids as well as minor compounds such as sugars, pigments, phenolics, phosphatides, saponins, non-protein nitrogen compounds and sterols, among other compounds (Beckel & Smith, 1944; Beckel et al., 1948a). It can be said that phase separation promotes a partial refining of the rich miscella (Sangaletti-Gerhard et al., 2014). Deacidification and phospholipid reduction of rice bran oil has been performed with ethanol by Rodrigues et al. (2014), based on this affinity to polar compounds. The high content of ethanol present in the poor miscela allows its direct use as solvent in a next extraction. Reusing the poor miscela as a solvent avoids unnecessary overuse of new anhydrous ethanol (Rao & Arnold, 1958; Regitano-d'Arce & Lima, 1987; Regitano-d'Arce, 1991).

Ethanol extraction produces light-colored soybean meal, due to the high affinity of this solvent to color compounds and phospholipids (Beckel et al., 1948b; Capellini et al., 2017). In addition to the visual benefits on the meal, ethanol eliminates the bitterness and "bean flavor", common in legumes such as soybeans, producing more pleasant flavors and aroma in the meal. Ethanol also removes flatulence agents (oligosaccharides) and antinutritional factors (lectins and protease inhibitors) (Beckel & Smith, 1948a; Regitano-d'Arce, 2006). These advantages over hexane extraction enable the production of a high-quality protein product directed for food and feed industries.

Many lab-scale studies carried out at the Laboratory of Oils and Fats at ESALQ/USP (Borges, 2014; Sangaletti et al., 2013; Sangaletti-Gerhard et al., 2014) have addressed the production of ethyl esters (biodiesel) from soybean oil-rich miscellae. The rich miscella has been directly inserted into the transesterification reaction without the need for any previous treatment, due to the partial refining properties resulting from phase separation (Figure 2.2). The use of the rich miscela as feedstock for alkali-homogeneous and enzymatic transesterification has demonstrated to be technically feasible (Sangaletti et al., 2013; Sangaletti-Gerhard et al., 2014). Since the presence of an alcohol is mandatory in biodiesel synthesis, there is no need for removal of the remaining ethanol in the rich-miscella. By simply adding more ethanol to make up for the correct molar ratio required by the reaction, the rich miscella can be used in transesterification directly after leaving the extractor. This way, the energy expenditure of solvent evaporation after extraction is avoided. In hexane extraction, the desolventization step is mandatory and, besides being a high energy-demanding step, submits the oil to another thermal process.

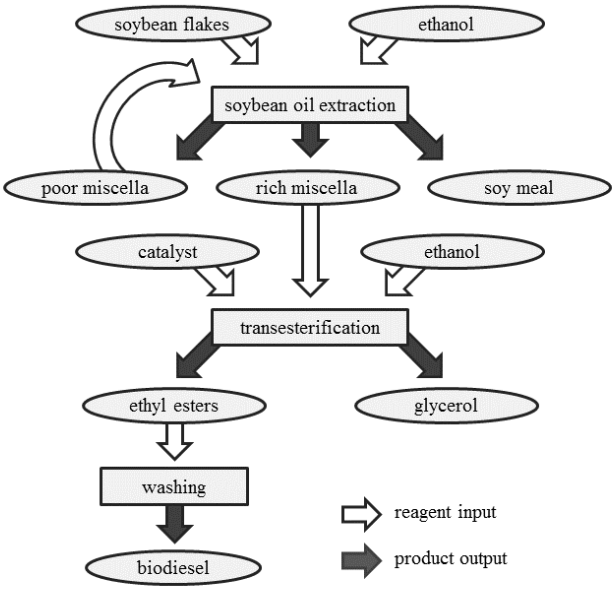


Figure 2.2. Flow chart of the materials and products involved in the production of biodiesel from soybean oil obtained by ethanol extraction (Regitano-d'Arce et al., 2017)

The same way that phospholipids and free fatty acids are carried by affinity, other polar compounds with antioxidant activity are removed from the seed matrix. This way antioxidant compounds, such as phenolics, are concentrated in the poor miscella, but also remain in smaller amounts in the rich miscella. Therefore, a range of antioxidants that would normally be restrained to the meal and meal-derived products can now be found in vegetable oils in significant quantities. This opens a new realm of opportunities to study the interactions between phenolics in lipid medium.

2.6. Antioxidants

Antioxidants are compounds that, in general, prevent or significantly delay the oxidation of molecules (Antolovich, 2002). They comprise many classes of molecules that, through different mechanisms, are capable of conferring protection against oxidation reactions.

These compounds are usually classified into two major categories, based on their protective mechanism: primary and secondary. The first relates to the ability of donating hydrogen atoms (Hydrogen Atom Transference - HAT) or transferring electrons, chain-breaking action. Secondary (preventative) antioxidants have the role of regenerating primary antioxidants. Some antioxidants can also have both primary and secondary antioxidant properties.

2.6.1. Tocopherols

Compounds known as tocopherols are derived from the methylation of tocols (benzopyrans), only found in the plant kingdom (Christie, 2014). Tocopherols are capable of providing a hydrogen atom to inhibit a peroxyl radical, which generates a hydroperoxyl compound and an unstable radical tocopherolyl. The latter will in turn react with a peroxyl radical again (Kamal-Eldin & Appelqvist, 1996). Thus, tocopherols interrupt chain reactions (chain-breaking activity) associated with lipid free radicals. They can be regenerated by ascorbic acid or other electron-donating antioxidants (El-Gendy et al., 2010). The structure of tocopherols is composed of the following components: a benzene ring, referred to as the chromanol head, holding a variable number of methyl groups, associated to a pyran ring connected to a saturated carbon chain of 16 carbons (phytyl chain) (Figure 2.3). The benzene ring of tocopherols has a hydroxyl group, resulting in tocopherols reactivity to the Folin-Ciocalteau reagent, used in quantitative analyzes of phenolic compounds (Evans et al., 2002). Tocopherols can be found as four homologues, namely α, β, γ, and δ. Structurally, they differ in the number and position of methyl groups on the chromanol head. The β and γ homologues present two methyl groups and can, therefore be called isomers. The δ and α homologues present one and three methylated carbons, respectively, at the chromanol ring. Each tocopherol homologue possesses three asymmetric carbons along the side chain, comprising three chiral centers or stereocenters. This feature results in the existence of eight stereoisomers for every homologue. The effects and importance of structural variations in the side chain on the antioxidant activity of tocopherols are not fully understood (Klein et al., 2007; Sontag & Parker, 2007). Tocopherol homologues present different reactivities within *in vitro* and biological systems but follow the general order of α > γ > β > δ. However, the designation of “vitamin E” can be, so far, only correctly attributed to the α-homologue (Azzi, 2017).

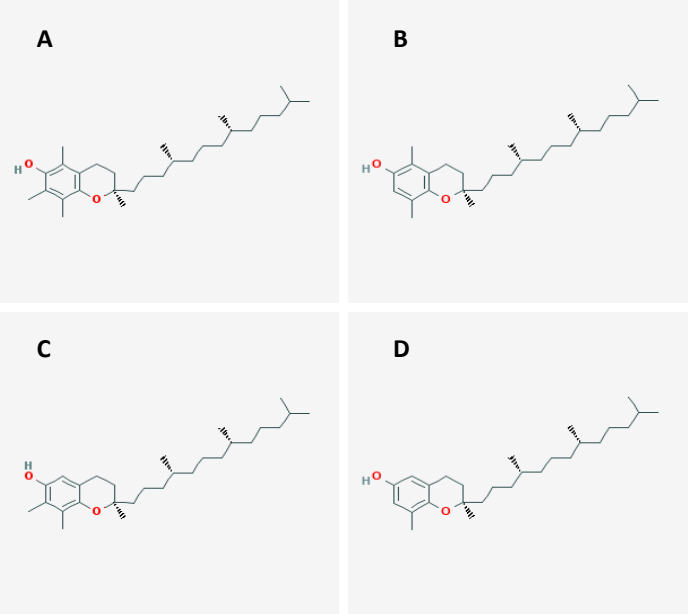


Figure 2.3. Molecular structure of the four tocopherol homologues. A. alpha-tocopherol; B. beta-tocopherol; C. gamma-tocopherol; D. delta-tocopherol. Adapted from: National Center for Biotechnology Information (2018)

2.6.2. Carotenoids

Carotenoids are antioxidants that present and confer color to fruits and other foods, being natural pigments synthesized only by vegetables and some microorganisms (Rivera et al., 2012). The terpenoid structure (40 carbons) derived from the attachment of isoprene units is the most frequently found and is composed, almost exclusively, of conjugated bonds of trans configuration (Skibsted, 2012). The presence of cyclic structures, at one or both ends, characterize the compounds as carotene, that is, of apolar nature and without the presence of oxygenated clusters (Figure 2.4). Carotenoids with oxygenated functions are lipophilic compounds that have a certain polarity and are called xanthophylls (Biehler et al., 2010). In plant structures, xanthophylls are commonly allocated at water/lipid interface regions (Skibsted, 2012). Carotenoids have an important role in the sequestration of activated oxygen species, associated with oxidation reactions catalyzed by light. In these reactions, compounds such as chlorophyll play the role of low energy oxygen activation to the singlet state. The action of carotenoids on singlet oxygen is limiting according to the diffusion of the compound, related to the number of double bonds in its chain. The energy of singlet oxygen is transferred to the carotenoid without further reaction. The carotenoid dissipates this energy to the medium (solvent), returning to its initial state (Stahl & Sies, 2003). Carotenoids also have sequestering properties of peroxyl radicals, generated in the lipid oxidation processes (Paiva & Russel, 1999). Unlike the action on singlet oxygen, radical quenching by carotenoids depends on the formation of intermediates and implies oxidation of the carotenoid, generating secondary compounds (Stahl & Sies, 2003). Among the

oxygenated carotenoids usually found in soybeans are lutein, cryptoxanthin and zeaxanthin (Slavin et al., 2009).

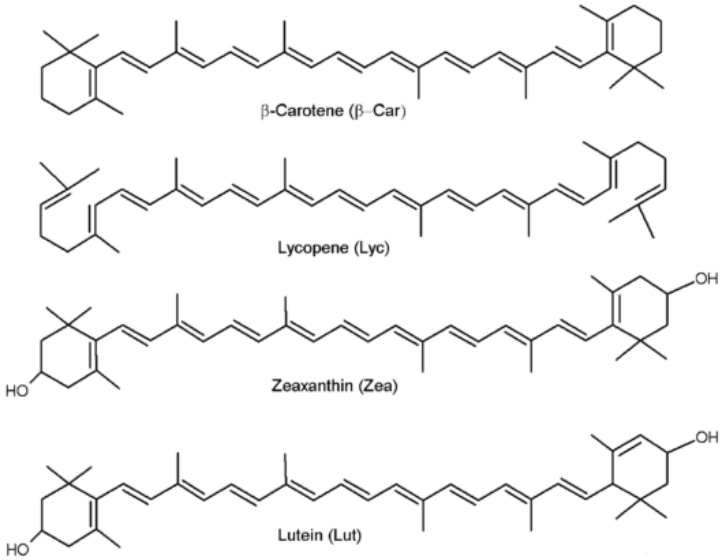
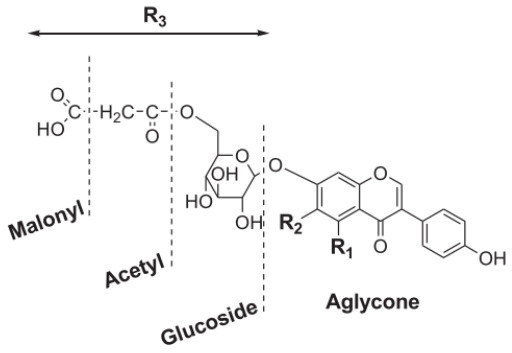


Figure 2.4. Structure of common carotenoids (Skibsted, 2012)

2.6.3. Isoflavones

Isoflavone distribution in plants is mainly restricted to the *Leguminosae* family, subfamily *Papilionoideae*, comprising soybeans, pulses, chickpea, beans and others (Villares et al., 2011). The presence of isoflavones is extensively associated to soybeans and its derivatives, such as tofu, tempeh, natto, soymilk and shoyu (Ikeda et al., 2006; Miura et al., 2016). These aromatic metabolites are a subcategory of the major group of flavonoids for their characteristic 3-ringed backbone structure. Isoflavones are more often found in nature as their glycosylated forms (Arora et al., 1998). They represent around 70% of the phenolic compounds existing in soybeans (Haron et al., 2009).

Three main isoflavones, daidzein, genistein and glycitein, are present in soybean varieties along with their glucoside, acetyl-glucoside and malonyl-glucoside derivatives, totalizing twelve known soybean isoflavones (Figure 2.5) (Cho et al., 2013). They are naturally converted to aglycones by β-glucosidase enzymes. However, glucoside-hydrolyzing activity can be enhanced with the use of microorganisms and enzyme extracts (Kuo et al., 2006; Wei et al., 2008; Prasad & Shah, 2011) or through processing of the grain and its derivatives (Jackson et al., 2002). Studies show enhancement on aglycone conversion during soaking of whole soybeans (Matsuura et al., 1995), hydrothermal treatments (Lima & Ida, 2014), ultrasound treatment prior to soaking (Falcão et al., 2018) and infrared drying of soybeans (Niamnuy et al., 2012).



Category	Isoflavone	R_1	R_2	R_3
Aglycone	Daidzein	H	H	H
	Genistein	H	OH	H
	Glycitein	OCH_3	H	H
Glucoside	Daidzin	H	H	$C_6O_5H_{11}$
	Genistin	H	OH	$C_6O_5H_{11}$
	Glycitin	OCH_3	H	$C_6O_5H_{11}$
Acetyl	Acetyldaidzin	H	H	$C_6O_5H_{11}$+$COCH_3$
	Acetylgenistin	H	OH	$C_6O_5H_{11}$+$COCH_3$
	Acetylglycitin	OCH_3	H	$C_6O_5H_{11}$+$COCH_3$
Malonyl	Malonyldaidzin	H	H	$C_6O_5H_{11}$+$COCH_2COOH$
	Malonylgenistin	H	OH	$C_6O_5H_{11}$+$COCH_2COOH$
	Malonylglycitin	OCH_3	H	$C_6O_5H_{11}$+$COCH_2COOH$

Figure 2.5. Twelve individual isoflavones present in soybeans (Lee et al., 2015)

The antioxidant activity of isoflavones in biological systems has been extensively studied, considered of utmost importance for the medical and nutritional science fields due to the association with prevention of degenerative diseases (Jenkins et al., 2002; Kulling et al., 2001). Researchers believe that the absorption of aglycones by humans is faster and occurs in higher amounts than that of the glycosylated counterparts (Izumi et al., 2000; Miura et al., 2016).

Isoflavones have shown to possess a greater protective role in metal-induced lipid peroxidation than in peroxidation systems induced by peroxyl radicals (Arora et al., 1998). Isoflavone presence is highly associated to the protein/hydrophilic fraction of legumes. It can be said that the antioxidant role of isoflavones in soybean oil and other lipids matrixes is still unexplored, since conventional methods of oil extraction render negligible amounts of these compounds.

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3. A HIGHLY STABLE SOYBEAN OIL-RICH MISCELLA OBTAINED BY ETHANOLIC EXTRACTION AS A PROMISING BIODIESEL FEEDSTOCK

Adapted with permission from BUENO-BORGES, L. B.; DE CAMARGO, A. C.; SANGALETTI-GERHARD, N.; ALENCAR, S. M.; SHAHIDI, F.; REGITANO-D'ARCE, M. A. B (2017) A highly stable soybean oil-rich miscella obtained by ethanolic extraction as a promising biodiesel feedstock. J Am Oil Chem Soc 94(8):1101-1109. DOI: 10.1007/s11746-017-3012-0.

ABSTRACT

Soybean oil is industrially obtained upon hexane extraction. In biodiesel production, soybean oil is submitted to phospholipid removal in order to improve its quality before transesterification. An extraction process was employed to produce ethanolic oil-rich miscella, which can be directly transesterified to produce biodiesel without prior refining. We assessed the oxidative stability of the miscella and three other soybean oils, namely degummed, alkali-refined and refined-bleached-deodorized (RBD) oil. *In vitro* antioxidant assays as well as the identification and quantification of tocopherols and isoflavones were also performed. Although hexane-extracted oils showed higher tocopherol contents than miscella, this latter sample and its direct biodiesel demonstrated superior stability in accelerated tests. Miscella also outperformed hexane-extracted oils in all *in vitro* assays. This behavior can be explained by the presence of phenolic compounds with higher affinity to ethanol than hexane, which was confirmed by the identification of isoflavones glycitein, genistin, and acetyldaidzin, found only in miscella. This study showed that the ethanolic extraction of soybean oil generated a highly stable lipid feedstock for biodiesel manufacture.

Keywords: bioethanol; Oxidative stability; Antioxidants; Esterification

3.1. Introduction

Vegetable oils are used in many applications, including the production of biodiesel fuel. The latter can be essentially defined as methyl or ethyl esters of fatty acids. When originated from seeds such as soybean, rapeseed and cotton, oils are industrially obtained through continuous extraction using hexane and its homologues as petroleum-based solvents employed worldwide (Beckel & Smith, 1944). Crude vegetable oils must be submitted to a series of processes known as refining (degumming, alkali-refining, bleaching and deodorization) in order to comply with market-required standards. Industrial refining eliminates impurities, minor compounds and prooxidants, thus improving vegetable oil shelf-life. Since transesterification is still largely performed at a facility other than that of oil extraction, lipid degradation reactions may happen during storage and transportation. Therefore, freshly extracted soybean oil must be at least submitted to degumming (removal of phospholipids) to ensure stability during this period. Removal of phospholipids as well as free fatty acids from the lipid feedstock is mandatory as they hinder transesterification catalyst's activity (Freedman et al., 1984).

Extraction of soybean oil with ethanol has been described initially by the pioneer work of Okatomo (1937). After cooling, the product of this process naturally separates into a solid phase consisting of gum (lecithin) and two liquid microemulsions: a dense, oil-rich phase and an upper phase, consisting mostly of ethanol. The lower phase is roughly 6-8% ethanol and 90% oil (oil-rich miscella). The upper phase has about 90% ethanol and 1.5% oil, known as poor miscella (Beckel et al., 1948; Sangaletti-Gerhard, 2014a). The poor miscella, collected after extraction, can be stored and further used as a solvent in the following extraction cycle, thus eliminating the need to distill the poor miscella to recover ethanol (Rao & Arnold, 1958). With the natural phase separation, poor miscella carries the majority of polar substances such as phospholipids, water and free fatty acids due to its high ethanol concentration. Therefore, it can be inferred that this phenomenon promotes a partial refining of the rich miscella (Sangaletti-Gerhard, 2014a; Nieh & Snyder, 1991). Ethanol carries phosphatides and free fatty acids based on affinity; however, the extraction of minor polar compounds with antioxidant capacity is also contemplated. In this sense, it is believed that the produced miscella may present higher resistance to oxidation than vegetable oils that have been subjected to refining following hexane extraction.

In continuation of our past research, we have focused on direct use of rich miscella in transesterification reactions without prior refining. The study of Sangaletti-Gerhard et al. (2014a) describes the optimized ethanolic extraction of soybean oil followed by direct production of fatty acid ethyl esters. This unified operation actively contributed to lower the overall energy demand for biodiesel production as ethyl esters, since the degumming step and related oil losses are avoided (Sangaletti-Gerhard et al., 2014b).

In contrast to hexane, ethanol is a low-toxicity solvent, produced from renewable sources. It has been demonstrated that ethanol usage in extraction processes improves organoleptic and functional properties of soybean meal (Beckel et al., 1948). Several advantageous aspects of the extraction using ethanol over hexane have been described before (Sangaletti-Gerhard et al., 2014a). However, although the composition of the oil-rich miscella has been previously determined with regard to its major components, there was still need to investigate the existing antioxidant compounds and their effects on the stability of this lipid feedstock and its direct biodiesel. Therefore, this work aimed to evaluate the resistance to thermal oxidation of a soybean oil ethanolic miscella in comparison to a degummed, an alkali-refined and a commercial-grade RBD soybean oil obtained by the conventional hexane extraction process as a potential feedstock for biodiesel production.

3.2. Materials and Methods

3.2.1. Materials

Soybean flakes were kindly provided by Granol S/A (Bebedouro, SP, Brazil). Degummed, alkali-refined soybean and RBD soybean oils were provided by Cargill S/A (Mairinque, SP, Brazil). Absolute (99.5%, v/v) ethanol, acetic acid, isooctane, potassium iodide (KI), potassium phosphate (K_3PO_4), sodium hydroxide (NaOH), sodium sulfate (Na_2SO_4), starch powder and hydrochloric acid (HCl) were analytical grade, purchased from Synth (Diadema, SP, Brazil). HPLC grade methanol, hexane and acetonitrile were purchased from JT Baker (Phillipsburg, NJ, USA). Formic acid was obtained from Chemco (Hortolandia, SP, Brazil). Tridecanoic acid and standard GLC-87 FAME mix were Nu-Check Prep (Elysian, MN, USA). 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman- 2-carboxylic acid (Trolox), fluorescein (30,60-dihydroxy-3H-spiro[2-benzofuran-1,90-xanthen]-3-one), α-tocopherol, rac-β-tocopherol, γ-tocopherol, δ-tocopherol were obtained from Sigma-Aldrich (St Louis, MO, USA).

3.2.2. Production of soybean oil ethanolic miscella

The oil extraction was carried out as described by Sangaletti-Gerhard et al. (2014a). The procedure was conducted in a double wall stainless steel 13 L capacity tank. A thermostat and a thermometer were used to program and monitor the temperature of the tank's interior at ethanol's boiling point. The system also comprised a 1.5 m long condenser for ethanol recovery. One kilogram of soybean flakes was placed in a cotton bag inside the tank followed by the addition of 1 L solvent for soaking during 30 min at room temperature. The extraction comprised four cycles, consisting of immersion in the solvent at 78 °C (Fig. 3.1). This 4-cycle process of oil-extraction of 1kg of soybeans is hereafter named batch. At each cycle, 2 L of solvent were added to the mass of flakes and the extract was collected at the end of one hour. For the first three cycles of extraction and the soaking stage, poor miscella was used as a solvent. Absolute ethanol was used in the fourth cycle to ensure complete extraction of the remaining oil. The collected output of the four cycles was combined and cooled down to room temperature allowing its contents to reach phase separation. At room temperature, the two phases (poor miscella and rich miscella), naturally separated by their individual densities, were collected separately, vacuum filtered (for gum removal) and stored under -4 °C. The upper layer (poor miscella) was reinserted in further batches as solvent. The collected denser oil-rich layer, hereafter named miscella, was the feedstock for ethyl esters production (biodiesel), using ethanol and alkali

catalyst. Since the amount of miscella rendered in one batch is approximately 150-160g, three batches of extraction were performed to generate the miscella used in this study.

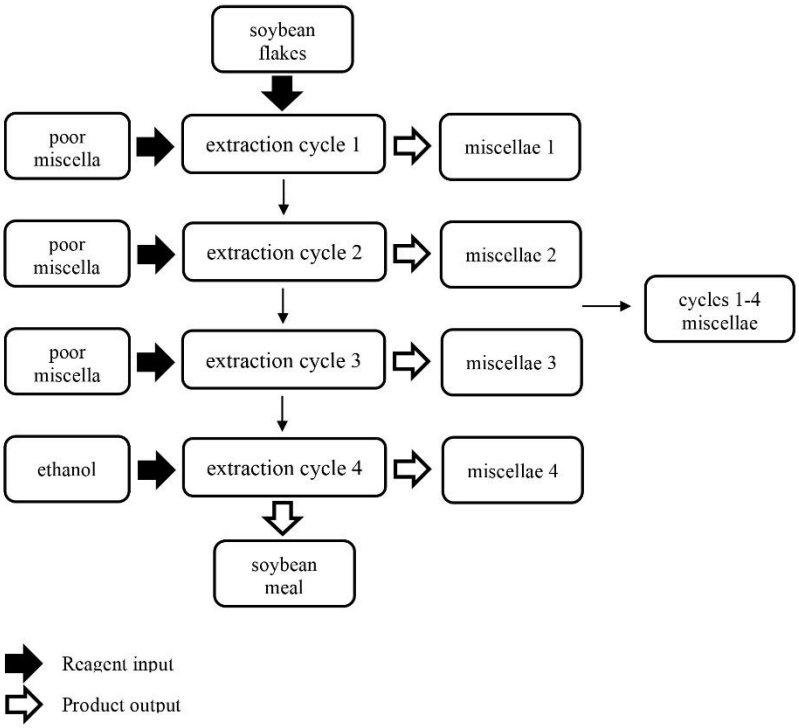

Figure 3.1. Soybean oil extraction with ethanol

With the exception of the oil-rich miscella, oil samples were obtained by an extraction process using hexane and then concentrated by evaporation until complete removal of the solvent. Following that, each of the hexane-extracted oils was submitted to one (degummed) or more refining steps (alkali-refined; RBD oil) and later used as feedstock in biodiesel production. Miscella, however, was obtained, stored and later used in the transesterification reaction without the need of solvent evaporation or any refining steps.

3.2.3. Characteristics of the soybean oil-rich miscella

Lipid content was evaluated based on the method described by Hara and Radin (1978). A mixture of 18 mL of 3:2 (v/v) hexane/isopropanol was added to a separation funnel containing one gram of the homogenized oil sample. The funnel was closed and vigorously shaken, releasing the pressure afterwards. Twelve milliliters of 0.5 M sodium sulfate were added to the funnel and, after mixing, phases were allowed to separate. The upper phase was transferred into a tared flat-bottomed

flask and the solvent evaporated at 45 °C to dryness. The flask was then placed in an oven at 105 °C for 1h and, after cooling inside a desiccator, lipid content was determined gravimetrically. Alcohol content of the miscella was determined by prior distillation of 25 mL samples. Distilled fractions were collected in 25 mL volumetric flasks and analyzed in a digital densimeter (DMA-48, Anton Paar, Graz, Austria). With the exception of the sample used in biodiesel production, ethanol was removed from miscella by rotary evaporation at 45 °C prior to further analyses.

3.2.4. Fatty acid composition of oil samples

Assessment of the oil samples' fatty acid profile was performed by means of gas chromatography. Esterification of fatty acids was prepared according to Hartman and Lago (1973) as described in details by de Camargo et al. (2012). Tridecanoic acid was used as an internal standard and the identification of compounds was based on a standard mix. A Shimadzu GC-2010 plus AF, equipped with RTX-Wax (30 m; 0.32mm; 0.25 μm) – Crossbond Carbowax polyethylene glycol column, FID detector, auto-injector and split injection port was used. Injector and detector temperatures were fixed at 250 °C. Nitrogen was employed as the carrier gas (1.2 mL/min). Sample was injected (1μL) at a 1:20 split ratio. Column oven was set as follows: 60°C (held for 0 min), 20 °C/min up to 210 °C (held for 7 min) and 30 °C/min up to 240 °C (held for 15 min).

3.2.5. Biodiesel production (fatty acid ethyl esters)

The conditions used in the transesterification of soybean oil samples were based in a previous study (Sangaletti-Gerhard et al., 2014a). All four oil samples reacted with ethanol and NaOH as catalyst to yield fatty acid ethyl esters (biodiesel). Transesterification was carried out in a 125 mL flat bottom boiling flask under reflux (Table 3.1). After complete dissolution of NaOH in ethanol, the oil sample was poured inside the flask and the reaction was conducted using a magnetic stirring. At the end of the reaction, the excess ethanol was removed by rotary evaporation at 50 °C followed by the addition of 5 g of pure glycerin to induce phase separation. The biodiesel layer was transferred to a separation funnel, washed thoroughly with HCl (0.05%) and water until neutralization. The biodiesel was subsequently filtered through layer of anhydrous sodium sulfate to remove residual water. Three replicates were performed for each sample. Ethyl esters yield was determined by gas chromatography according to the Standard ABNT NBR 15764:2012 (2012).

Table 3.1. Conditions used in the transesterification of soybean oil samples

Variables	Miscella[a]	Hexane-extracted oils[b]
Quantity of oil used	50 g	45 g
NaOH % (m/m oil)	0.6	0.3
Ethanol to oil molar ratio	12:1	12:1
Stirring speed	1000 rpm	1000 rpm
Reaction time	1 h	1 h
Temperature	30 °C	30 °C

[a] Conditions showed a satisfactory yield (>95%) for all samples

[b] Degummed, degummed and alkali-refined, and refined-bleached-deodorized oils

3.2.6. Oxidative stability of vegetable oils using Rancimat test

To evaluate the resistance of samples to oxidation, the Rancimat method and an accelerated oven test were used. The former was conducted using a 743 model Rancimat equipment (Metrohm Ltd, Herisau, Switzerland) according to the AOCS method Cd 12b-92 (2003). Vegetable oil samples (3 g) were weighed in test tubes and heated at 110 °C with an airflow of 10 L/h. Similarly, the same equipment and parameters were used to analyze biodiesel. The induction period was assessed in quadruplicates, expressed in hours.

3.2.7. Oxidative stability under Schaal oven conditions

Accelerated oxidation test was conducted at 62 °C ± 2 in a forced air oven over a period of 8 days. Oil samples were placed in open clear glass vials and each was removed every 48 h. Peroxide value analyses were performed according to the AOCS method Cd 8b-90 (2003), as follows. In an Erlenmeyer flask, 1g of oil sample was dissolved in 50 mL 3:2 (v/v) acetic acid/isooctane and 0.5 mL of a saturated KI solution was added. After exactly 1 minute, 30 mL water were transferred to the flask and the sample was titrated with 0.1 N $Na_2S_2O_3$ solution, using 1% starch solution as an indicator until complete disappearance of the blue color.

3.2.8. Tocopherol content

The identification and quantification of tocopherols were carried out for the oil samples according to the AOCS method Ce 08-89 (2003). Details of the procedure have appeared elsewhere (de Camargo et al., 2016). In short, determinations were carried out by normal phase HPLC, model LC-6AD, using a model RF-10AXL fluorescence detector (Shimadzu Scientific Instruments Inc., Columbia,

MD, USA) set at excitation and emission of 290 and 330 nm, respectively. A Lichrospher Si60 column (5µm, 25cm × 4mm i.d., E. Merck, Darmstadt, Germany) was used. Injections of 10µL per run were used and elution was carried out at a flow rate of 1.0 mL/min using an isocratic hexane-isopropanol (99:1, v/v) mobile phase. Tocopherol homologues (alpha, beta, delta, and gamma) were quantified using external calibration with authentic standards.

3.2.9. HPLC-DAD-ESI-MS^n analysis

The identification and quantification of major phenolics present in the oil samples was performed according to minor modifications of the method published elsewhere (de Camargo et al., 2015). Analyses were performed on an Agilent 1100 system (Agilent) equipped with a G1311A quaternary pump, a G1379A degasser and a G1329A ALS automatic sampler, a G1130B ALS Therm, a G1316 Colcom column compartment, A G1315B diode array detector (DAD) and a system controller linked to Chem Station Data handling system (Agilent). Separations were conducted with a SUPERLCOSILTM LC-18 column (4.6 × 250 mm × 5 µm, Merck, Darmstadt, Germany). The binary mobile phase consisted of 0.1% formic acid (A) and 0.1% formic acid in acetonitrile (B). The flow rate was adjusted to 0.5 mL/min and the elution gradient used was as follows; 0 min, 100% A; 5 min, 90% A; 35 min, 85% A; 45 min, 60% A; held at 60% A from 45 to 50 min; afterward mobile phase A was increased to 100% at 55 min, followed by column equilibration from 55 to 65 min. The compounds were detected at 280 nm, and the samples were filtered before injection using a 0.45 µm PTFE membrane syringe filter (Thermo Scientific, Rockwood, TN, USA). HPLC-ESI-MS^n analysis was carried out under the same conditions as described above using an Agilent 1100 series capillary liquid chromatography/mass selective detector (LC/MSD) ion trap system in electrospray ionization (ESI) in both the positive and negative modes. The data were acquired and analyzed with an Agilent LC/MSD software (Agilent). The scan range was set in a range from m/z 50 to 600, using smart parameter setting, drying nitrogen gas at 350 °C, flow 12 L/min, and nebulizer gas pressure of 70 psi. Phenolic compounds were tentatively identified according to their ion fragmentation patterns using tandem mass spectrometry (MS^n), UV spectral and literature data.

3.2.10. Total phenolic content (TPC)

Extracts were prepared using two grams of each oil sample, which were thoroughly mixed with 4 mL of ethanol, centrifuged and the supernatant collected. This procedure was repeated three more times. The solvent was then evaporated to dryness at 40 ºC and the extract was dissolved in 5

mL methanol. Extracts for antioxidant determination were prepared in triplicate. TPC determination was performed by the Folin-Ciocalteau method using a microplate reader SpectraMax M5 (Molecular Devices, Sunnyvale**,** CA, USA) according to Al-Duais et al. (2009). To each well, 20 µL of extract or ethanol as a blank or standard solution were added along with 100 µL Folin-Ciocalteau (10% aqueous solution), followed by mixing. After 5 min 75 µL of 7.5% solution of sodium carbonate were added and the plate was mixed once more. After 40 min in the dark, the absorbance of the samples was read at 730 nm. The results were expressed as mg gallic acid equivalents per gram of sample.

3.2.11. Trolox equivalent antioxidant capacity assay

The ability of previously prepared extracts to scavenge the ABTS radical cation was measured in comparison with different concentrations of trolox (Re et al., 1999). The method described by de Camargo et al. (2015) was used with minor modifications. In brief, 88µL sodium persulfate (140 mM) were added to every 5 mL of 7 mM ABTS solution in 75 mM potassium phosphate buffer, thus generating the radical for a period of 16 hours. The $ABTS^{\bullet+}$ solution was then diluted to 0.7 ± 0.02 abs at 734 nm. Twenty microliters of sample, potassium phosphate (blank) or standard were mixed with 220 µL of $ABTS^{\bullet+}$ solution, after 6 min of reaction the absorbance was read at 734 nm using a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA).

3.2.12. Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay (Chiste et al., 2011) was performed using a 96-well microplate and to each well 30µL of extract, 60µL of 508.25 nM fluorescein solution and 110µL 76mM AAPH solution were added. Both fluorescein and AAPH solutions were prepared in 75mM potassium phosphate buffer (pH 7.4). Phosphate buffer was used instead of the sample to compose the blank. Using a SpectraMax M3 microplate reader (Molecular Devices, Sunnyvale, CA, USA), emission and excitation wavelengths were set at 528 and 485 nm, respectively, and readings were carried out every minute for 2 h at 37 °C. Areas under the fluorescence decay curve (AUC) were calculated using the equation below (Eq. 1). Trolox was used as standard to build a calibration curve with concentrations ranging from 12.5 to 400 µM. The net integrated AUC from the standard curve and samples was obtained after subtraction of the AUC from blank.

$$\text{Eq. 1} \quad AUC = 1 + \left(\frac{f_1+f_2+f_3+\cdots f_i}{f_0}\right)$$

Where: f_0 is fluorescence at t_0 and f_i is fluorescence at t_i.

3.2.13. Statistical analysis

Unless stated otherwise, all analyses were executed in triplicate and results are expressed as means ± standard deviation. Statistical significance among means was assessed by one-way ANOVA at the level of $p < 0.05$. Tukey's test for multiple comparisons was performed using Minitab 17 statistical software (Minitab Inc., State College, PA, USA).

3.3. Results and Discussion

The oxidative stability of a vegetable oil may be analyzed as a function of its fatty acid composition, degree of unsaturation, protection granted by natural antioxidants, and effects of existing compounds with direct and indirect antioxidant/prooxidant action (Hamilton et al., 1997). Fatty acid profile of the samples is shown in Table 3.2. Results indicate that the fatty acid profile should not play a major role in oxidative stability of different samples because the contents of saturated and unsaturated samples were quite similar among all samples tested. Miscella produced in this study contained 7.2% ethanol and 91.1% lipids, which is consistent with the literature (Sangaletti-Gerhard et al., 2014a).

Table 3.2. Fatty acid profile of soybean oils extracted with ethanol and with hexane from different industrial refining steps (g/100 g of oil)

Fatty acid	Soybean oils			
	miscella	degummed	alkali-refined	RBD
C16:0	11.4 ± 0.2a	11.1 ± 0.1a	11.0 ± 0.0a	11.0 ± 0.2a
C18:0	3.78 ± 0.0c	3.86 ± 0.0d	3.47 ± 0.0a	3.66 ± 0.0b
C18:1	22.6 ± 0.1a	23.1 ± 0.0b	23.9 ± 0.2c	23.4 ± 0.1b
C18:2	54.8 ± 0.1a	54.5 ± 0.4a	54.2 ± 0.3a	55.6 ± 0.0b
C18:3	6.48 ± 0.0ab	6.51 ± 0.0b	6.38 ± 0.1a	5.26 ± 0.0c
C20:0	0.34 ± 0.0a	0.34 ± 0.0a	0.36 ± 0.0a	0.36 ± 0.0a
C20:1	0.26 ± 0.0a	0.24 ± 0.0a	0.30 ± 0.0b	0.23 ± 0.0a
C22:0	0.36 ± 0.0a	0.40 ± 0.0a	0.39 ± 0.0a	0.39 ± 0.0a
Total saturated	15.88	15.71	15.24	15.43
Total unsaturated	84.11	84.29	84.76	84.58

Data represent mean ± standard deviation for each sample (n = 3). Means followed by different letters within a row show difference among samples ($p < 0.05$). RBD stands for refined-bleached-deodorized oil

Induction periods of oil samples and their respective ethyl esters, obtained by the Rancimat test, are shown in Fig. 3.2. This assay accelerates the oxidation rates to a higher extent than Schaal's

test, and has been established as an official method to assess biodiesel oxidative stability. The ethanolic miscella displayed a high induction period (25.13 h), showing up to 3.5-fold higher stability as compared with its counterparts. Likewise, ethyl esters obtained from the oil-rich miscella showed up to 2.1-fold higher stability than that of the remaining samples. Ethyl esters derived from RBD oil presented greater resistance than those of degummed and alkali-refined oils. This may have been caused by the presence of prooxidant compounds that remained in these samples after biodiesel conversion and purification steps. For all samples, there was a significant reduction of the induction period from the oil to its ethyl ester derivative. This difference is also found when oils are converted into methyl esters (Pereira et al., 2013). The structural aspect between oils and alkyl esters could play an important role on this matter. PUFA are usually located at the sn-2 position of naturally occurring triacylglycerols, therefore sn-1 and sn-3 fatty acids may confer protective effects against oxidation due to steric hindrance (Shahidi & Zhong, 2010), which could contribute to the higher resistance found in triacylglycerols. In addition, processing steps following biodiesel production, such as washing, drying and distillation (when present), are known to reduce the content of natural antioxidants originated from the feedstock (McCormick et al., 2007), ultimately contributing for a lower induction period of the derivative biodiesel.

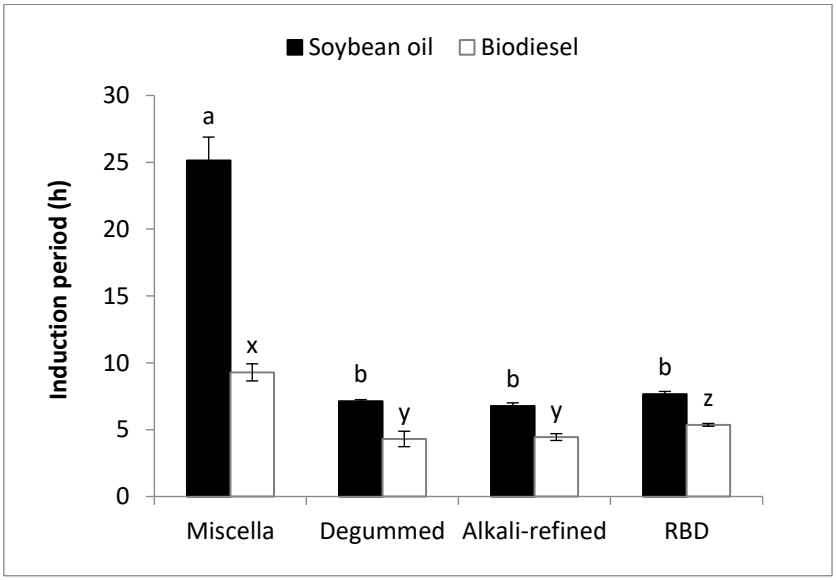

Figure 3.2. Oxidative stabilities of soybean oil and biodiesel samples in Rancimat test. The higher the induction period (h) the higher the oxidative stability. Data represent mean ± standard deviation for each sample (n = 3). Lower-case letters above bars represent significant difference ($p < 0.05$). RBD stands for refined-bleached-deodorized oil

Tocopherols are among the most studied natural antioxidants present in vegetable oils, including soybean oil (Shahidi & de Camargo, 2016). Pereira et al. (2013) demonstrated that tocopherol degradation occurs at a higher rate for biodiesel in comparison to oil samples during the Rancimat test,

which suggests that the lower resistance displayed by ethyl esters may be related to faster loss of antioxidant capacity. Overall, miscella and its direct ethyl esters were notably more resistant to oxidation than the other samples upon Rancimat test.

By inducing oxidation under milder conditions than Rancimat, Schaal oven test (Fig. 3.3) can give a broader perspective of samples' stability. Miscella kept its stability throughout the entire period of the experiment whereas hexane-extracted oils rapidly oxidized. Among this group, degummed oil stability was only slightly better. As mentioned before, oil extraction and ethyl esters production may, but not necessarily, be performed in the same facility. However, in order to improve oil stability and the yield of transesterification reaction, degumming step must be compulsorily performed after oil extraction. This experiment may indicate miscella's ability to better endure storage than hexane-extracted soybean oils, without refining. After eight days in the oven, miscella's peroxide value increased from 3.33 to 4.24 meq O_2/kg while values for the RBD oil increased from 3.78 to 137.9 meq O_2/ kg. From an industrial viewpoint, the use of ethanolic extraction of soybean oil is able to generate a product that can be stored, transported or transesterified without prior refining steps while still maintaining its quality over time. Results obtained in the oven test clearly support the data obtained in the Rancimat test.

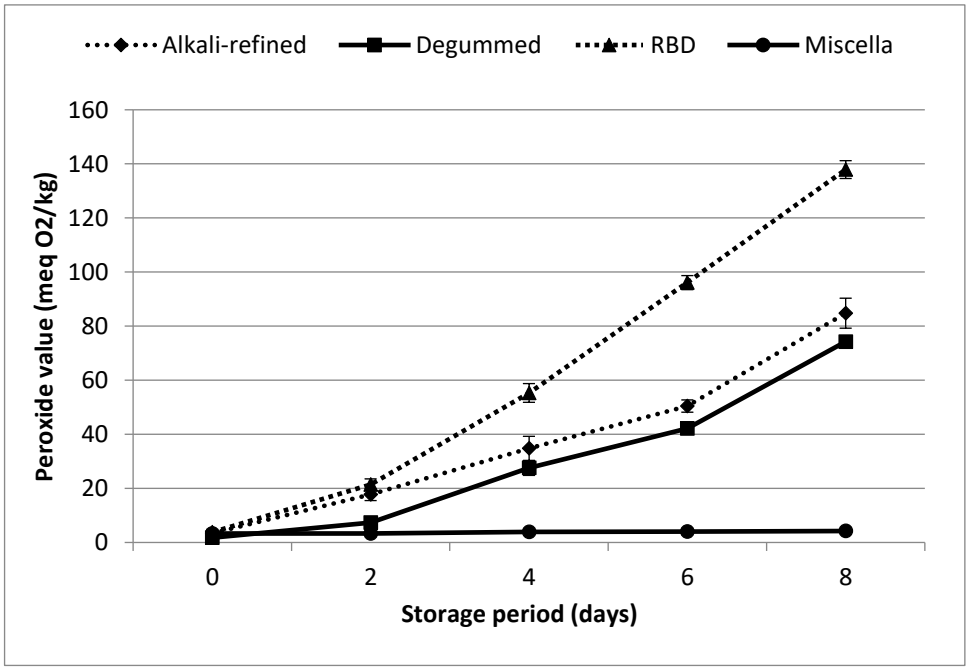

Figure 3.3. Peroxide value of oil samples during storage at 62°C. Data represent the mean ± standard deviation of each sample (n = 3). RBD stands for refined-bleached-deodorized oil

The total phenolic content, the identification and quantification of tocopherols and isoflavones as well as the radical scavenging activity were performed to endorse the results obtained in Rancimat and Schaal oven test conditions. Upon heating, ethanol is able to achieve the same oil extraction efficiency found for the conventional process with hexane (Beckel et al., 1946). Therefore, the use of this solvent allows for a combined action of the affinity for oil matrixes and the ability of carrying a broader range of compounds with varied polarity. Additionally, ethanol carries phosphatides and free fatty acids based on their affinity. Likewise, minor polar compounds with antioxidant capacity may also be transferred to the oil during the extraction process. This became clear with the results of TPC (Table 3.3). Among the ethanolic extracts, miscella's presented the highest ability to reduce the phosphomolybdic and phosphotungstic acids mixture in Folin-Ciocalteau reagent, showing up to 1.7-fold phenolic content as compared to that of the degummed oil. The presence of higher amounts of phenolic compounds found for this sample (0.42 GAE/g oil), compared to hexane-extracted ones, was expected due to the polar nature of the solvent used in the oil-extraction process.

Table 3.3. Radical scavenging activity and total phenolic content of soybean oil samples

	Miscella	Degummed	Alkali- Refined	RBD
ABTS (µmol TE/g oil)	0.63 ± 0.0c	0.55 ± 0.0b	0.60 ± 0.0c	0.49 ± 0.0a
ORAC (µmol TE/g oil)	1.22 ± 0.1c	0.89 ± 0.0a	1.09 ± 0.1b	1.06 ± 0.0b
TPC (mg GAE/g oil)	0.42 ± 0.0d	0.25 ± 0.0a	0.28 ± 0.0b	0.29 ± 0.0c

Data represent mean ± standard deviation for each sample (n = 3). Different lower-case letters within each row are significantly different ($p < 0.05$). RBD stands for refined-bleached-deodorized oil

The ABTS assay is known to be influenced by both polar and apolar compounds (Re et al., 1999), which may allow for a better assessment of the variety of compounds present in the ethanol extracted oil samples. Although many factors can influence antioxidant reaction with $ABTS^{\bullet+}$, the extent of the reaction generally correlates with the number of phenol groups available for electron/hydrogen transfer (Tian et al., 2013). In TEAC assay, miscella (0.63 TE/g oil) presented the highest content of ABTS radical cation-reducing antioxidants whereas in accordance to the degree of refining, RBD oil showed the lowest activity (0.49 TE/g oil). In the ORAC assay, antioxidant compounds existing in the prepared extracts react with peroxyl radicals via hydrogen atom transfer, thus delaying the fluorescence decay. In contrast to the ABTS assay, ORAC employs a rapidly generated peroxyl species involved in oxidation reactions that take place in lipid matrixes (Schaich et al., 2015). Miscella afforded the best results (1.22 µmol TE/g oil) and again outperformed its counterparts.

The tocopherol content (Table 3.4) of the hexane-extracted oils was higher than that of the miscella, which may be due to the higher affinity of the apolar solvent with this class of antioxidants. However, the presence of all tocopherol homologues in the miscella demonstrates that ethanol is also

able to extract different tocols as evidenced by the concentration of beta- and delta-tocopherols in this sample, which were at least comparable to two of the remaining samples. Among the oils tested, RBD had the lowest level of total tocopherols, arising from the extra refining steps, comprising bleaching and deodorization. Both of these processes contribute to the removal of undesirable compounds, but the latter also leads to decomposition of peroxides and tocopherols due to the high temperatures experienced (Jung et al., 1989). Degummed oil, which was the sample with the lowest exposure to refining, showed the highest content of alpha- and beta-tocopherols. With the exception of the delta-homologue, found in similar quantities in alkali-refined and degummed oils, miscella displayed the lowest levels of all tocopherols among the samples tested. However, it is known that high tocopherol concentrations may not guarantee the expected protection to oils under favorable oxidation conditions (Kulås & Ackman, 2001a; Huang et al., 1994). Such prooxidant effects have been reported for α-tocopherol at concentrations above 100 mg/kg and for γ-tocopherol above 500 mg/kg (Evans et al., 2002; Kulås & Ackman, 2001b). Therefore, it is possible that the lower induction period presented by hexane-extracted oils may have been partially due to prooxidant effects caused by high tocopherol concentrations.

Table 3.4. Contents of tocopherols (mg/kg) and isoflavones (mg/kg) in soybean oil samples

	Miscella	Degummed	Alkali-Refined	RBD
Tocopherols (mg/kg)				
α-Tocopherol	15.3 ± 1.8ª	98.9 ± 1.1c	78.9 ± 0.2b	73.3 ± 6.0b
β-Tocopherol	7.40 ± 0.6ª	13.9 ± 2.6b	6.70 ± 2.5a	5.70 ± 0.9a
γ-Tocopherol	329 ± 11ª	758 ± 5.6c	739 ± 8.8c	527 ± 14b
δ-Tocopherol	119 ± 7.6c	110 ± 1.5cb	100 ± 3.0b	41.0 ± 3.9c
Total Tocopherols	466 ± 12a	980 ± 9.8d	925 ± 5.9c	647 ± 22b
Isoflavones (mg/kg)				
Daidzein	tr	nd	nd	nd
Genistein	tr	tr	tr	nd
Glycitein	1.14 ± 0.1	nd	nd	nd
Genistin	21.1 ± 2.2	tr	tr	nd
Acetyldaidzin	9.71 ± 0.2	nd	nd	nd

Data represent mean ± standard deviation for each sample (n = 3). Means followed by different lower-case letters within a row show difference among samples ($p < 0.05$). RBD stands for refined-bleached-deodorized oil. Tr = traces; nd = not detectable.

The data presented thus far demonstrate that miscella showed higher oxidative stability and radical scavenging activities despite its lower content of alpha-, gamma- and total-tocopherols compared to the other samples. Likewise, ethyl esters obtained from miscella were more stable as shown in the Rancimat test. To lend support to these findings all feedstocks were subjected to determination of other phenolic compounds using HPLC-DAD-ESI-MS^n (Table 3.4). Two aglycones,

namely, daidzein and genistein, were tentatively identified according to their deprotonated molecular ions at m/z 253 and at 269, respectively. Their respective ions at m/z 224 and 225 were also consistent with the literature values (Chen et al., 2005). Daidzein was detected only in the miscella, but not quantified, whereas a trace amount of genistein was detected in the miscella as well as in the degummed and the neutralized oils. In contrast, no isoflavone was detected in the RDB oil. Glycitein was tentatively identified in its deprotonated molecular ion at m/z 283 and m/z at 269 in MS^2 due to the loss of hydrogen and methyl group $[M - H - H - CH_3]^-$ which is similar to the fragmentation pattern reported by Lee et al. (2015). Genistin, $[M - H]^-$ at m/z 431, gave a product ion at m/z 269 due to the loss of a glucose moiety $[M - H - 162]^-$ (Chen et al., 2005). Isoflavones exist in the aglycone form as well as in the glucoside, glucoside malonate, and glucoside acetylate forms. Accordingly, the compound that showed an m/z at 457 in MS and characteristic ions at m/z 253 in MS^2 was tentatively identified as acetyldaidzin (Chen et al, 2005). The major isoflavone quantified in the miscella was genistin, followed by acetyldaidzin and glycitein.

To the best of our knowledge, this is the first report on the production of soybean oil with significant amounts of isoflavones. The presence of such phenolic compounds in the tested sample demonstrates that ethanol was able to extract both tocopherols and isoflavones. Thus, the present study demonstrates that the oxidative stability of oil-rich miscella and the remaining samples was not correlated to their tocopherol contents and that the presence of other compounds with antioxidant effect should not be underestimated. The antioxidant effect of isoflavones on lipid matrixes has been addressed previously in the literature. Using a Rancimat apparatus, Dziezic and Hudson (1983) have showed induction period increases in isoflavone-added samples of lard. Yue and others (2008) demonstrated the protective effect of a soy flour extract with high isoflavone content on fish oil. Other studies have reported the effects of isoflavone incorporation against the oxidation of the LDL particle (Ruiz-Larrea et al., 1997; Kerry & Abbey, 1998). Therefore, we suggest that isoflavones present in the miscella may be responsible for its higher oxidative stability and thus serving as a better feedstock for processes involving esterification reactions.

3.4. Conclusion

The ethanolic extraction of soybean oil rendered an oil-rich miscella with a higher resistance to oxidation compared to its hexane-extracted counterparts, as demonstrated by accelerated oxidation tests. Ethyl esters produced from this feedstock better resisted oxidation conditions than those produced using degummed, alkali-refined, or refined-bleached-deodorized oils. Such features allow for better preservation of both oil and biodiesel quality during storage and transportation. All

tocopherol homologues were identified and quantified in the oil-rich miscella, degummed oil, alkali-refined oil, and refined-bleached-deodorized oil but only the oil-rich miscella showed a significant content of isoflavones. The high oxidative stability of the oil-rich miscella was primarily influenced by the presence of isoflavones rather than tocopherols. Degummed oil has been the primary feedstock for biodiesel production from soybean; however, the details presented in our findings demonstrates that the ethanol oil-rich miscella is a promising substitute for degummed soybean oil and should be further considered for biodiesel manufacture.

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4. IMPROVING WASTE COOKING OIL QUALITY FOR BIODIESEL PRODUCTION WITH THE ETHANOLIC BYPRODUCT OF SOYBEAN OIL EXTRACTION

Adapted with permission from BUENO-BORGES, L. B; SANTOS, G. C. P.; ALENCAR, S. M.; REGITANO-D'ARCE, M. A. B.

ABSTRACT

Waste cooking oils (WCOs) can be used as feedstock for biodiesel production. Their usual high acidity, high moisture and low stability can impair the reaction yield and generate a low-quality biodiesel. Extraction of soybean oil using ethanol instead of hexane generates an ethanolic by-product with great unexplored antioxidant potential. Here, we performed liquid-liquid washings with the goal of generating oil-rich miscellae as biodiesel feedstocks with higher quality than WCOs. Treatments with three different solvents were applied to WCO: 99% ethanol, 95% ethanol and the soybean oil extraction ethanolic phase (SEP). Washings were performed at 78°C, 1:2 oil/solvent ratio, 1200 rpm, 10 min. Treatments reduced acid value in 40-61% and peroxide value in 15-50%. Improvements in feedstock quality generated biodiesel yields 24-54% higher. The oil-rich phase produced with SEP was 15% more resistant to oxidation than WCO. This increase in oxidative stability was attributed to the transference of isoflavones from the SEP. On the other hand, biodiesel from treated samples presented equal or lower oxidative stability than WCO-derived ethyl esters. HPLC-DAD analysis showed that no isoflavones remained in the final biodiesel. Pretreatment of WCO with ethanol-based solvents such as the SEP has great potential to improve WCO quality for biodiesel production. However, transesterification methods call for advancements that will allow for the preservation of natural polar antioxidants in purified biodiesel.

Keywords: Bioethanol; Soybean oil; Frying; Liquid-liquid extraction; Deacidification; Ethyl ester

4.1. Introduction

Waste cooking oils (WCOs) are residual lipid feedstocks that have been used in the process of frying foods. At the same time, refined vegetable oils can raise biodiesel production costs up to 80% (Moecke et al., 2016). Thus, industrial waste, such as used cooking oil, becomes a potential alternative low-cost raw material, with great integration value between food and biofuel industries.

The contact with hydrated foods and the high temperatures used in frying (160-200 °C) leads to the increase in moisture and free fatty acid contents in WCO (Mittelbach & Gangl, 2001). Such features impose challenges to the transesterification reaction via alkaline catalysis. For that reason, many techniques have been developed for overcoming yield-related issues in the production of biodiesel from acid oils (Lam et al., 2010). One of them consists in deacidifying lipid feedstocks through liquid-liquid extraction with polar solvents (Tunc et al., 2010). However, besides being highly acidic, WCO also presents high oxidation levels and lacks thermal stability, which ultimately renders low-quality final biodiesel.

Alternatively to the use of hexane, a petroleum-based solvent, extraction of vegetable oils can be performed using ethanol. This process generates two miscellar liquid phases that separate naturally, by density, after cooling: the oil-rich phase with approximately 7% ethanol, and the ethanol-rich phase, a byproduct composed of roughly 3% oil and 90% ethanol (Sangaletti-Gerhard et al., 2014). This phase separation concentrates polar components, such as free fatty acids, phospholipids, water and antioxidants in the byproduct. Applications and potentialities of this latter byproduct have not yet been explored.

In an attempt to improve the quality of WCO for biodiesel production, it was submitted to three different solvent washings, so WCO-derived miscellae were generated using 95%, 99% ethanol and the ethanol-rich phase from the extraction of soybean oil (SEP). The antioxidant capacity, oxidative stability and overall composition of these miscellae and their fatty acid ethyl esters were assessed and compared to WCO.

4.2. Material and Methods

4.2.1. Material

Approximately 3L of WCO were obtained from a local collection center (household use). The oil was filtered using paper coffee filter to remove solids and was later stored in freezer (-18 °C). HPLC grade methanol and hexane as well as Karl Fisher reagent were purchased from Panreac (Barcelona, Spain)**.** Reagent-grade absolute (99%) and 95% ethanol were purchased from Dinamica (Indaiatuba, Brazil). DPPH, AAPH, FAME standard mix, fluorescein, Folin reagent, trolox, isoflavone standards and tocopherol homologues were obtained from Sigma-Aldrich (St Louis, MO, USA).

4.2.2. Production of ethanolic miscellae

Production of miscellae was analogous to a liquid-liquid washing process, between the WCO and the chosen solvent. Three different solvents were used to generate WCO miscellae: absolute ethanol, 95% ethanol and SEP. The SEP was the byproduct of soybean oil extraction using ethanol, obtained according to procedure described in Bueno-Borges et al. (2017) (Fig. 4.1). Characteristics of the SEP are shown in Table 4.1. WCO and the solvent were added to a 1000 mL flat bottom flask in a ratio of 1:2 (oil/solvent), under magnetic stirring at 1200 rpm for 10 min, under reflux and water bath at 78 °C. After this washing period, the mixture separated upon cooling to room temperature, resulting in an ethanol-rich top layer and an oil-rich bottom layer. This procedure was conducted three times for each solvent: three replicates of each type of miscella were produced. Both phases (top and bottom

layers) were collected separately, stored in amber bottles, frozen (-18 °C) and further analyzed. Oil rich miscellae generated using 99% ethanol, 95% ethanol and the soybean oil extraction ethanolic phase are hereafter named M99, M95 and MSEP, respectively.

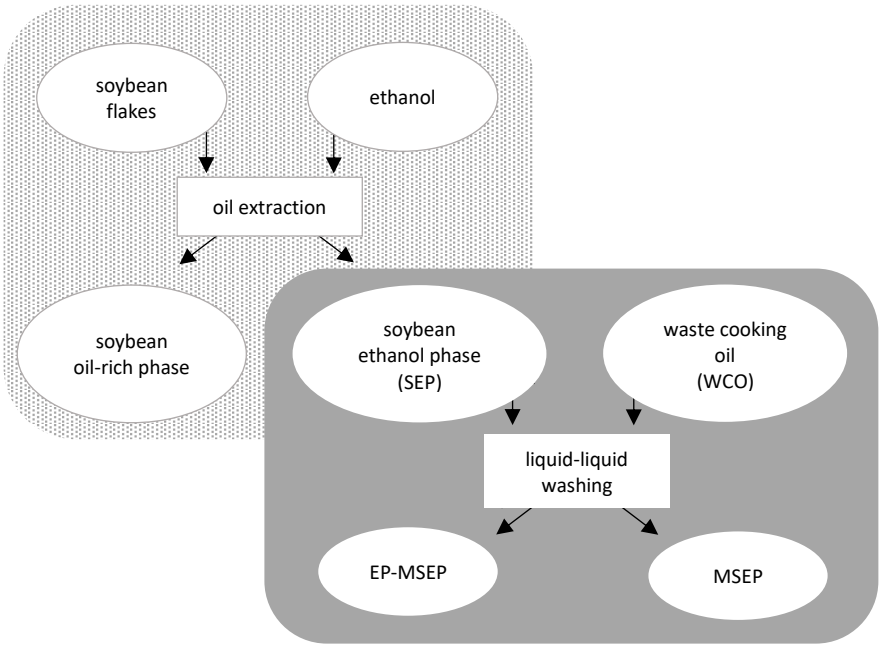

Figure 4.1. Production and use of the SEP with waste cooking oil, to generate the miscella used in this study. SEP = soybean oil extraction ethanolic phase. MSEP = oil-rich miscella generated with the SEP. EP-MSEP = ethanolic phase generated in the washing of WCO with the SEP

Table 4.1. Characteristics of the SEP used as solvent to generate MSEP

Analyses	SEP
Oil content (% m/m)	1.71 ± 0.0
Ethanol content (% m/m)	85.0 ± 0.3
Water content (% m/m)	10.24 ± 0.1
Acid value - mg KOH/g miscella (mg KOH/g oil)	1.11 (64.9)
Peroxide value (meq O_2/kg oil)	1.07 ± 0.0
DPPH (µmol Trolox/g sample)	2.40 ± 0.3
ORAC (µmol Trolox/g sample)	14.6 ± 0.6

Values are means of a least three determinations ± SD. SEP = soybean oil extraction ethanolic phase; MSEP = oil-rich miscella generated with the SEP. DPPH = 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay, expressed as trolox equivalents (µmol Trolox/g sample); ORAC = Oxygen Radical Absorbance Capacity assay, expressed as trolox equivalents (µmol Trolox/g sample)

4.2.3. Biodiesel production (fatty acid ethyl esters)

WCO and miscellae were used as feedstock to yield fatty acid ethyl esters (FAEE/biodiesel) through alkaline homogeneous transesterification. Ethanol was the acyl acceptor and NaOH was the catalyst. Transesterification was performed twice for each replicate of oil/miscella under reflux. Sodium ethoxide was prepared by dissolving NaOH in ethanol to the concentration of 1%. The oil sample was poured into the same flask and the reaction was conducted under magnetic stirring (900 rpm) at 30°C. The reaction oil-to-ethanol molar ratio was 1:12, therefore ethanol content of the three miscellae was taken into account for calculations. At the end of the reaction, phase separation was induced by rotary evaporation (40 °C) to remove excess ethanol. After phase separation into glycerol (lower) and ester (upper phase), samples were washed with distilled water (60 °C) until neutralization was achieved - pH verification of the wash water was accomplished with phenolphthalein. FAEEs were then filtered over anhydrous sodium sulfate. Yield was calculated using Eqs. 1 and 2,

$$Crude\ Yield\ (CY) = \frac{mFAEE}{mOil} * 100 \qquad (1)$$

$$Real\ Yield\ (RY) = \frac{CY * pFAEE}{100} \qquad (2)$$

where $mFAEE$ is the mass of ethyl esters obtained after filtration on sodium sulfate, $mOil$ is the mass of oil used in the reaction and $pFAEE$ is the pure ethyl ester content (%) of the FAEE fraction after filtration, which was determined by gas chromatography according to method NBR 15764 (ABNT, 2012).

4.2.4. Oil/miscellae and biodiesel characteristics

The oil content in miscellae was determined gravimetrically after hexane/isopropanol liquid-liquid extraction, followed by washing with 7% Na_2SO_4 (Hara & Radin, 1978). Ethanol content was determined by distilling 25 mL of the samples. The collected distillate was then read in a digital densimeter. Water content was performed using a volumetric Karl Fisher equipment. Peroxide and acid values were determined by titration methods Cd 8b-90 and Ca 5a-40 (AOCS, 2003), respectively, after removal of ethanol and water contents from miscellae. All characterization analyses were performed in triplicates.

4.2.5. Oxidative stability in Rancimat

The induction period of oil and biodiesel samples was determined by means of the Rancimat apparatus. Parameters were: 3 g sample, 110 °C, 10 $L.h^{-1}$ air flow. For each sample replicate, three Rancimat determinations were carried out. Results are the average induction period expressed in hours.

4.2.6. Fatty acid profile

WCOs and WCO-derived miscellae were analyzed for their fatty acid profile by gas chromatography after preparation of fatty acid methyl esters (Hartman & Lago, 1973). 50mg of sample reacted with 2mL 0.5 N methanolic potassium hydroxide to generate methyl esters. This reaction was conducted in screw cap glass tubes (boiling water bath, 5min). After cooling the tubes under tap water, 2.5 mL of a solution of ammonium chloride and sulfuric acid solution in methanol (2:3:60 m/v/v, prepared under reflux) were added and incubation was repeated (boiling water bath, 5min). Tubes were cooled once more and 2mL of HPLC-grade hexane were added, followed by thorough mixing. Hexane phase was collected, washed with saline solution (36%), distilled water and stirring with sodium sulfate (0.5g). Hexane phase was transferred to glass vials and analyzed for fatty acid profile. FAEE (biodiesel samples) were analyzed directly after dilution with HPLC-grade hexane (0.05 g FAEE/mL). Gas chromatograph was a Shimadzu GC-2010 plus AF system with FID detector, and RTX-Wax (30 m; 0.32mm; 0.25 µm) – Crossbond Carbowax polyethylene glycol column (Restek Corp., Bellefonte, PA, USA). Inlet and detector temperatures were 250 °C, 1µL was injected by split mode (1:20). Column oven program started at 60°C (hold for 0 min), 20 °C/min to 210 °C (hold for 7 min) and 30 °C/min to 240 °C (hold for 15 min). Nitrogen was the carrier gas (1.2mL/min). Identification was performed by comparing retention times with methyl/ethyl ester mix standard. Sample composition was determined by normalization, expressed as a percentage of total peak area.

4.2.7. Antioxidant assays

The antioxidant activity of samples was determined by means of polar extracts prepared from oils and biodiesel. Oil/miscellae were vortexed (1 min) with ultrapure water (1:2 oil to solvent) in Falcon tubes, centrifuged (2min/5000 rpm) and the supernatant was collected. This procedure was repeated 3 more times. The combined water phases were washed with hexane to remove oil residues, followed by lyophilization. Residues were suspended with HPLC grade ethanol and stored. Biodiesel extracts were prepared by adding methanol to biodiesel (1:2 ester-to-solvent ratio), followed by

thorough mixing (1 min) and freezing for 30 min. Tubes were centrifuged (room temp., 5000 rpm, 5 min) and the methanolic supernatant was collected. This procedure was repeated 3 more times. Combined phases were frozen and centrifuged once more. The supernatant was transferred to a flask, dried under N_2, and lyophilized. Residues were suspended in ethanol.

Antioxidant capacity by the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity assay was determined by adding 66 µL of sample extract, standard or ethanol (control) to the microplate well (Al-Duais et al., 2009), followed by addition of 134 µL of 150 µM DPPH in ethanol. Readings were performed at 517 nm after room temperature incubation in the dark for 45min. Trolox was used as standard to build a calibration curve.

The Oxygen Radical Absorbance Capacity (ORAC) assay was performed by adding 30µL of the extract, fluorescein solution (60µL) and AAPH solution (110µL) to each microplate well (Chisté et al., 2011). Solutions of 76mM AAPH and 508.25nM fluorescein were prepared from 75mM potassium phosphate buffer. The latter was used as blank. Readings were carried out at 37 °C every minute for a period of 2 hours, registering the area under the curve. Wavelengths were 528nm and 485nm for emission and excitation, respectively. A standard curve was built using trolox to express the results.

4.2.8. HPLC-DAD analysis of extracts

Identification and quantification of major phenolics in sample extracts were performed by reversed-phase high-performance liquid chromatography (HPLC) using a Shimadzu SCL-10Avp equipment. Column was a Shimadzu ODS-A (4.6 mm, 250 mm, 5 µm). The system comprised a photodiode array detector (SPD-M10Avp, Shimadzu Co., Kyoto, Japan), LC-6AD pumps and a SIL-10AF auto injector. The binary mobile phase gradient consisted of A: water (99.75%)/formic acid (0.25%); B: acetonitrile (80%)/water (19.75%)/formic acid (0.25%) and it was programed as follows: 0 min – 20% B; 10 min – 30% B; 20 min – 50% B; 30 min – 60 min B; 32 min – 100%; 35 min – 100%; 40 min – 20% B; 45 min – 0% B. Oven temperature was kept at 30 °C.

Sample extracts were filtered (0.22µm) and injection flow was 1.0 mL/min, using 20µL volume. Chromatograms were analyzed by Class-VP software and phenolics were identified by comparing their retention times and UV spectral data with authentic standards under the same analysis conditions. Calibration curves were built for the available standards for quantification purposes.

4.2.9. Tocopherol content

Tocopherol content determination was carried out by normal phase HPLC by isocratic elution, according to method Ce 08-89 (AOCS, 2003). A Shimadzu LC-6AD equipment was used, with RF-10AXL fluorescence detector. Excitation and emission wavelengths were 290 and 330 nm, respectively. Column was Lichrospher Si60 (5 µm, 25 cm × 4 mm i.d., E. Merck, Darmstadt, Germany). Injections (10 µL) were conducted at 1.0 mL/min and mobile phase was hexane–isopropanol (99:1 v/v). Quantification of homologues alpha, beta, delta, and gamma was performed using external calibration with authentic standards.

4.2.10. Statistical analysis

Data was analyzed statistically using one-way ANOVA followed by Tukey's multiple comparison test, using Minitab 18 1.0.0 software. Significance at the 95% confidence interval and $p<0.05$ were considered statistical difference.

4.3. Results and Discussion

The production of biodiesel from ethanol-in-oil miscellae has been demonstrated to be both technically and economically feasible as an alternative to the use of refined vegetable oils (Sangaletti-Gerhard, 2014b). Preliminary tests of this study have helped defining the conditions used for miscellae production, which showed that the use of temperature at 78 °C (ethanol boiling point) was crucial for improving acidity reduction (data not shown).

Table 4.2 shows some characteristics of the oil samples, the correspondent ethanolic phase generated in the process and FAEE yield. Overall, oil miscellae were similar in composition, in agreement with previous studies (Sangaletti-Gerhard, 2014a). Production of miscellae using hydrated and pure ethanol resulted in reduction of the water content in WCO by up to 47.5% and 75%, respectively. On the other hand, due to its composition, the use of the SEP (Table 4.1) resulted in water content increase. Oil samples M99, M95 and MSEP have lower specific gravities than WCO as a result of the presence of ethanol and water miscellae inside the oil. This feature reduces oil viscosity and improves alcohol diffusion through the lipidic sample, which are valued properties for biodiesel production.

Table 4.2. Oil/miscellae, ethanolic phases and ethyl ester (biodiesel) properties

	Oil samples			
	WCO	M95	M99	MSEP
Oil content (% m/m)	-	85.3 ± 1.0[b]	82.7 ± 0.8[a]	85.5 ± 0.4[b]
Ethanol content (% m/m)	-	10.2 ± 0.9[b]	9.63 ± 0.8[b]	6.09 ± 0.2[a]
Water content (% m/m)	0.41 ± 0.0[c]	0.21 ± 0.0[b]	0.10 ± 0.0[a]	1.04 ± 0.1[d]
Specific Gravity	0.9198 ± 0.000[c]	0.8986 ± 0.000[a]	0.8988 ± 0.001[a]	0.9094 ± 0.000[b]
		Ethanolic phase		
		EP-M95	EP-M99	EP-MSEP
Oil content (% m/m)		2.90 ± 0.0[a]	2.85 ± 0.0[a]	1.55 ± 0.0[b]
Ethanol content (% m/m)		95.3 ± 0.0[b]	95.9 ± 0.2[b]	85.2 ± 0.3[a]
Water content (% m/m)		2.21 ± 0.1[b]	0.73 ± 0.0[a]	10.8 ± 0.2[c]
		FAEE		
	BWCO	BM95	BM99	BMSEP
pFAEE content (%)	91.6 ± 0.1	97.0 ± 0.7	94.8 ± 0.0	93.4 ± 0.6
Real FAEE yield (%)	48.5	69.7	74.8	60.3

[a, b, c] Different letters within rows represent significant difference. Values are means of three determinations ± SD. FAEE = fatty acid ethyl esters (biodiesel); *pFAEE* = pure fatty acid ethyl ester; WCO = waste cooking oil; SEP = soybean oil extraction ethanolic phase; MSEP = oil-rich miscella generated with the SEP; M95 = 95% ethanol oil-rich miscella; M99 = 99% ethanol oil-rich miscella; EP-M95 = ethanolic phase generated in the washing of WCO with 95% ethanol; EP-M99 = ethanolic phase generated in the washing of WCO with 99% ethanol; EP-MSEP = ethanolic phase generated in the washing of WCO with the SEP; BWCO = biodiesel from waste cooking oil; BMSEP = biodiesel from oil-rich miscella generated with the SEP; BM95 = biodiesel from 95% ethanol oil-rich miscella; BM99 = biodiesel from 99% ethanol oil-rich miscella

Depending on the extent to which the oil has been heated and used, existing hydroperoxides break down into volatile compounds, and are no longer detectable as peroxides. That may explain why WCO peroxide values found in literature are so variable (higher and lower than 10 meq O_2/kg of oil) (Karakaya & Simsek, 2011). Both acid and peroxide values reduced significantly after WCO treatment with all solvents. Percentage decrease was more evident in M95 (60.4% in AV; 50.4% in PV) and M99 (61.2% AV; 41.4% PV) than in MSEP (40.1% AV; 14.8% PV). MSEP acid value was higher than for the other miscellae due to the original high FFA content in the SEP, as shown in Table 4.1 and Fig 4.2.a. Nevertheless, all miscellae produced presented AV ≤ 1 mg KOH/g oil (≤ 0.5% FFA expressed as oleic acid). Rodrigues et al. (2014) used hydrated ethanol to perform the deacidification step of rice bran oil by means of a liquid-liquid equilibrium system between oil and solvent. Five cycles were performed to achieve a 98.1% reduction in FFA content.

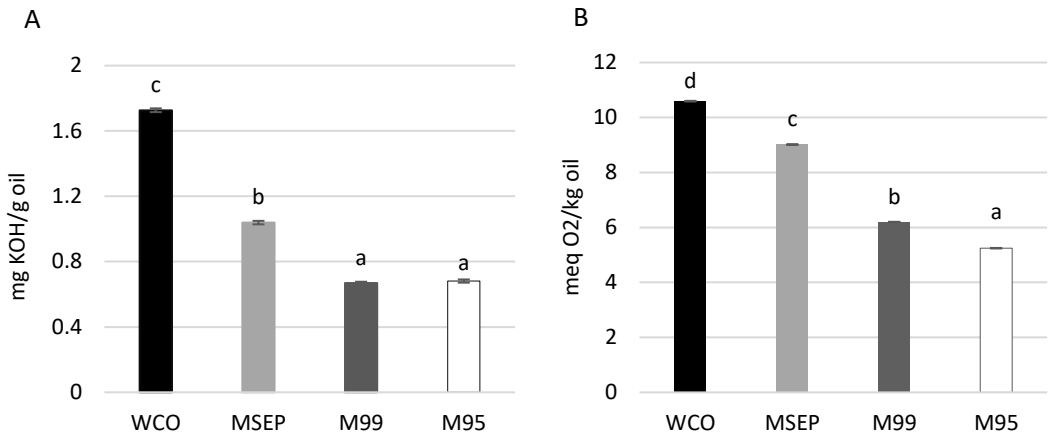

Figure 4.2. a. Acid value of oil-rich miscellae after washing treatment with 95% ethanol, 99% ethanol and SEP, in comparison to WCO. **b.** Peroxide value of oil-rich miscellae after washing treatment with 95% ethanol, 99% ethanol and SEP, in comparison to WCO

Ethanol, at both concentrations, provided a ~60% reduction in acid value, compared to the original acidity in WCO. SEP efficiently reduced AV by 40%. For biodiesel production, this reduction is important to avoid saponification reactions when alkalis are used as catalyst. Formation of soaps hinder the separation between biodiesel and glycerol, reduce reaction yield and the rate of conversion to alkyl esters (Jacobson et al., 2008). In this study the conservative type of transesterification process was used as a standard procedure for comparison of conversion yields between samples. Increases in FAEE conversion are most likely related to improvements in feedstock quality. Utilization of MSEP resulted in 24.3% increase in FAEE conversion, whereas M95 and M99 generated a 43.7% and 54.2% increase, in comparison with the original WCO. The lower water and FFA contents in the latter are most likely responsible for the improved result.

Table 4.3. Fatty acid profile of oil rich phase samples after washing treatment with 95% ethanol, 99% ethanol and SEP and their correspondent FAEE samples expressed as percentage (%)

	Oil				FAEE			
	WCO	MSEP	M99	M95	BWCO	BMSEP	BM99	BM95
$C_{12:0}$	0.54^{a}	0.52^{b}	0.46^{c}	0.47^{c}	0.65^{a}	0.61^{a}	0.56^{a}	0.59^{a}
$C_{14:0}$	0.35^{a}	0.33^{a}	0.32^{b}	0.32^{b}	0.43^{a}	0.49^{a}	0.39^{a}	0.42^{a}
$C_{16:0}$	9.97^{ab}	10.0^{a}	9.92^{b}	9.96^{ab}	10.3^{a}	10.1^{a}	10.3^{a}	10.2^{a}
$C_{16:1}$	0.28^{a}	0.28^{a}	0.28^{a}	0.28^{a}	0.32^{a}	0.32^{a}	0.32^{a}	0.32 ^{a}
$C_{18:0}$	3.44^{a}	3.42^{a}	3.45^{a}	3.46^{a}	3.59^{ab}	3.56^{a}	3.67^{c}	3.63^{bc}
$C_{18:1}$	42.5^{b}	42.5^{b}	42.8^{a}	42.8^{a}	43.5^{b}	43.5^{b}	44.1^{a}	43.6^{ab}
$C_{18:2}$	37.2^{ab}	37.3^{a}	37.0^{c}	37.0^{bc}	38.7^{c}	38.6^{b}	38.4^{b}	38.2^{a}
$C_{18:3}$	4.45^{a}	4.46^{a}	4.49^{a}	4.44^{a}	0.28^{a}	0.29^{a}	0.23^{a}	0.26^{a}
$C_{20:0}$	0.45^{a}	0.44^{a}	0.47^{a}	0.44^{a}	0.71^{a}	0.76^{a}	0.65^{a}	0.70^{a}
$C_{20:1}$	0.48^{a}	0.48^{a}	0.49^{a}	0.48^{a}	0.93^{a}	0.97^{a}	0.84^{a}	0.93^{a}
$C_{22:0}$	0.28^{a}	0.28^{a}	0.31^{a}	0.29^{a}	0.50^{ab}	0.52^{ab}	0.46^{a}	0.71^{b}
$\sum_{SFA}$	15.0^{a}	15.0^{a}	15.0^{a}	15.0^{a}	16.2^{a}	16.4^{a}	16.0^{a}	16.3^{a}
$\sum_{MUFA}$	43.3^{a}	43.3^{a}	43.6^{b}	43.6^{b}	44.8^{a}	44.7^{a}	45.3^{b}	44.8^{ab}
$\sum_{PUFA}$	41.7^{ab}	41.8^{a}	41.5^{b}	41.5^{b}	39.0^{d}	38.9^{c}	38.7^{b}	38.4^{a}

$^{a, b, c}$ Different letters in the same row indicate significant difference. Means of two injections ± SD. SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids. WCO = waste cooking oil; SEP = soybean oil extraction ethanolic phase; MSEP = oil-rich miscella generated with the SEP; M95 = 95% ethanol oil rich miscella; M99 = 99% ethanol oil-rich miscella; BWCO = biodiesel from waste cooking oil; BMSEP = biodiesel from oil-rich miscella generated with the SEP; BM95 = biodiesel from 95% ethanol oil-rich miscella; BM99 = biodiesel from 99% ethanol oil-rich miscella

Fatty acid composition of oil and FAEE samples is shown in Table 4.3. A wide range of fatty acids was found in the WCO, illustrating the complex mixture that results from the use of different oil types and food matrixes within a domestic environment. Noteworthy, fatty acid composition was not strongly affected by miscellae production with ethanol-based solvents, except for linolenic acid contents that consistently reduced with the treatments as would be expected. Differences in fatty acid composition among samples were stronger after conversion into FAEE. After transesterification, C18:3 suffered a major reduction, consequently decreasing PUFA content in FAEE as compared to oil samples. PUFA content was higher in BWCO, followed by BMSEP, BM99 and BM95.

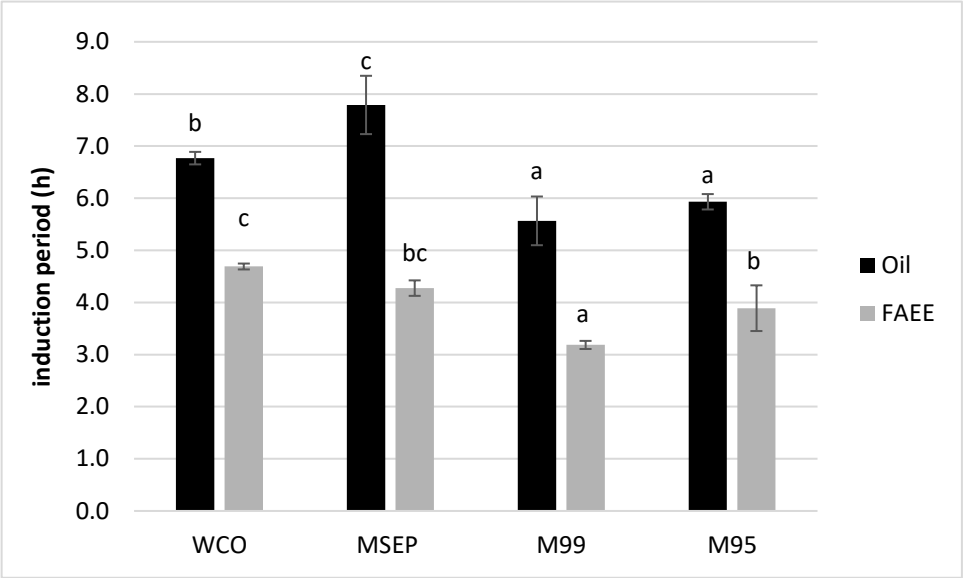

Figure 4.3. Induction period (hours) of oil samples and their correspondent FAEE (biodiesel) by the Rancimat test, at 110 °C. Values are the mean of four replicates and error bars are standard deviation (SD); different letters indicate a significant difference between the samples by the Tukey test ($p < 0.05$). WCO = waste cooking oil; SEP = soybean oil extraction ethanolic phase; MSEP = oil-rich miscella generated with the SEP; M95 = 95% ethanol oil-rich miscella; M99 = 99% ethanol oil-rich miscella

Rancimat induction periods are shown in Fig. 4.3. Although peroxide value reduction was not as pronounced as for M99 and M95, MSEP presented the highest induction period among oil samples (7.8h ± 0.5), surpassing WCO by roughly 15% (6.8h ± 0.1). This agrees with the findings in DPPH and ORAC assays (Fig. 4.4), suggesting the presence of soybean antioxidant compounds (phenolics) carried by the SEP to the MSEP. Such compounds, extracted along with the oil when ethanol is the solvent, concentrate in the ethanolic phase. The oxidative stability for M99 and M95 was statistically similar, 13-17% lower as compared to WCO. Pure and hydrated ethanol removed free fatty acids with high efficiency, but likely also removed antioxidant compounds from the oil, decreasing its protection against oxidation reactions.

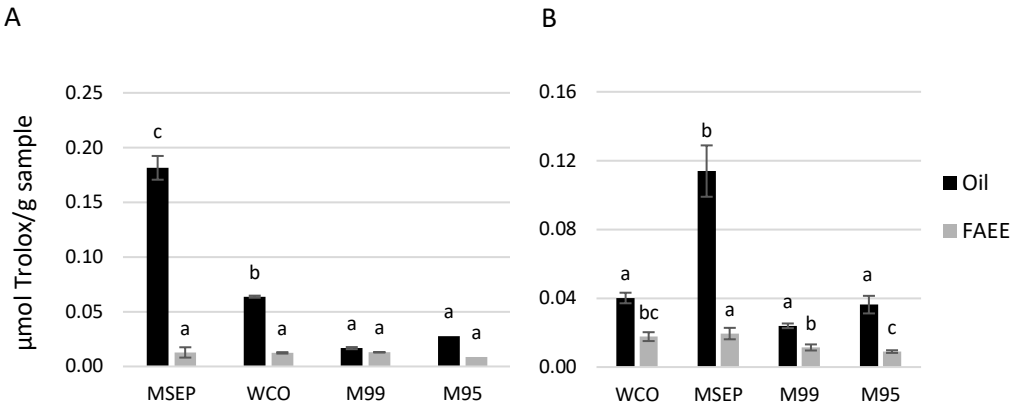

Figure 4.4. Antioxidant activity of hydrophilic extracts from oils and FAEE (biodiesel) samples. **a.** ORAC test expressed in µg of trolox/g oil or biodiesel. **b.** DPPH test expressed in µg of trolox/g oil or biodiesel. Results are means of triplicates and error bars show SD. WCO = waste cooking oil; SEP = soybean oil extraction ethanolic phase; MSEP = oil-rich miscella generated with the SEP; M95 = ethanol 95% oil rich miscella; M99 = 99% ethanol oil-rich miscella; BWCO = biodiesel from waste cooking oil; BMSEP = biodiesel from oil-rich miscella generated with the SEP; BM95 = biodiesel from 95% ethanol oil-rich miscella; BM99 = biodiesel from 99% ethanol oil-rich miscella

Production of miscellae using pure and hydrated ethanol decreased the antioxidant activity of extracts by the ORAC assay in comparison to the original WCO (Fig 4.4). Results suggest that whilst removing free fatty acids from WCO, phase separation from M95 and M99 was also responsible for stripping the oil from antioxidant compounds with affinity to ethanol. MSEP, however presented the highest antioxidant activity in both assays, indicating that transference of polar antioxidants from the SEP to the oil might have taken place. For that reason, the antioxidant activity of MSEP was higher than that of the WCO, since antioxidant compounds were added to the sample from an external source. The antioxidant activity of FAEE (biodiesel) samples was considerably lower than their correspondent oils. Our preliminary data indicate that polar antioxidants are mostly removed from the feedstock during transesterification than during biodiesel purification steps.

Fig. 4.5. A and B show the contents of each tocopherol in oil and FAEE samples, respectively. Tocopherol content strongly decreased after production of miscellae from WCO using 99% and 95% ethanol. This marked effect was not observed in MSEP. This suggests that ethanol phase generated in the production of miscellae using 95% and 99% ethanol removed existing tocopherols in WCO. As for MSEP, the presence of tocopherols and oil in the SEP contributed to preserve the levels of these compounds after miscellae production. Slight changes in the content of homologues between WCO and MSEP also indicate tocopherol transference from SEP to MSEP.

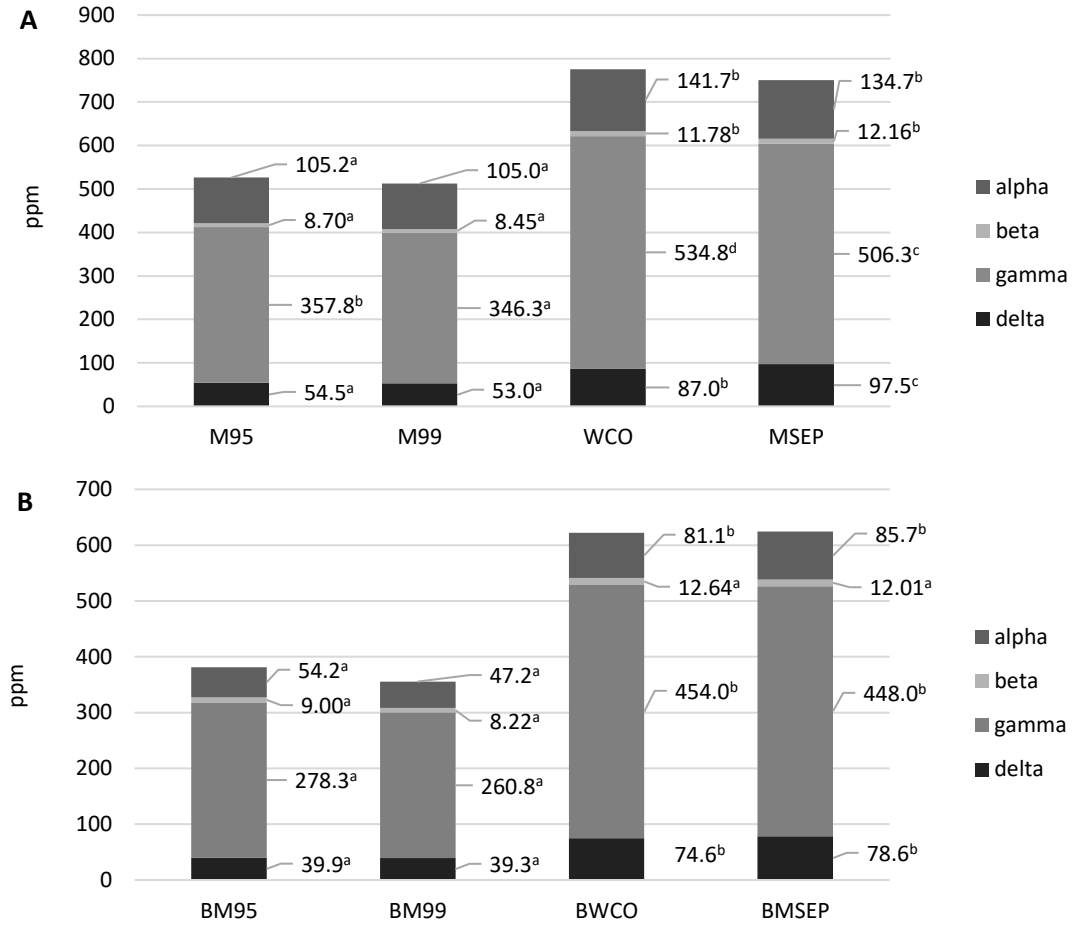

Figure 4.5. a. Tocopherols (alpha, beta, gamma, delta homologues) in oil/miscellae samples. **b.** Tocopherols in FAEE samples. Values in ppm (µg Toc/g oil or FAEE) are means of three replicates. Lowercase letters represent significance between same type homologues ($p<0.05$). WCO = waste cooking oil; SEP = soybean oil extraction ethanolic phase; MSEP = oil-rich miscella generated with the SEP; M95 = 95% ethanol oil rich miscella; M99 = 99% ethanol oil-rich miscella; BWCO = biodiesel from waste cooking oil; BMSEP = biodiesel from oil-rich miscella generated with the SEP; BM95 = biodiesel from 95% ethanol oil-rich miscella; BM99 = biodiesel from 99% ethanol oil-rich miscella

Except for the beta homologue, transesterification of oils into FAEE resulted in significant loss of all tocopherols. Since eight stereoisomers for each tocopherol homologue can be found in nature (Shahidi; de Camargo, 2016), the observed preservation of beta-tocopherol after production of miscellae and biodiesel synthesis could be related to these structural differences. After biodiesel synthesis, total tocopherol reduction was 27.5% and 30.6% for BM95 and BM99, 19.7% and 16.8% for BWCO and BMSEP. It is clear that the transesterification process and purification steps have been the cause for reduction in antioxidant levels in biodiesel, ultimately reducing its oxidative stability in comparison to the correspondent oil.

Isoflavones detected in SEP and MSEP are shown in Table 4.4. No phenolics were detected in the polar extracts of other samples. Isoflavones are a subclass of phenolic compounds belonging to the flavonoid group. Twelve isoflavones exist in soybeans and they can be divided into two groups: aglycones (daidzein, glycitein, genistein) and their glycosylated forms with glucoside, malonylglucoside and acetylglucoside groups.

Table 4.4. Major phenolics in samples (μg/g sample) by HPLC-DAD

	SEP	MSEP	BMSEP	WCO	BWCO	M95	BM95	M99	BM99
Daidzin	166.9	1.53	nd	nd	nd	nd	nd	nd	nd
Glycitin	71.40	0.67	nd	nd	nd	nd	nd	nd	nd
Genistin	236.5	3.82	tr	nd	nd	nd	nd	nd	nd
Malonyldaidzin[b]	9.688	nd	nd	nd	nd	nd	nd	nd	nd
Malonylglycitin[a]	0.178	nd	nd	nd	nd	nd	nd	nd	nd
Malonylgenistin[c]	10.89	0.07	nd	nd	nd	nd	nd	nd	nd
Acetyldaidzin[b]	13.07	0.21	nd	nd	nd	nd	nd	nd	nd
Acetylglycitin[a]	11.43	0.24	nd	nd	nd	nd	nd	nd	nd
Acetylgenistin[c]	16.04	0.44	nd	nd	nd	nd	nd	nd	nd
Daidzein	84.36	3.44	tr	nd	nd	nd	nd	nd	nd
Glycitein	31.84	1.20	nd	nd	nd	nd	nd	nd	nd
Genistein	332.5	13.2	tr	nd	nd	nd	nd	nd	nd

[a]Expressed as glycitin equivalents; [b]Expressed as daidzin equivalents; [c]Expressed as genistin equivalents. Results are means of three injections. nd = not detected; tr = traces. WCO = waste cooking oil; SEP = soybean oil extraction ethanolic phase; MSEP = oil-rich miscella generated with the SEP; M95 = 95% ethanol oil-rich miscella; M99 = 99% ethanol oil-rich miscella; BWCO = biodiesel from waste cooking oil; BMSEP = biodiesel from oil-rich miscella generated with the SEP; BM95 = biodiesel from 95% ethanol oil-rich miscella; BM99 = biodiesel from 99% ethanol oil-rich miscella

The detected isoflavones were, in their majority, glycosylated compounds. However, both in SEP and MSEP, genistein was the major compound. It is known that phenolic compounds in their aglycone forms possess higher antioxidant activity than their glycosylated counterparts, and that has been verified specifically for isoflavones (Kwak et al., 2007). We suggest that isoflavones existing in SEP and passed on to MSEP might have been responsible for the increased oxidative stability displayed by MSEP, as compared to WCO. The protective role of isoflavones on oil matrixes has been appointed in a previous work our group (Bueno-Borges et al., 2017). As no phenolics were detected in BMSEP, it is believed that such compounds may have been eliminated during the transesterification and the subsequent purification washing steps. In order to achieve commercialization standards, FAEE must undergo several washing steps, until traces of catalyst, water, glycerol and many other specifications

reach established standardized limits. Current biodiesel production methods are therefore incompatible with preserving polar antioxidant compounds in the final product.

4.4. Conclusion

The use of ethanol-based solvents can generate ethanol-in-oil miscellae as a means to significantly improve the quality of WCOs for transesterification. It is possible to produce a value-added feedstock for the biodiesel production chain at a lower cost.

The use of the SEP to generate WCO-derived miscellae with improved oxidative stability has demonstrated to be technically feasible. Provided that free fatty acids and water transference to the miscella could be avoided, the antioxidant potential of this byproduct could be further explored. However, for the biodiesel industry to benefit from the presence of naturally occurring antioxidants in the feedstock, novel production processes should be developed, with emphasis on transesterification methods that require less post-production processing steps.

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5. BIOACTIVE COMPOUNDS IN THE PRODUCTS OF SOYBEAN OIL ETHANOLIC EXTRACTION

ABSTRACT

Extraction of soybean oil using ethanol as solvent can be an advantageous alternative to hexane-based extraction. Besides making use of a renewable non-toxic solvent, ethanolic oil-extraction can generate oil-rich miscellae (ORM) with great stability and an oil-poor miscella (OPM) that can be reused successfully as solvent in further extractions. However, the effects of consecutively reutilizing the oil-poor miscella on oil quality are still unknown. Here, we aimed to determine the changes in antioxidant composition and oil stability throughout 50 consecutive ethanolic extractions. Contents of tocopherols, total carotenoids and isoflavones significantly changed over extractions. Poor correlation with lipophilic parameters indicated that OPM and ORM could have a strong presence of xanthophylls. Average tocopherol content was 17-fold higher in ORM than OPM, but isoflavone contents were up to 6.5-fold higher in OPM than ORM. ORM exhibited a remarkable affinity for the isoflavone aglycone genistein. OPM has shown to be a great concentrator of hydrophilic antioxidants. Reusing the OPM largely improved ORM's stability, as expected. However, this increase was not kept after the first ten extractions, and ORM's stability, at the end of the study was comparable to the first ORM. OPM reuse, performed in the conditions of this study, has shown great potential for application, but its limitations must be better understood and overcome.

Keywords: Aglycone; Isoflavone; Ethanolic miscella; Oil stability; Soybean

5.1. Introduction

The increasing global awareness of the importance of environmental protection, paves the way for the development of cleaner industrial processes. Large-scale extraction of canola, soybean, rapeseed, safflower and sunflower oils is currently performed with hexane by means of a well-stablished counter current process. This petroleum-derived solvent is, in fact, a mixture of isomers, namely commercial hexane, and it is the worldwide accepted solvent for industrial oil extraction. Hexane, however, is highly flammable and explosive, strongly associated to chronic toxicity (Chen et al., 2018) and is a hazardous air pollutant listed on the Clean Air Act (EPA, 1995). In this regard, sustainably-oriented approaches have been investigated as substitutes to hexane oil extraction (Abraham et al. 1991; Loyao Jr et al., 2018).

Ethanol is one of the most promising alternatives for oil extraction, as this low-cost renewable solvent has less handling and health risks than hexane. Ethanol has a maximum allowed concentration in the workplace (MAK value) 10-fold higher than hexane (50 ppm) and can be obtained from several vegetable sources and processes (Goldemberg et al., 2008). The theoretical basis for the solid-liquid extraction of vegetable oils using ethanol has been established by many authors (Beckel et al., 1948a; Magne & Skau, 1953). At the boiling temperature (78 °C), it shows the same oil extraction

efficiency as petroleum-derived solvents (Rao & Arnold, 1958). After ethanol extraction, once cooled to room temperature, miscella (oil/solvent mixture) naturally separates into three phases – an oil-rich phase containing some level of ethanol (4-10%), gum deposits, and an ethanolic phase with 1-4% oil. The oil-poor miscella concentrates, besides ethanol and moisture from the solid material, phospholipids, sugars and other ethanol soluble compounds, thus promoting a partial refining of the oil-rich miscella (Sangaletti-Gerhard et al., 2014a). The ethanolic phase, hereafter named oil-poor miscella, has been reused in further extractions upon some degree of processing (e.g. oil removal by reverse osmosis) (Abraham et al. 1991) or directly applied to fresh feedstock (Magne & Skau, 1953).

Recently, our research group has demonstrated the technical and energetic feasibility of direct transesterification of the soybean oil-rich miscella for biodiesel production (Sangaletti-Gerhard et al., 2014b). However, knowledge on the antioxidant profile of oils extracted with ethanol and its effects on the stability remains scarce. This work sheds some light on the antioxidant composition of two main products of the ethanolic extraction of soybean oil – the oil-rich and oil-poor miscella. This is the first report on the changes in the antioxidant profile derived from consecutive extractions of soybean oil, performed with the reuse of solvent (oil-poor miscella).

5.2. Material and Methods

5.2.1. Materials

Flaked soybean was furnished by Cargill (Mairinque, SP, Brazil). Isopropanol and hexane were HPLC-grade, purchased from Panreac (Barcelona, Spain). HPLC-grade methanol, ethanol and acetonitrile were J.T. Baker (Phillipsburg, NJ, USA). AAPH, fluorescein, methyl-β-cyclodextrin powder, trolox, formic acid, isoflavone and tocopherol standards were obtained from Sigma-Aldrich (St Louis, MO, USA). Absolute ethanol used in the soybean oil extraction as well as all other reagents were analytical grade.

5.2.2. Ethanolic extraction of soybean oil

The apparatus used in this work was a custom-made immersion type extractor (Fig. 5.1). It is composed entirely of stainless-steel containing eight individual extraction vessels with two stoppered openings on the lid, one of which, coupled to a glass-condenser, and a drainage faucet on the bottom of the vessel. These are fixed to an oil-bath tank, equipped with heating element and a thermostat. The oil was constantly mixed by a propeller to ensure optimal heat diffusion. The temperature used

for extraction was set to 78 °C ± 2 (boiling point of ethanol). Soy-to-solvent ratio was 1:2 m/v. Soybean flakes were weighed (50 g) in a cotton bag, supported by an internal cast basket which was accommodated inside each individual vessel and closed with the lid and stoppers. One volume of solvent (50 mL) was added through the upper opening for soaking of the flakes for 30 min. After the soaking period, heat was turned on and 100 mL of solvent was added. After 30 min, the content was drained and collected in Falcon tubes, to conclude extraction cycle 1. The tubes, containing the collected from cycle 1, were cooled in tap water and refrigerated (-20 °C) for 10 min, then centrifuged (3 min, 3200xg). After cooling and centrifugation (Fig. 5.1), phase separation was visible and the upper layer (oil-poor miscella) was collected and used as solvent for cycle 3. Centrifugation allowed full separation between oil-rich and poor phases as well as formation of gum deposits at the bottom of the tube. The same was performed for cycle 4, using the oil-poor phase from cycle 2. If needed, absolute ethanol was used as make-up solvent to complete the volume of poor miscella to 100 mL before entering each extraction cycle. In the first extraction, anhydrous ethanol was used as the solvent for cycles one and two. In the following extractions, the solvent used was the oil-poor miscella produced in the previous extraction.

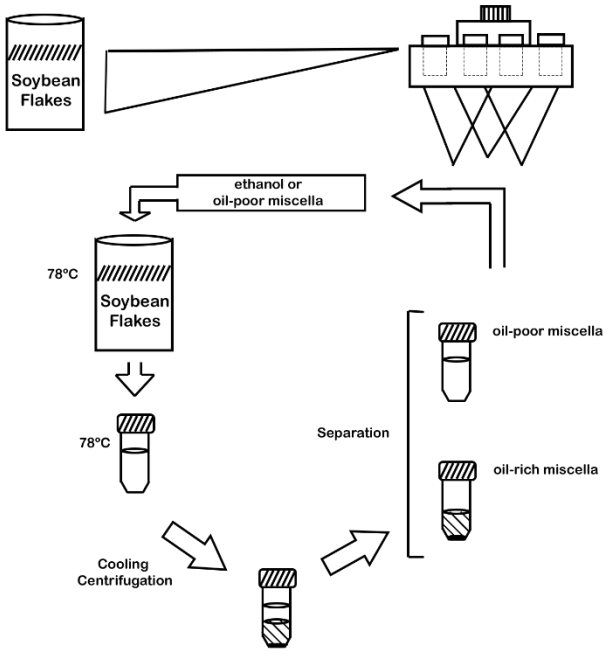

Figure 5.1. Ethanolic extraction of soybean oil from flaked soybeans with the apparatus used in the study

An aliquot (2 mL) of the poor miscella produced in each extraction was stored for analysis and the remainder (approx. 200 mL) was used as solvent in subsequent extractions. The oil-rich

miscella (lower phase) obtained in each extraction, was stored (-20 °C) until analysis. In this study, two extraction processes were conducted independently, concomitantly, for 50 times. Therefore, two samples of each product (i.e. oil-rich miscella and oil-poor miscella) were obtained at each extraction.

Flaked soybean was analyzed for total oil content using the Soxhlet apparatus with n-hexane, and for moisture content (AOAC, 2006). Oil-poor and rich miscellae were analyzed for their non-volatile matter (NVM) gravimetrically after oven-drying.

5.2.3. Oil-stability tests

Oil-stability tests were performed for evaluating changes in the oxidative stability of the oil-rich miscella throughout the consecutive extractions.

5.2.3.1. Oil Stability Index

Oil-stability of the oil-rich miscellae was determined by means of the Rancimat equipment, according to method Cd 12b-92 (AOCS, 2003), using a 743 model Rancimat system (Metrohm Ltd, Herisau, Switzerland). Analyses were performed three times for each sample, at 110 °C with airflow of 10L/h. Samples obtained in extractions no. 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 were assessed. Approximately 2.5 g of oil-rich miscella was used for each replicate. Results are expressed as the induction period (hours).

5.2.3.2. Oven-test

Oil-rich miscellae from extractions no. 4, 7, 13, 19, 26, 32, 38, 44, 48 were evaluated. Each oil sample was transferred into seven screw top clear-glass vials (2.0 mL; 12 x 32 mm). These were placed, without caps, into a non-ventilated oven at 62 ± 1 °C for 14 days. Every second day, a vial from each sample was removed from the oven and stored in freezer. Its content was analyzed for conjugated dienes according to method Ch 5-91 (AOCS, 2003). In brief, 0.01-0.05g of each oil sample was dissolved in isooctane into 10-25mL volumetric flasks to obtain absorbances between 0.2 and 0.8 at 232nm. A UV-VIS spectrometer (model UV-1203, Shimazu, Japan) spectrophotometer with deuterium lamp was used and the analysis was performed twice for each sample duplicate. Specific extinction at 232nm (K_{232}) was calculated by the formula,

$$K_{232} = \frac{A}{C}$$

where *A* is the absorbance of the diluted sample at 232nm and *C* is the sample final concentration expressed in g sample/100 mL isooctane.

5.2.4. In-vitro antioxidant assays

5.2.4.1. Preparation of extracts

The hydrophilic (HF) and lipophilic fraction (LF) of antioxidants were extracted from the oil-rich and oil-poor miscellae. Three grams of each duplicate of oil-rich miscella were weighted in a Falcon tube, to where 6 mL 80 % methanol and 3 mL hexane were added. The tube was vortexed for 30 s and centrifuged (10 °C; 6800xg; 5 min). The bottom layer (methanol phase) was pipetted and transferred to a different tube avoiding collecting any portions of the thick white interface (strong presence of phospholipids). Another 3 mL of 80% methanol was added to the first tube, repeating the procedures thereafter. In total, three extractions with 80% methanol were performed. To the combined collected methanol phases, 3 mL of hexane was added to remove all oil residues and lipophilic antioxidants. After thorough stirring, followed by centrifugation, the hexane phase was collected and added to the first tube. Combined methanol extracts were dried by rotary evaporation (35 °C) followed by lyophilization. These were later resuspended with 1 mL of pure HPLC-grade ethanol to compose the hydrophilic fraction of the oil-rich miscella (HF-ORM). Residual hexane in the first tubes, containing the oil, was dried under nitrogen and 3 mL hexane was added, composing the lipophilic fraction for each oil-rich miscella (LF-ORM). Both LF and HF were stored under -20 °C until analysis. Extracts of the oil-poor miscella were similarly obtained with some modifications: 1 mL of poor miscella was added to an Eppendorf tube and dried under nitrogen gas flow. After that 500 µL of MilliQ water and 400 µL HPLC-grade hexane were added to the tube. Tubes were mixed thoroughly, centrifuged (10 °C; 8600xg; 5 min) and the bottom layer (aqueous) was collected and transferred to a 15 mL Falcon tube. Another 500 µL of MilliQ water were added, repeating the procedures to complete 3 water extractions. The combined water extracts were frozen, lyophilized, and resuspended in 10mL ethanol to compose the antioxidant hydrophilic fraction of the oil-poor miscella (HF-OPM). The remaining hexane phase in the Eppendorf tubes comprised the lipophilic fraction of the oil-poor miscella (LF-OPM).

5.2.4.2. Oxygen Radical Absorbance Capacity – ORAC

The ORAC assay was performed for the HF and LF of rich and poor miscellae using a microplate reader and 96-well black microplates. HF analysis was performed by adding 30 µL of the extract (or standard), 60 µL of 508.25 nM fluorescein solution, and 110 µL 76 mM AAPH solution to the microplate well. Trolox was diluted in 75 mM potassium phosphate buffer (pH 7.4) to build a standard curve (12.5, 25, 50, 100, 200, 400 µM). The blank was only phosphate buffer (200 µL) and the control sample was the buffer (30 µL) used instead of the sample. The analysis of the antioxidants contained in the LF was performed based on the methodology described by Huang et al. (2002) and Chirinos et al. (2013), with modifications. The LF (30 µL) was pipetted in the microplate well, dried under nitrogen gas flow and resuspended in 30 µL 7% randomly methylated β-cyclodextrin (RMβCD) in acetone. Dilutions of trolox were prepared in 7% RMβCD in acetone (50, 75, 100, 200, 400 µM) to compose another standard curve. For the analysis of both the HF and LF, fluorescein and AAPH solutions were prepared in phosphate buffer. Using a SpectraMax M3 microplate reader (Molecular Devices, Sunnyvale, CA, USA) and emission and excitation wavelengths set at 528 and 485 nm, respectively, the microplate was stirred, and readings were carried out every minute for 2 h (37 °C), recording the fluorescein decay area under the curve (AUC). The net integrated AUC from the standard curve and samples was obtained after subtraction of the AUC from blank and control. ORAC analysis was performed in triplicates for each oil-rich and poor miscellae duplicates.

5.2.5. Total carotenoid content

Carotenoid content was measured directly in the oil-rich miscella, dried under nitrogen gas and diluted in hexane to generate absorbances between 0.2 and 0.8. As for determinations in oil-poor miscellae, the ethanol content was completely removed under nitrogen gas and the residue resuspended in hexane. Readings were performed at 470nm in a UV–VIS spectrometer (model UV-1203, Shimazu, Japan). A calibration curve was built using several concentrations (0.2-0.06 mg/mL) of β-carotene in hexane as the standard. Results were expressed as mg β-carotene/g of oil-rich miscella or mg β-carotene/mL of oil-poor-miscella.

5.2.6. Tocopherols by HPLC

Tocopherols were quantified and identified by normal phase HPLC according to method Ce 08-89 (AOCS, 2003). A Shimadzu HPLC system (Columbia, MD, USA) was used, model LC-6AD, with RF-

10AXL fluorescence detector, and LiChrospher Si60 column (5 µm, 25 cm × 4 mm i.d., E. Merck, Darmstadt, Germany). Excitation and emission wavelengths were 290 and 330 nm, respectively. Isocratic elution was carried out at a flow rate of 1.0 mL/min using mobile phase of hexane–iso-propanol (99:1, v/v). Injection volume was 10 µL. External standards of α, β, γ and δ tocopherol homologues were used for identification, by comparing retention times, and quantification through calibration curves. Briefly, 0.5g of oil-rich miscellae were dissolved in HPLC-grade hexane in 5 mL volumetric flasks, whereas oil-poor miscellae (1mL) were dried under nitrogen gas and resuspended in hexane (1 mL). Hexane-diluted samples were filtered (Millex PTFE 0.22 µm pore size, Millipore, Billerica, MA, USA) and placed in screw-cap glass vials for injection.

5.2.7. Phenolics by HPLC-DAD analysis

Major phenolics quantification was determined in HF extracts of the oil-rich and oil-poor miscellae (see section 5.2.4.1). Additionally, determination of phenolics was performed in soybean flake extracts. These were prepared following the same procedure for ORM and OPM HF extracts, except using 2g of flaked soybeans without prior treatments. Analyses were performed on a Shimadzu HPLC system (Shimadzu Scientific Instruments Inc., Columbia, MD, USA) equipped with LC-30AD pumps and a SIL-30AC Auto Injector/Auto Sampler, CTO-20A column oven (30°C), SPD-20 A diode array detector, and a CBM-20A system controller linked to Class-VP software. Column was Shimadzu ODS-A (4.6 mm, 250 mm, 5 µm). The binary solvent system comprising: A - Milli-Q water acidified at 0.25% (v/v) with formic acid, and B - acetonitrile/water/formic acid (80:19.75:0.25 v/v/v). Flow rate was 1 mL/min, using the following programming for solvent B concentration: 0 min – 20%; 10min – 30%; 20 min – 50%; 30 min – 60%; 32-35 min – 100%; 40-45 min – 20%. Sample HF extracts were filtered (Millex PTFE 0.22µm, Millipore, Billerica, MA, USA) and injected at 20µL volume. Phenolics were identified by RT and UV spectra comparison with authentic standards as well as confirmation through mass spectrometry. Calibration curves were prepared from isoflavone aglycones (daidzein, glycitein, genistein) and their glycosylated forms, daidzin, glycitin and genistin, for quantification purposes.

5.2.8. Statistical analysis

All analyses were processed by one-way variance analysis (ANOVA) using Minitab® 18.1 software (Minitab, Inc; State College, PA, United States). Tukey test was used to determine statistical difference among means at the level of 0.05. Unless stated otherwise, analyses were performed in triplicates and are reported as means ± standard deviation.

5.3. Results and Discussion

The consecutive extractions generated oil-poor and oil-rich miscellae, as well as defatted soybean meal and gums. Soybean flakes used in this study presented an average oil content of 22.6 ± 0.4 (% m/m) and moisture content of 8.4 ± 0.0 (% m/m). Extractions, each one using approximately 50 g soybean flakes, yielded an average of 9.0 ± 0.9 (g) oil-rich miscella and 2.0 ± 0.4 (g) of total gums. Oil-poor miscella recovery ranged from 185 to 208 mL per extraction. Mean residual oil in soybean meal after extraction was 3.0 ± 1.2 (% m/m), indicating an average extraction yield of 86.72% for the process employed in this study. Fig. 5.2 shows the contents of non-volatile matter (NVM) for OPM and ORM obtained in ethanolic extractions. This parameter has been used by previous authors and is often a good indication of the lipid content for the oil/ethanol miscellae (Beckel et al. 1948b).

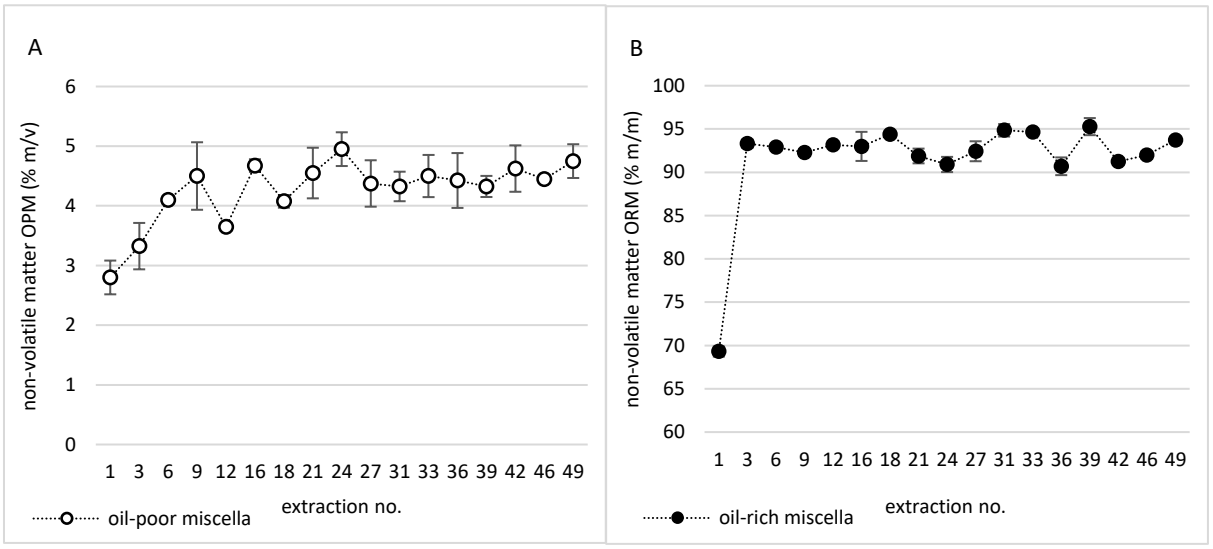

Figure 5.2. A. Non-volatile matter (% m/v). **B.** Non-volatile matter (% m/m) in ORM. Error bars stand for standard deviation

In OPM, NVM increased on a smaller rate than in ORM. However, for both extraction products variations occurring after extraction no. 1 were generally not statistically significant. Throughout extractions, OPM averaged 4.2% NVM, and ORM 91.5%. Sangaletti-Gerhard et al. (2014b) have reported 91.5% NVM for the soybean oil rich miscella, obtained by a similar process. This agrees with the findings by Rao and Arnold (1958), who produced rich miscellae with 91.1-92.9% oil content and poor miscellae with 2.7-3.3% oil content. Beckel, Belter and Smith (1948b) have found between 4.3 and 5.5% NVM in the poor miscella after reusing it for 85 extractions, by means of a continuous countercurrent extractor. Consistently, is has been demonstrated that the composition of these

extraction products remains stable regardless of the method used for ethanolic extraction. Such findings strengthen the argument for ethanolic oil extraction as a large-scale viable alternative.

Rancimat generates an environment of intense pro-oxidant conditions to assess the oil's resistance. In the oven-test, milder thermal conditions are used over an extended time period, which provides more information on the oil's oxidation profile. These accelerated oxidation tests can help pinpoint the ideal oil composition for increased stability.

ORM were evaluated using the Rancimat equipment (Fig. 5.3). The induction period changed significantly throughout the consecutive extractions, increasing gradually from extraction #1 (5.54h ± 0.5) to reach its maximum observed value at #10 (21.35h ± 1.7). At extraction #15, the induction period decreased 65% (7.36h ± 0.4), increasing gradually up to 14.14h ± 0.7 at #35, and decreasing once more at #40 (9.27h ± 0.7) to remain statistically constant until #50 (11.00h ± 0.8). These findings were partially endorsed by the results from the accelerated oven-test (Fig. 5.4), in which similar oxidation patterns have been observed.

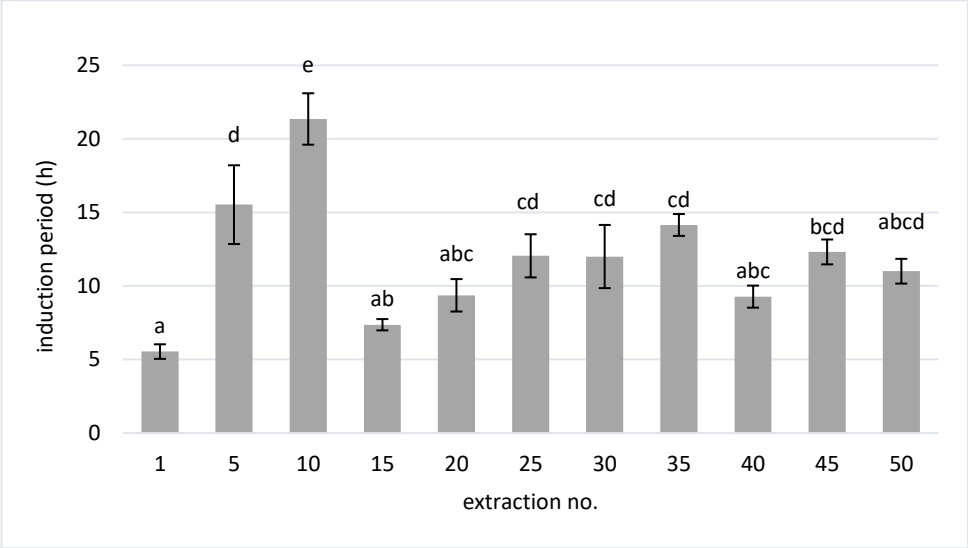

Figure 5.3. Induction period (h) of oil-rich miscellae by Oil Stability Index, using the Rancimat equipment. Results are means of three repetitions. Error bars stand for standard deviations. Letters represent statistical difference to the 0.05 level

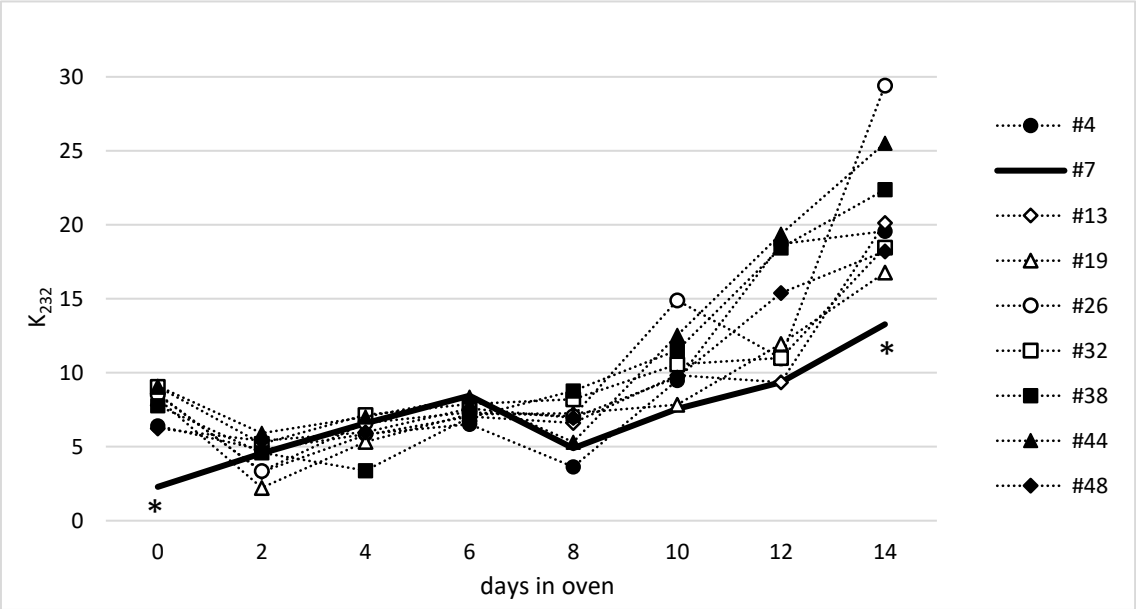

Figure 5.4. Absorptivity at 232 nm of oil-rich miscellae submitted to accelerated oven-test at 62 °C. Measurements were taken twice for each sample duplicate on indicated days; lines were drawn for illustrative purposes. * stands for statistical difference at the level of 0.05

Under oven-test conditions, oil samples undergo oxidation reactions, generating conjugated dienes, peroxides and hydroperoxides, which in turn breakdown into volatile compounds. For that reason, absorptivity at 232 nm can decrease at some points during the experiment. ORM #7 showcased greater oxidative stability in the longer-term in comparison to other samples. Prior to the oven-test (day 0) #7 presented the lowest measured values for conjugated dienes, indicating good oxidative status.

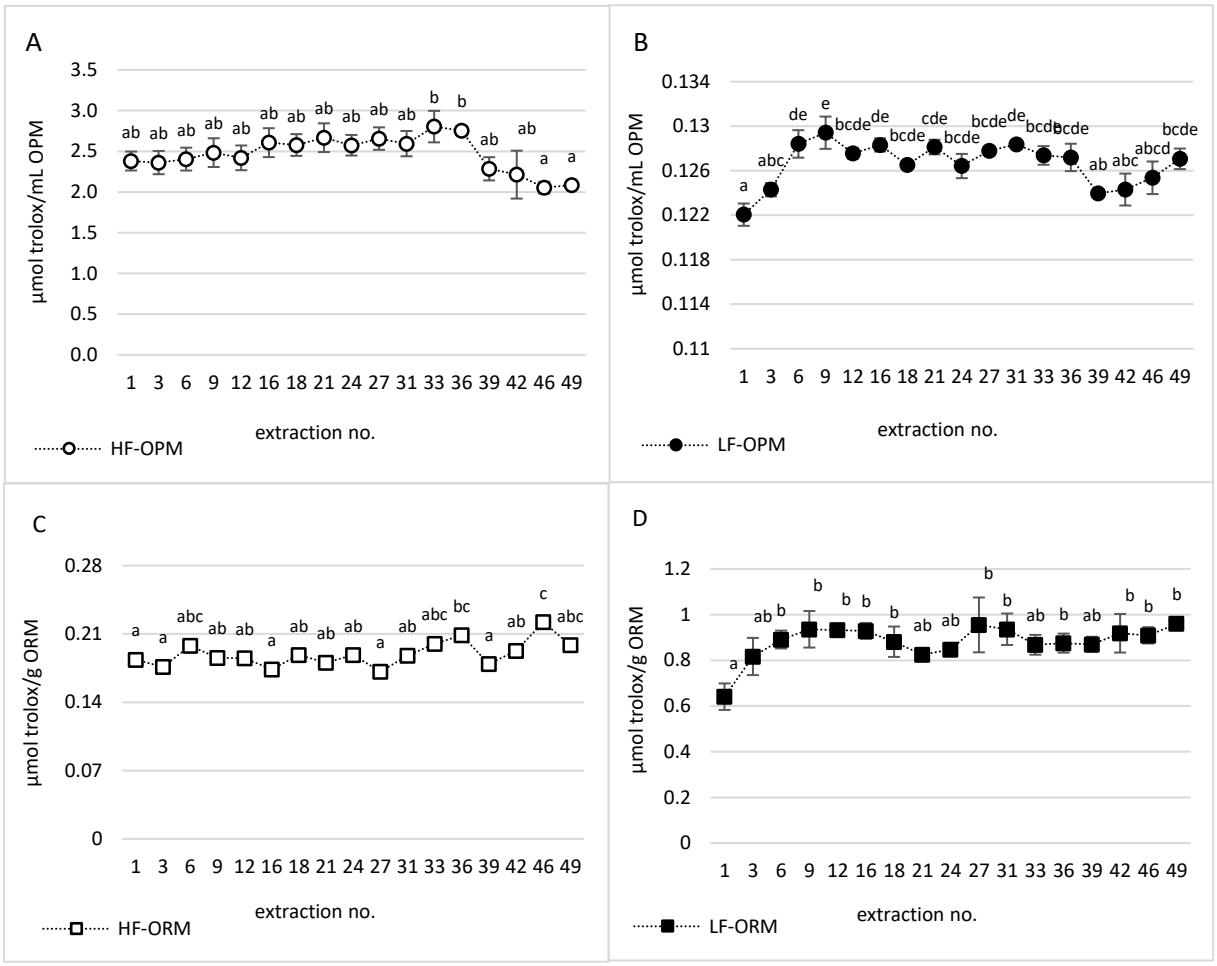

Figure 5.5. Oxygen antioxidant capacity (ORAC) **A.** ORAC for the hydrophilic (HF) fraction of oil-poor miscellae (OPM), expressed as µmol trolox/mL of OPM. **B.** ORAC for the lipophilic fraction (LF) of oil-poor miscellae (OPM), expressed as µmol trolox/mL of OPM. **C.** ORAC for the hydrophilic (HF) fraction of oil-rich miscellae (ORM), expressed as µmol trolox/g of ORM. **D.** ORAC for the lipophilic fraction (LF) of oil-rich miscellae (ORM), expressed as µmol trolox/g of ORM

In the ORAC assay, AAPH decomposition at 37 °C acts as a peroxyl radical generator in the reaction medium. For the analysis of the lipid fraction of rich and poor miscellae RMβCD was used for improving the solubility of lipophilic antioxidants in the test solution. RMβCD is composed of seven glucopyranose units, cyclically linked, conferring hydrophilic character to the external surface and moderate hydrophobicity to the internal structure. HF-OPM-ORAC (Fig. 5.5a) reduced significantly from extraction #33 to #46-49. Average HF-OPM-ORAC was nearly 20-fold higher than LF-OPM-ORAC (Fig. 5.5b), which should be expected given the high ratio between alcoholic/lipid fractions (22.5) in this product. OPM-LF-ORAC correlated moderately with non-volatile matter (0.67). As for ORM, LF-ORAC (Fig. 5.5d) was 4.6-fold higher than ORM-HF-ORAC, given that the composition of the rich miscella in this study is comprised of, mostly, lipids. The highest activity for deactivation of the ORAC radical in HF-ORM was observed for sample #46, which was not statistically different from samples #6,

#33, #36 and #49 (Fig. 5.5c). Correlation between ORM-LF-ORAC and non-volatile matter was strong (0.81). Correlations between LF-ORAC and NVM for both OPM and ORM reveal that, as expected, non-volatile matter may be more representative of lipid content for the rich miscella than for the poor-miscella. In LF-ORAC, RMβCD was used as a facilitator to improve solubilization of hydrophobic antioxidants in aqueous solutions. Still, there's a gap in techniques that allow proper assessment of lipophilic antioxidants' activity in lipid-soluble and other complex media.

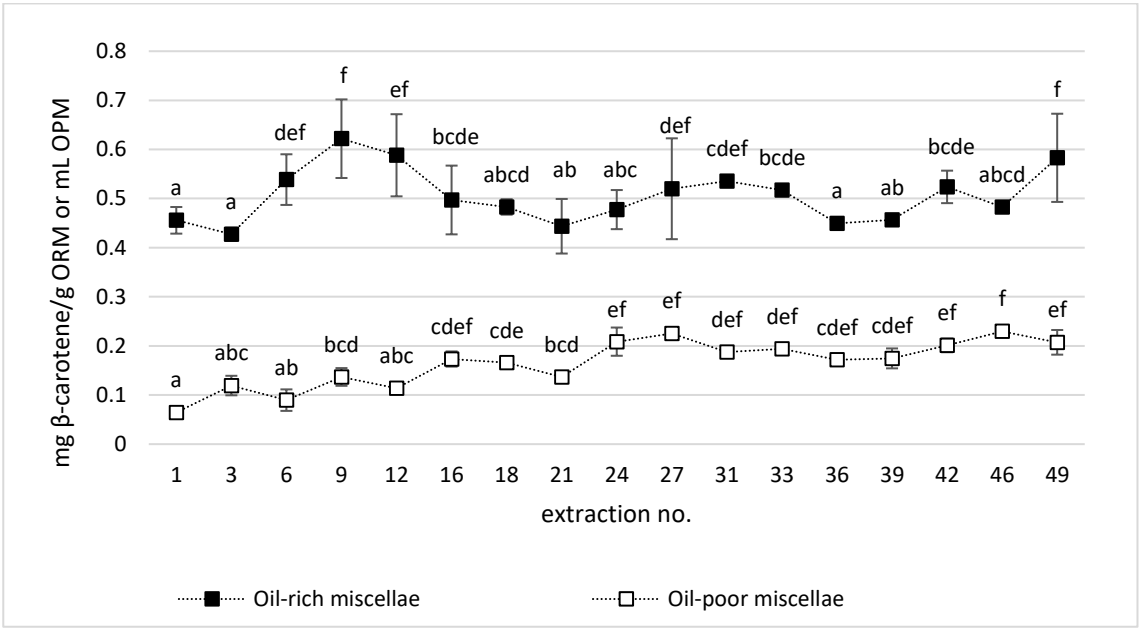

Figure 5.6. Total carotenoid content in oil-rich and oil-poor miscellae, expressed as mg β-carotene/g ORM or mL OPM

Total carotenoid content (Fig. 5.6) for both OPM and ORM varied significantly along extractions. In oil-poor miscella, total carotenoid content (mg β-carotene/mL) started at 0.06 ± 0.0, increasing significantly over extractions, until reaching stabilization at #16 (0.17 ± 0.0). As for the oil-rich miscella, carotenoid content fluctuated thoughout the study, concentrating among samples #6 to #16 and again at #27-33. Although the oil-rich miscella can reach over a 45-fold higher lipid content than the oil-poor miscella, average carotenoid content between these two products was similar. This could indicate that the majority of carotenoids concentrated in the OPM were oxocarotenoids, also known as xanthophylls. As opposed to carotenes – hydrocarbon carotenoids – xanthophylls present at least one oxygen atom in the structure, greatly increasing their solubility in polar media as compared to carotenes. Lutein is the major xantophyll found in soybeans, followed by smaller contents of zeaxanthin and cryptoxanthin (Slavin et al., 2009). Total carotenoids in OPM correlated with non-volatile matter at the level of 0.75 and with LF-OPM-ORAC at 0.15. Good correlation with NVM could be related to the increase in hydrophilic compounds in OPM along extractions. These ethanol-soluble

solids would be embeded in the increasing NVM content since the oil-poor miscella is mostly comprised of ethanol. Carotenoids in ORM presented a moderately positive correlation with LF-ORM-ORAC (0.62) but correlated weakly to non-volatile matter in ORM (0.25). These findings indicate a poor relationship between total carotenoids and lipid content in the ORM, which could point to the presence of xantophylls. Also, LF-ORAC indicated that the antioxidant activity of lipid-soluble compounds in the rich miscella is not restricted exclusively to carotenoids. Oil-stability tests and total carotenoids exhibited strong similarities. This could indicate that the unusual oxidative resistance presented by the soybean oil-rich miscella in comparison to hexane-extracted soybean oils (Bueno-Borges et al., 2017) may be influenced by the carotenoid fraction obtained in ethanolic oil extraction.

Tocopherols were determined in the OPM and ORM (Fig. 5.7). Correlation between total tocopherols and NVM was 0.74 for OPM and 0.89 for ORM. As expected, tocopherol content was strongly, positively affected by non-volatile matter, which is directly linked to oil content in both miscellae. This effect was stronger in ORM, for its much higher oil content. Changes in tocopherol content were characterized by increasing concentration until attaining a peak concentration from where reduction continued to occur at a smaller rate. Tocopherols in the oil-poor miscella varied significantly along extractions. Total tocopherols increased continuously starting at 31.9 ppm ± 0.3 (#1) to reach the maximum observed concentration at 62.9 ppm ± 0.4 (#9). From #12 to #42 tocopherol content decreased gradually in 15% (62.9-53.3 ppm). In rich miscellae, total tocopherols at #1 were 157.7 ppm ± 20.7, to reach a 2-fold increase at #3 (315.5 ppm ± 6.44). From extraction #3, ORM increased non-significantly up to the maximum observed value at #18 (372.5 ppm ± 29.3), decreasing non-significantly thereafter to 305.5 ± 7.7 (#33). Total tocopherols correlated strongly with LF-ORAC in OPM and ORM: 0.81 and 0.80, respectively.

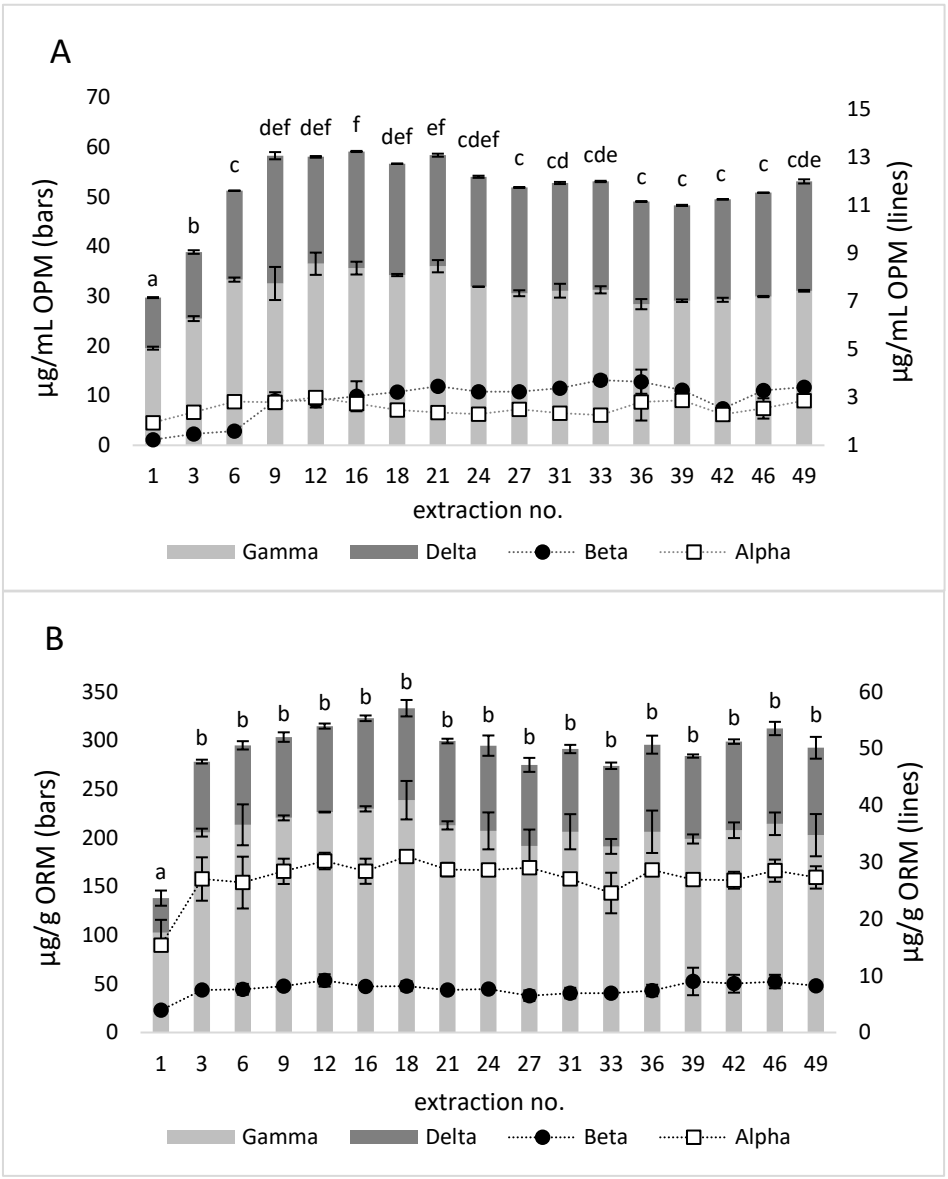

Figure 5.7. A. Tocopherol content in oil-poor miscella (µg/mL OPM). **B.** Tocopherol content in oil-rich miscella (µg/g ORM). Letters represent statistical diference of total tocopherols between samples

In average, tocopherol content was 17-fold higher in ORM than OPM. Despite that difference, tocopherol profile between rich and poor miscellae was similar: alpha homologue corresponded to 8.4 and 8.1%, beta was 2.4 and 4.1%, gamma was 63.2 and 59.9% and delta, 26.0 and 27.9%, respectively.

Figures 5.8a and 5.8b show the isoflavone (aglycone x glycosylated) contents in oil-poor miscella and oil-rich miscella, respectively. Total isoflavones in OPM did not increase over the study, showing no clear trend. Instead, glycosylated isoflavones in OPM increased in 16-77%. Strongest correlations involving isoflavones in OPM occurred between total glycosylated and total carotenoids (0.63), as well as non-volatile matter (0.62). Total OPM aglycones correlated inversely to HF-ORAC in (-0.59). In oil-rich miscellae, aglycone content increased significantly over extractions, reaching

stabilization around extraction no. #16, maintained until #49. Glycosylated isoflavone content did not vary significantly in ORM, showing no clear trend. Ratio between total isoflavones in OPM and ORM was 4.5 to 10.3, demonstrating the stronger affinity of OPM for phenolic compounds. Highest ORM correlations occurred between total aglycones and NVM (0.57), as well as HF-ORAC (0.41). These results indicate the strong affinity of isoflavone aglycones for the oil-rich miscella. Such compounds are highly valued for their enhanced absorption features in comparison to their glucoside counterparts (Izumi et al., 2000). Sophisticated approaches have been applied to the processing of soybeans and soy foods with the aim of increasing aglycone content, given the great commercial and nutritional importance of these compounds (Wei et al., 2008; Prasad & Shah, 2011; Lima & Ida, 2014). The ethanolic extraction of soybean oil, however, has shown to naturally promote the concentration of such important bioactives.

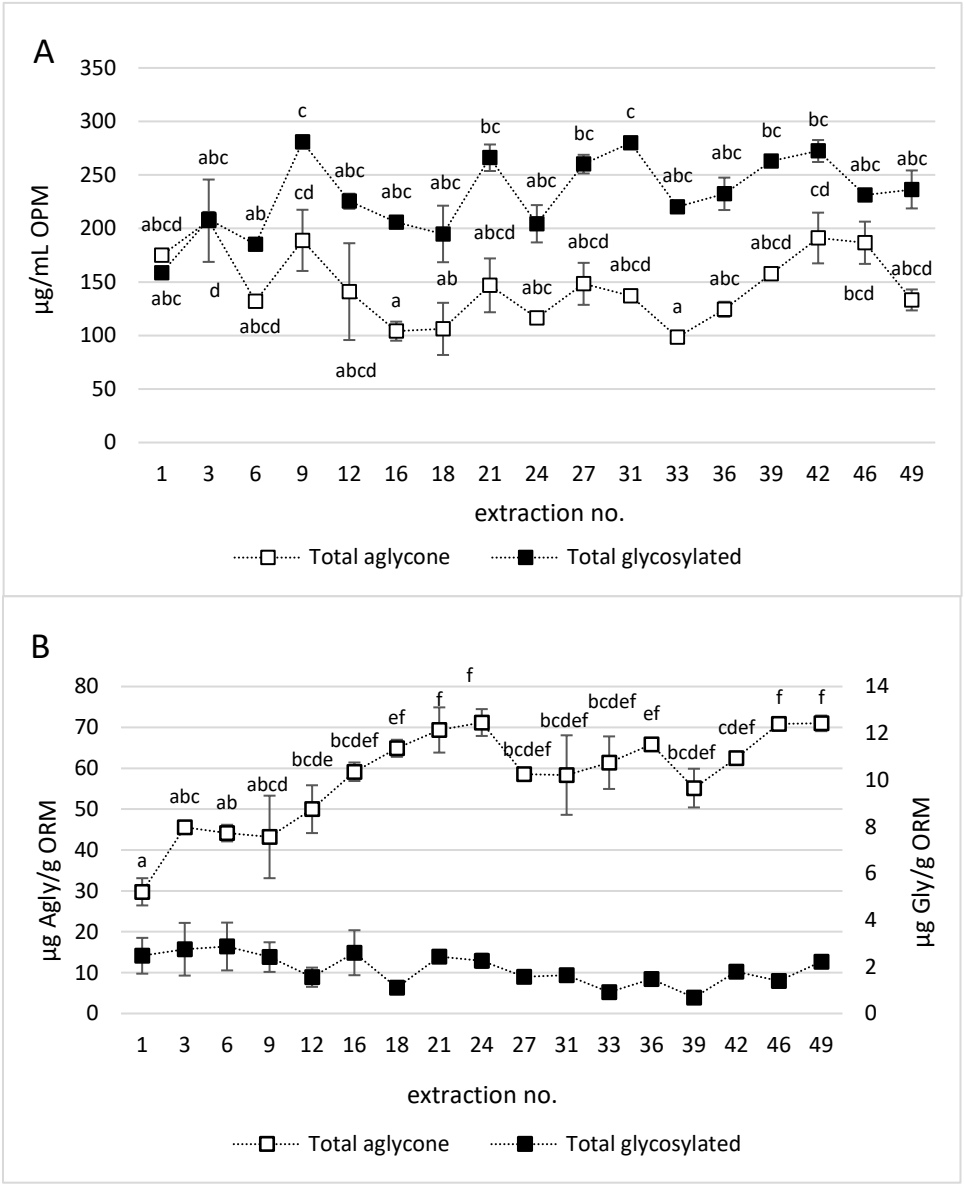

Figure 5.8. A. Total aglycones (Agly) and total glycosylated (Gly) isoflavones in oil-poor miscella (µg/mL OPM). **B.** Total aglycones (Agly) and total glycosylated (Gly) isoflavones in oil-rich miscella (µg/g ORM). Letters represent statistical diference between samples

Soybean flakes used in this study presented 8.4% moisture content. Since extractions were carried out without drying the flakes before extraction, moisture content would be expected to increase in the produced miscellae. For that reason, Rao and Arnold (1958) had predicted that, by reusing the oil-poor miscella there would be a buildup of alcohol-soluble nonglyceride compounds in the extraction products. This effect was confirmed by observing the content of non-volatile matter of the HF extracts prepared from OPM and ORM (data not shown), which increased along the study. This reveals that alcohol-soluble compounds increased over extractions in both miscellae, which was reflected on the isoflavone content of the ORM as observed in Fig. 5.9.

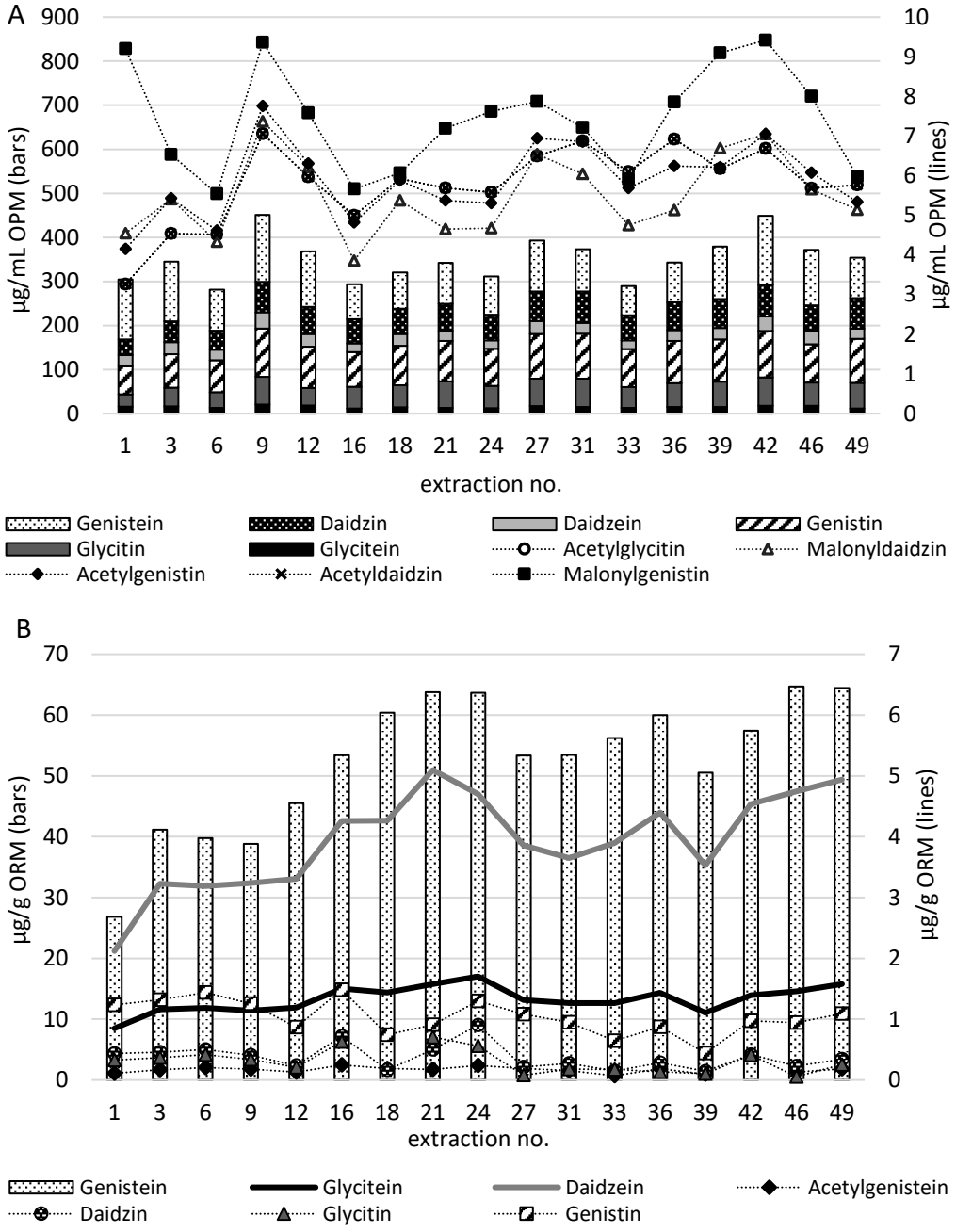

Figure 5.9. A. Isoflavones in oil-poor miscella (µg/mL OPM). **B.** Isoflavones in oil-rich miscella (µg/g ORM)

Table 5.1. Average isoflavone profile in soybean flakes (μg/g, %), oil-poor miscella (%) and oil-rich miscella (%)

	Soybean flakes		Oil-poor miscella	Oil-rich miscella
	μg/g*	%	%	%
Daidzin	4.90 ± 0.0	6.8	10-18	0.2-1.4
Glycitin	4.94 ± 0.1	6.9	8.4-16	0.1-1.0
Genistin	8.34 ± 0.0	12	19-27	0.8-3.8
Malonyldaidzin	2.25 ± 0.0	3.1	1.2-1.6	-
Malonylglycitin	1.39 ± 0.0	1.9	-	-
Malonylgenistin	4.01 ± 0.0	5.6	1.5-2.7	0-0.3
Acetyldaidzin	0.38 ± 0.0	0.5	1.0-1.9	0-0.3
Acetylglycitin	2.50 ± 0.0	3.5	3.0-3.8	0-0.8
Acetylgenistin	0.82 ± 0.0	1.1	1.2-1.7	0-0.4
Daidzein	6.20 ± 0.0	8.6	5.6-7.6	6.1-7.1
Glycitein	2.35 ± 0.0	3.3	3.0-4.6	2.0-2.6
Genistein	33.8 ± 0.5	47	20-41	83-90
Total isoflavones	71.8 ± 0.4			

* determined on wet basis

In OPM, aglycones (daidzein, glycitin, genistein) and β-glycosides (daidzin, glycitin, genistin) were the highest in content. Genistein was the most abundant isoflavone in OPM, followed closely by genistin. Malonylglycitin was not detected in OPM. In ORM, genistein was also the most present isoflavone, 13-fold higher in concentration than the second most abundant (daidzein). Malonyldaidzin and malonylglycitin were not detected in ORM. Kinoshita et al. (1998) have reported that soy sauces produced from whole soybeans contained more phenolics - specifically daidzein, genistein derivatives and ferulic acid - than those produced from defatted soybeans. This enhanced affinity for lipid-based matrixes, displayed by some soy phenolics, could help explain the increasing aglycone and, specially, genistein content in ORM. The interaction or even the accumulation of other components such as moisture, phospholipids and saponins may be involved in the aglycone-increased effects observed here.

Table 5.1 shows the isoflavone composition in soybean flakes in comparison to OPM and ORM. All 12 soybean isoflavones were found in the ethanolic extracts prepared from soybean flakes, revealing a strong presence of aglycones - approximately 60% of total isoflavones. OPM displayed a similar isoflavone composition to soybean extracts, but with a higher balance between aglycone and glycoside fractions. Soybeans presented total isoflavone content (71.8 μg/g soybean flakes)

comparable to ORM (32.2-73.8 μg/g ORM). This demonstrates the strong ability, displayed by OPM, of concentrating phenolics (336-498 μg/mL OPM).

Overall, whole soybeans presented higher malonyl-glycosides than OPM and ORM, respectively. OPM, presented higher β-glycosides than soybeans and ORM, respectively. The detailed isoflavone composition of ORM (Fig. 5.9) reveals that the aglycone increase presented in Fig. 5.8 was almost entirely due to genistein accumulation, since daidzein and glycitein contents remained similar between soybeans, OPM and ORM. Isoflavones are well-studied phytoestrogens with many known benign effects on biological systems (Sirotkin & Harrath, 2014). Isoflavones are usually regarded as having poor antioxidative activity in comparison to compounds such as quercetin, ascorbic acid and kaempferol. However, among known isoflavones, genistein has the strongest *in vitro* antioxidant capacity (Mitchell et al.,1998). Kwak et al. (2011) have found increased radical scavenging activity for a fermented soy paste with higher contents of isoflavone aglycones in comparison to other soy products.

The effects and interactions between lipophilic and hydrophilic compounds on the stability of lipid systems have yet to be fully understood. The presence of water and surface-active molecules, such as phospholipids and saponins, has also to be considered in the overall oil-stability (Laguerre et al., 2016). It still unclear if oil-stability was more intensely influenced by one group of molecules or their interactions in the complex lipid matrix, either generating positive effects against oxidation or undesired pro-oxidant ones.

5.4. Conclusion

The present study addressed the variety of antioxidant groups existing in the soybean and that can be transferred to soybean oil by means of the ethanolic extraction. The soybean oil-rich miscella showcased a very strong affinity for the isoflavone genistein, which increased significantly over extractions, to some extent. The production of a high genistein-containing soybean oil presents great potential for product development, since isoflavone aglycones are highly valued bioactive compounds. This finding could help raise awareness on the many advantages of the ethanolic extraction of soybean oil, given the biological importance of isoflavone aglycones. The ORM also displayed a stable composition, reflected on its tocopherol content and non-volatile matter, in accordance to other findings throughout the literature.

The oil-poor miscella revealed to be a great concentrating agent for hydrophilic antioxidant compounds. Its reuse as solvent in the soybean oil extraction allowed to improve the oxidative resistance of the generated oil-rich miscella to a great extent. However, it also showed that this application, as performed in the conditions of this study, has limitations, since oil-stability not only

stopped improving but decreased after a certain number of reuses. Pro-oxidant effects, caused by the interaction between high concentrations of antioxidant compounds, may have taken place in this marked reduction in ORM's oxidative resistance. If so, isoflavones could have played a major role in this reduction, considering that this class of antioxidant compounds increased greatly over extractions, with emphasis on the compound genistein.

More studies on the composition of soybean oil ethanolic miscellae will allow to pinpoint the direct effects of specific antioxidants on oil-stability.

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6. FINAL CONSIDERATIONS

The ethanolic extraction of soybean oil has demonstrated to possess interesting features and great potential for many applications. The general composition of the extraction products – oil-poor miscella and specially the oil-rich miscella – was consistently stable, as observed here and by previous researchers. This aspect of the ethanolic oil extraction delivers a great level of assurance to the possibility of large-scale applications of this technique.

As observed in this study, the oil-poor miscella worked as a concentrating agent for antioxidants with affinity to polar solvents. The contents of isoflavones and antioxidant activity assays revealed a much higher presence of such compounds in the OPM in comparison to the original matrix (soybean flakes) and the oil-rich product. The OPM acts as an antioxidant repository, which could be used to improve the oxidative stability of low-quality oils, such as waste cooking oil. The solvent reuse element of the ethanolic extraction enables the oxidative stability of the rich miscella to be improved, since antioxidant compounds are gradually added at each extraction. This way, the pool of antioxidants is much higher than it would be possible to garrison in a single extraction. Consecutively reusing the oil-poor miscela, however, has exhibited limitations, since the oxidative stability of the ORM reduced largely after a certain number of extractions. This could be a strong indicative of pro-oxidant activity taking place when determined antioxidants reach a limit concentration in the oil-rich miscella. Further studies are demanded on this matter, allowing the full comprehension on the oxidative stability regulation of the oil-rich miscella. Increasing knowledge on the ethanolic oil extraction of vegetable oils can, therefore, improve the general understanding of the underlying mechanisms involved in the complex antioxidant interactions within lipid systems and their role in providing resistance against oxidation reactions.