Production of Biodiesel and Protein Isolates from Dehulled Yellow Mustard Flour

by

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Abstract

Aqueous extraction is an emerging alternative to hexane-based oilseed extraction since it eliminates the dangers associated with processing, and allows the simultaneous recovery of high-quality protein products and vegetable oils. Five different non-enzymatic and enzymatic aqueous extraction processes (AEP/EAEP) were developed for dehulled yellow mustard flour with the aim of producing food-grade protein isolates and fuel-grade biodiesel. The oil released in these processes was tied up in oil-in-water emulsions that must be destabilized to recover free oil prior to biodiesel production. The remarkable stability of the released emulsion was due to the presence of protein emulsifiers of high molecular weight along with the mixed phospholipid-oleosin layer. pH adjustment and enzymatic treatments with different proteases and phospholipases for emulsion destabilization were relatively inefficient; therefore, a novel chemical approach was developed based on dissolution of the emulsion in tetrahydrofuran (THF) or dioxane to recover the released oil in the form of single-phase oil-solvent-water miscella suitable for direct conversion to biodiesel through single-phase base-catalyzed transmethylation after reducing the water content to the quality standards required for biodiesel feedstock. The emulsion destabilization using organic solvents was optimized, based on experimentally prepared ternary phase diagrams of THF/oil/water and dioxane/oil/water, and the results suggest that this technically viable approach can successfully recover essentially all of the oil from the emulsion. The resulting miscella phases were successfully dehydrated by adsorption over zeolite 4A using either batch or fixed-bed systems. The dehydrated miscella phases were finally reacted with methanol in a single-phase transmethylation process with high yields (99.3 wt%) to fatty
acid methyl esters (FAME). The resulting FAME satisfied the international standards for use as biodiesel fuel.

Finally, our integrated process consisting of aqueous contact of mustard flour to recover protein as the main product followed by dissolution of the resulting emulsion with THF could produce high-quality protein products free of solvent contact/residues, while biodiesel could be efficiently recovered using a less flammable solvent than conventional hexane extraction. We believe that this study will help Canada to balance food and biofuel production that can lead to lower food prices, benefiting many poor people in the world.

Keywords dehulled yellow mustard flour; aqueous extraction process (AEP); enzyme-assisted aqueous extraction process (EAEP); yellow mustard emulsion; emulsion destabilization; tetrahydrofuran (THF); 1,4-dioxane; dimethylformamide (DMF); oil-solvent-water miscella; ternary phase diagram; miscella dehydration; single-phase base-catalyzed transmethylation; standard fatty acid methyl esters (FAME)
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CHAPTER 1

1 Introduction

This project endeavors to recover both protein and biodiesel from dehulled yellow mustard flour by combining two technologies: ultrafiltration/diafiltration membrane separation process and single-phase transmethylation process, which is based on the rapid reaction between the oil and alcohol in a solvent that dissolves both the non-polar oil and the polar components.

“Food or Fuel” is an international controversy about the use of agricultural land to produce biofuels which results in higher food prices and adversely affects poor people. Therefore, it is essential to find a compromise that satisfies the needs for both food, and green transportation fuel. Mustard seeds are sources of oil and protein, and potentially could be used to produce both biodiesel and high quality protein products. Canada is the world's second-largest producer and largest exporter of mustard seed. Its production can be readily increased without adversely affecting any existing food crops such as canola since mustard seed has the advantage of being tolerant to drought, heat and frost. Yellow mustard contains ~30% oil and 27% of very high quality nutritious protein. The proteins are equivalent to soybean in nutritional quality, as they have a well-balanced amino acid distribution for human use. Unfortunately, the oil is very high in erucic acid, which is known to cause heart health problems, and is at present banned for use in food products in much of the developed world. The oil is potentially useful for the production of biodiesel since high erucic acid oils can make biodiesel containing erucic acid alkyl esters, which have superior lubricating properties. Therefore, this study focused on recovering both protein and biodiesel from dehulled yellow mustard flour by combining two novel technologies: membrane processing and single-phase transmethylation process.

To achieve this goal, the Food Engineering Group at University of Toronto initiated a program to produce protein isolates from dehulled yellow mustard flour using an aqueous extraction process (AEP) since desolventizing after solvent extraction can denature the protein extracted (Balke, 2006, Soltero, 2013). AEP, in which water is used as an extraction medium to extract protein and other soluble components present in oilseeds and to release the oil as a separate phase, is an
emerging alternative to hexane extraction since it does not leave undesirable solvent residues in the resulting food products. During AEP, an oil-rich and a protein-rich aqueous fractions were produced simultaneously with minimal protein damage; however, most of the oil extracted was bound in a stable oil-in-water emulsion due to the stabilizing effect of the oleosin protein and phospholipids surrounding the oil bodies (Chen and Diosady, 2003, De Moura et al., 2008, Rosenthal et al., 1996). Clearly, new techniques for recovering the oil from the emulsion are desirable.

The previous demulsification methods identified in the literature, including heat treatment and freeze-thaw treatment were unable to break emulsions formed by aqueous extraction processes. Heat treatment alone did not modify the free oil recovery from the emulsion produced from aqueous processing of soybean flour, and freeze-thaw treatment resulted in oil yields of only up to 22% (Chabrand et al., 2008). Total emulsion destabilization has been recently achieved through the addition of various proteases and phospholipases to the soybean oil emulsions formed in the enzymatic aqueous processing of extruded soybean flakes and soybean flour (Chabrand and Glatz, 2009, De Moura et al., 2008, Jung et al., 2009, Wu et al., 2009). However, due to the cost of enzyme treatment, we decided to concentrate on enzyme-free processes in this project. One of the most successful chemical approaches in the literature is still the work of Li et al. (1977), who developed a process to break emulsions by adding a mixture of isopropyl alcohol (IPA) and cyclohexane, one miscible with oil and one miscible with water, and separating the emulsion into two separate layers; however, this technique is too expensive and dangerous to be effectively used in industrial oil production. Therefore, members of the Food Engineering Group investigated the use of organic solvents with solubilities in both oil and water to solubilize yellow mustard emulsion through single and multiple stage extractions. Isopropyl alcohol’s (IPA) complete miscibility with water and relatively good solubility in oil made it an excellent candidate for emulsion destabilization. The highest oil recoveries, 96.3%, were achieved using four-stage extraction at 2:1 IPA:oil weight ratio (Ataya Pulido, 2010, Jung, 2011). The use of other organic solvents with solubilities in both oil and water including dimethylformamide (DMF), tetrahydrofuran (THF) and 1,4-dioxane was also evaluated in this investigation.
Once the oil is extracted from the emulsion with these organic solvents, the solution of oil and solvent (miscella) can be mixed with methanol and used directly in the production of biodiesel through transesterification after water removal.

Transesterification is a chemical reaction between triglycerides and an alcohol in the presence of a catalyst to produce the corresponding alkyl esters of the fatty acid mixture that is found in the parent vegetable oil. Methanol is the prevalent alcohol for the production of methyl esters for use as biodiesel due to its low cost. However, the solubility of oil in methanol is low at ambient temperature and the reaction presents mass transfer limitations. In a novel process developed in our department by Professor Boocock’s group, simple ethers such as tetrahydrofuran (THF) or methyl tertiary butyl ether (MTBE) were used as co-solvents that dissolved all reagents in the transmethylation to greatly enhance the rate of the reaction (Boocock et al., 1998, Boocock et al., 1996a, Boocock et al., 1996b).

The overall goal of the program was to develop an integrated process consisting of an aqueous extraction step (AEP) to recover high-quality food protein, free of solvent contact/residues, as the main product, followed by destabilization of the resulting oil-in-water emulsion for direct conversion to biodiesel. To achieve this goal, first, an optimal aqueous extraction process (AEP) for dehulled yellow mustard flour was designed with improved oil and protein extractabilities while addressing the quality of the protein extracted and the stability of the emulsion released. Secondly, the use of cyclic ethers including THF or 1,4-dioxane was evaluated to solubilize the emulsion obtained during AEP through single- and multi-stage destabilization processes to produce oil-solvent-water miscella. Finally, the adsorptive dehydration of miscella was investigated and the dehydrated miscella was used as the medium for a single-phase transmethylation to produce standard fatty acid methyl esters (FAME).

Based on the goal of this investigation, the following literature review (Chapter 2) introduces the structure and composition of the yellow mustard seed. It then compares the conventional oil recovery methods with aqueous extraction and considers the advantages and disadvantages of the aqueous extraction process. The next section reviews the characteristics of the food emulsions and previously published methods for demulsification. Finally, biodiesel production and the effect of water on the transesterification process, and water removal strategies are reviewed.
CHAPTER 2

2 Literature Review

2.1 Mustard Seeds

The term “mustard” is believed to be derived from the use of seeds as condiments; the sweet “must” of old wine was mixed with crushed seeds to form a paste, “hot must” or “mustum ardens”, hence mustard (Hemingway, 1995). Apart from the use of mustard in the preparation of condiments, the seeds have considerable potential as sources of edible oil and protein. In this respect, efforts have been made for several years to develop mustard seeds as potential oilseed crops in Canada (Woods et al., 1991).

Western Canada has been a major producer of mustard seeds since World War II, when supplies from Western Europe - the historic base of production - were disrupted. The industry began in a very small way, with a modest 40 hectares planted in southern Alberta in 1936. The production was dramatically increased in Canada since the excellent cultivation conditions of the Prairies grew mustard seeds with high quality and relatively low risk of crop failure. Canadian mustard seed production is now concentrated in the Prairies, particularly in Saskatchewan, where over 80% of the domestic crop is produced. Demand for mustard seeds is expected to grow during the next decade as the world population and the use of spices increase. Canada can consistently meet the international demand for this valuable and widely consumed commodity.

One reason for the high quality of mustard seeds in Canada is the hot and dry weather of the growing regions in July, which contributes to the reduction of oil concentration, and increase in the concentration of protein and glucosinolates. The second advantage is characterized by strong winds which allow the crop to dry prior to harvesting. In addition, the cold and dry winters provide suitable storage conditions to preserve the seed quality. Mustard seed also has the advantage of being tolerant to drought, heat and frost. Research priorities for mustard breeding include investigation of the health and nutritional benefits and the new food and non-food applications such as the development of bio-products from mustard (Agriculture and Agri-Food Canada, 2011).
Canada is now the world's second-largest producer of mustard seeds. The major producers of mustard seeds in 2012 were Nepal (35.8 %), Canada (29.2 %), Myanmar (16.8 %), Russian Federation (10.2 %), and Ukraine (8.6 %) (FAOSTAT, 2012). Canada is also considered the largest exporter of mustard seed in the international market accounting for about 50% of global exports. The largest market for Canadian mustard seeds is the United States, where around 43% of the shipments were destined in 2007. Other important markets include Belgium, Germany, the Netherlands, and Japan (Agriculture and Agri-Food Canada, 2012).

The most significant mustard species are yellow mustard (*Brassica hirta*), brown and oriental mustard (*Brassica juncea*), and black mustard (*Brassica nigra*). About 44% of western Canadian mustard production in 2013 was estimated to be the yellow type, followed by 41% brown and 15% oriental mustard (Canadian Grain Commission, 2013).

Yellow mustard (*Brassica hirta*) is also known as white mustard (*Sinapis alba*) (Weber et al., 1974). Yellow mustard seeds are slightly larger than the other types, about 3 mm in diameter, and they are flattened laterally. As the name implies, they are mostly pale straw in color, and this light color allows easier food processing. Yellow mustard seed, the mildest of the three types, is suitable for a wide range of applications, including spice mixes, processed meats, and other food products. It is also used more extensively in cooking due to its less aggressive flavor. The most recognizable product made from yellow mustard seed is yellow table mustard or hotdog mustard (Balke, 2006).

Brown and oriental mustard seeds (*Brassica juncea*) share the same chemical make-up with seed diameter of 2 mm. Oriental mustard seeds are golden yellow, while the brown types are reddish brown to dark brown in color. They are used extensively for cooking oil in Pakistan and India, and globally as a spice for a variety of hot prepared mustards. Brown and oriental mustard seeds contain more oil (39.5-44.9%) than yellow mustard seeds (32.3%), while the protein content in yellow mustard seeds (29.5%) is slightly higher (24.2-26.3%) (Canadian Grain Commission, 2013). Currently yellow mustard seed is only used as a condiment in Western markets. While the traditional food uses for mustard are saturated, there is a huge potential for using the protein for human food and recovering the oil for industrial applications, the ultimate goal of this investigation.
Black mustard seeds (*Brassica nigra*) are about 2 mm or less in size, and they are similar to brown mustard seeds in outward appearance. Black mustard seed has been largely replaced by *Brassica juncea* since it has the same dominant flavor compounds with better agronomics and color. However, black mustard seed is still important as a spice and oil plant in India (Balke, 2006).

### 2.2 Yellow Mustard Seed Structure

In order to gain a better understanding of the simultaneous oil and protein extraction from dehulled yellow mustard flour, it is essential to present the structure of the yellow mustard seed.

Yellow mustard seed belongs to the botanical family Cruciferae, of which several species are presently utilized as oilseed crops. The mustard seeds occur in pods carrying about 10-40 seeds each, similar to their rapeseed cousins (Appelqvist, 1971).

The seed of mustard contains a seed coat or hull, 15-20% of the total seed weight, two cotyledons, and a very small endosperm with only few cells in thickness (Appelqvist, 1971). The yellow mustard seed structure and the cotyledon microscopic structure are presented in Figure 2-1a, b.

![Figure 2-1 a Structure of yellow mustard seed; b Structure of each cotyledon cell (Huang, 1992)](image-url)
2.2.1 Yellow Mustard Cotyledon Microstructure

The main feature of oilseed cotyledon cells is the existence of discrete cellular organelles called oil and protein bodies which contain, respectively, most of the oil and protein in the seed. As it is shown in figure 2-1b, the spaces between protein bodies in cotyledon cells are filled with the oil bodies which are enmeshed in a cytoplasmic network presumably composed of proteins (Wolf and Baker, 1975). Yellow mustard seed oil bodies, the principal repository sites of triglycerides (TG), have a spherical shape and possess diameters ranging from 0.1 to 1.5 µm, with the average diameter of 0.73 µm and a composition of 94.64% TG, 3.25% proteins, 1.97% phospholipids (PL), and 0.17% free fatty acids (FFA) (Tzen et al., 1993). Tzen and Huang (1992) proposed a model for an oil-body of maize in 1992 (Figure 2-2), and showed one year later that the model is in consistent with diverse species including mustard seed (Tzen et al., 1993). The model describes a spherical particle of a TG matrix surrounded by a shell of PL embedded with proteins called oleosins. The PL form a single layer with the hydrophobic acyl moieties facing the matrix and the hydrophilic head groups being exposed to the exterior. Eighty percent of this layer is filled with PL, and the remaining 20% is occupied by oleosins. Of each oleosin molecule, about 20% of the amino acid residues are embedded in the PL layer, 30% are located in the TG matrix, and 50% are exposed to the exterior. Therefore, on the surface of seed oil bodies, amphiphilic oleosins and PL work cooperatively to stabilize the organelles, prevent them from coalescing (Tzen and Huang, 1992), and make the oil extraction extremely difficult.

![Figure 2-2: A proposed model of a maize oil body (Tzen and Huang, 1992)](image-url)
2.2.2 Yellow Mustard Seed Coat Structure

Mustard seed coats or hulls are substantially different from the cotyledon of the seed. The hulls in *Sinapis alba* typically comprise 15-20% of the total seed weight with about 5-10% oil and 15% protein (Appelqvist, 1971). Only three cell layers are clearly detectable in the mature seed coat: (i) the dead outer epidermis, giving rise to the mucilage sheath, (ii) the dead palisade layer which is responsible for the mechanical properties of the seed coat and (iii) the aleurone layer which demonstrates all features of typical storage cells surviving seed desiccation (Bergfeld and Schopfer, 1986). The seed coats’ primary economic importance is as an emulsifier and filler, and their emulsifying capacity is due to the presence of cold-water swelling mucilage on the outer surface that has been shown to complicate oil extraction in aqueous process. The mucilage in yellow mustard seed accounts for approximately 5% of the mass of the whole seed, corresponding to ~25% of the mass of the seed coat (Balke, 2006, Balke and Diosady, 2000). The seed coat, including the mucilage can be removed readily by mechanical dehulling. Accordingly, this study was performed using dehulled yellow mustard flour.

2.3 Major Components of Yellow Mustard Seed

Yellow mustard oil and protein are the major components of the seeds. The compositions of the oil and protein presented in the following sections (2.3.1 and 2.3.2) show the reason why the protein is suitable for human use and the oil is desirable for biodiesel production.

2.3.1 Oil

Yellow mustard seed contains 30-32% oil that is stored inside small spherical oil bodies. Oil is a mixture of 96-98% fatty acid triacylglycerols (commonly referred to as “triglycerides”), with the balance consisting of other fat soluble compounds. Triglycerides consist of three fatty acids, which are substituted in the hydroxyl (alcoholic) sites of a glycerol backbone. The construction of a simple triglyceride is shown in Figure 2-3, where each fatty acid is represented as a different “R”. In plant oils, the fatty acids consist of a long unbranched aliphatic chain, which can be either saturated or unsaturated. Depending on the number of double bonds on the chain, it is referred to as mono-, di-, or polyunsaturated.
The remaining 2-4% of oil consists of non-triglyceride components including phospholipids, free fatty acids (FFA), diglycerides, monoglycerides, tocopherols, tocotrienols, sterols, sterol esters, volatile and non-volatile compounds, color compounds, and trace metals (Gupta, 2008).

Table 2-1 presents the typical fatty acid profile of the triglycerides of yellow mustard seed. Yellow mustard oil is low in saturated fats, high in erucic acid, and low in linoleic acid. In addition to culinary use that was mentioned previously, it is a potential source of erucic acid for industrial applications.

Table 2-1: Fatty acid composition (%) of yellow mustard oil (Canadian Grain Commission, 2013)

<table>
<thead>
<tr>
<th>Fatty Acids</th>
<th>Yellow Mustard, Canadian Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stearic (C18:0)</td>
<td>1.1</td>
</tr>
<tr>
<td>Oleic (C18:1)</td>
<td>26.0</td>
</tr>
<tr>
<td>Linoleic (C18:2)</td>
<td>9.2</td>
</tr>
<tr>
<td>α-Linolenic (C18:3)</td>
<td>10.9</td>
</tr>
<tr>
<td>Erucic (C22:1)</td>
<td>34.2</td>
</tr>
<tr>
<td>Saturated Fatty Acids¹</td>
<td>5.1</td>
</tr>
<tr>
<td>Iodine Value</td>
<td>103</td>
</tr>
</tbody>
</table>

Data from Canadian Grain Commission report on Quality of Western Canadian Mustard 2013 (CGC, 2013). Data were calculated based on the average fatty acid compositions of yellow mustard, Canadian samples No. 1, 2, 3 and 4. ¹Saturated Fatty Acids are defined as the sum of Palmitic (C16:0), Stearic (C18:0), Arachidic (C20:0), Behenic (C22:0), and Lignoceric (C24:0)
2.3.1.1 **Erucic Acid**

Erucic acid, also known as cis–13-docosenoic acid, is a 22–carbon monounsaturated fatty acid with a single double bond at the omega 9 position. Erucic acid is found in the seeds of the *Cruciferae* and *Tropaeolaceae*. It constitutes about 30–60% of the total fatty acids of rapeseed, mustard seed and wallflower seed and up to 80% of the total fatty acids of nasturtium seeds (FSANZ, 2003). It is believed to contribute to certain heart conditions such as myocardial lipidosis, which is accumulation of triglycerides in the heart (Beare-Rogers et al., 1972). In response to health concerns, canola was developed as a variety of rapeseed with very low erucic acid content (less than 2%).

Since high erucic acid oil has negative effects on the health of humans, it has been banned in the European Union and North America and it is considered unsuitable for human consumption. However, it is recognized for its excellent lubricating properties (Nieschla and Wolff, 1971). High erucic acid oils can make biodiesel containing erucic acid alkyl esters which have superior lubricating properties that may bring a price premium.

According to table 2-1, yellow mustard oil contains ~34% erucic acid, making it unsuitable for human consumption but potentially useful for production of biodiesel, the final goal of this investigation.

2.3.2 **Protein**

Yellow mustard seed contains considerable amount of protein that accounts for around 30 % of the seed dry weight. Proteins are considered as the basis of life as they play an essential role in the cellular maintenance, growth and functioning of the human body and they are also essential components in different food systems. Proteins are organic compounds made of amino acid residues arranged in a linear chain. The chemical structure of a main-chain amino acid is depicted in Figure 2-4a. Generally the side chains may be conveniently classified as: aliphatic, aromatic, acidic, basic, polar uncharged, and sulphur containing. Amino acids can react with the elimination of water molecules to produce a polypeptide. The structure of a protein is presented in Figure 2-4b. The 20 naturally occurring amino acids are the primary building units of proteins and possess a range of chemical properties which collectively endows each protein molecule with a unique set of physicochemical characteristics. A protein molecule is constantly changing
conformation in response to changes in environmental conditions, such as, pH, temperature, salts, and nature of solvents (Phillips et al., 1994).

All but nine of the twenty naturally amino acids that constitute proteins can be synthesized by the body; the remaining essential amino acids must be consumed from external sources. Since the protein composition of human flesh is essentially the same as animal flesh, meat provides a well-balanced source of the essential amino acids. Unfortunately, the high cost of meat protein makes obtaining a balanced protein intake difficult for much of the world’s population (Balke, 2006). Thus, plant proteins have become popular food ingredients mostly because of the recognition that foods from plant sources are equally, if not more, beneficial to human health (Shih, 1998).

![Chemical structure of a main-chain amino acid (R: side chain group); Chemical structure of a protein (CO-NH: peptide bond)](image)

Table 2-2 presents the amino acid composition of yellow mustard seed, soybean, and rapeseed compared to FAO/WHO/UNU and MIT amino acid requirements for adults. As shown here, the proteins of rapeseed and mustard are essentially equivalent to soybean in quality, and they are well balanced relative to the recommended intakes for human use.

The majority of mustard seed proteins are storage proteins, while there is also a small fraction of oleosin proteins (oil body proteins). The predominant storage proteins are legumin-type globulins (11S or 12S or cruciferin,) and napin-type albumins (2S or napins) accumulated in special organelles called protein bodies (Figure 2-1b). The protein bodies generated from the protein storage vacuoles, spherical organelles with an average diameter of 1.5 to 8 μm, which provide sufficient protection for storage proteins against premature breakdown by cytoplasmic enzymes (Wanasundara, 2011).
### Table 2-2: Literature values for mustard, soybean, and rapeseed amino acids compared to MIT and FAO/WHO/UNU recommended levels (mg amino acid/ g protein)

<table>
<thead>
<tr>
<th>Essential Amino Acids</th>
<th>Amino Acid Requirements for Adults</th>
<th>Yellow Mustard</th>
<th>Soybean</th>
<th>Rapeseed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoleucine</td>
<td>30</td>
<td>35</td>
<td>40</td>
<td>38</td>
</tr>
<tr>
<td>Leucine</td>
<td>59</td>
<td>65</td>
<td>79</td>
<td>81</td>
</tr>
<tr>
<td>Lysine</td>
<td>45</td>
<td>50</td>
<td>69</td>
<td>54</td>
</tr>
<tr>
<td>Methionine &amp; cystine*</td>
<td>16</td>
<td>25</td>
<td>43</td>
<td>---***</td>
</tr>
<tr>
<td>Phenylalanine &amp; tyrosine**</td>
<td>38</td>
<td>65</td>
<td>84</td>
<td>92</td>
</tr>
<tr>
<td>Threonine</td>
<td>23</td>
<td>25</td>
<td>33</td>
<td>45</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>6</td>
<td>10</td>
<td>---***</td>
<td>17</td>
</tr>
<tr>
<td>Valine</td>
<td>39</td>
<td>35</td>
<td>57</td>
<td>35</td>
</tr>
</tbody>
</table>

*Total sulphur containing amino acids
**Total aromatic amino acids
*** Not available

Although some allergens (Sin a 1 and Bra j 1) in the 2S albumin class of yellow mustard seed storage proteins have been characterized (Monsalve et al., 2001), the cross-reactivity relative to the soy protein allergens is low (Mustorp et al., 2008). Food allergy is a relatively rare and sometimes violent reaction of the immune system to food proteins. While European Union has listed mustard as an allergenic food ingredient, the incidence of mustard allergies in animals seems to be low, since canola and mustard meals have long been used as a proteinaceous feed. Yellow mustard protein is currently used for livestock feeding and has limited uses in the food industry due to the presence of anti-nutritional components and due to protein denaturation during industrial oil extraction. However, the well balanced amino acid profile (Table 2-2) and the low allergenicity of yellow mustard protein along with the ability of mustard to grow in dry
areas make it interesting to investigate technologies for producing food-grade protein. Therefore, the members of Food Engineering Group initiated a program to produce food-quality protein products from yellow mustard flour using aqueous extraction of solvent extracted meal (Xu et al., 2003). Market and environmental pressures for the elimination of hydrocarbon solvents in food processing makes aqueous extraction processes (AEP) attractive. Therefore, the utilization of friendly AEP processing conditions and proper membrane separation processes (Balke, 2006, Soltero, 2013) opened the opportunity for value increase of yellow mustard seeds. The yellow mustard oil which is also produced by AEP as a by-product of protein isolate production is valuable as a source of erucic acid for industrial applications.

2.3.2.1 Oleosins

As shown in Figure 2-2, the oil bodies contain proteins, termed oleosins, which play a major role in stabilizing these bodies. Oleosins are alkaline proteins of small molecular mass, ranging from 15 to 26 kD. As deducted from computer model of the amino acid sequences, each oleosin consists of three structural domains, including an amphipathic NH2-terminal domain, a central hydrophobic stretch of 72 amino acid residues, and an amphipathic α-helical domain at or near the COOH-terminus. These secondary structures apparently enable the protein to reside stably on the surface of the oil bodies. The oleosins seem to be decisive in maintaining the integrity of the oil bodies during desiccation that accompanies seed maturation by preventing interaction and possible coalescence (Huang, 1994, Murphy, 1993, Tzen and Huang, 1992).

As described in section 2.2.1, on the surface of seed oil bodies, amphiphilic oleosins and PLs work cooperatively to stabilize the organelles inside the cells. Oil bodies isolated by aqueous floatation have a very strong hydrophilic surface due to the presence of oleosins and PLs (Tzen and Huang, 1992). Therefore, in aqueous extraction the water soluble components disperse easily into the water, while the released oil is emulsified due to the strong hydrophilic surface surrounding the hydrated oil bodies in aqueous medium.

2.4 Minor Components of Yellow Mustard Seed

Apart from the major constituents, yellow mustard seeds also contain undesirable compounds: glucosinolates, phenolics and phytates. These anti-nutritional compounds bind to the protein and hinder the bioavailability of amino acids and minerals. Thus, it is essential to remove them from
the mustard meal before consumption by humans. Membrane-based processes were developed to produce protein isolates from mustard seeds with no detectable glucosinolates, phytates, or phenolic compounds (Diosady et al., 2005, Marnoch and Diosady, 2006).

2.4.1 Glucosinolates

The glucosinolates are a large group of sulphur-containing compounds which occur in all the economically important varieties of Brassica species (Mithen et al., 2000). The basic structure of all glucosinolates consists of three building blocks: a β-thioglucose moiety, a sulfonated oxime moiety, and a variable side chain (Hopkins et al., 2009, Mithen, 2001). Yellow mustard seed contains 140 µmol/g glucosinolates. The main glucosinolate in Sinapis alba is sinalbin or p-hydroxybenzyl glucosinolate (Matthaus, 1997).

A diet rich in glucosinolates can be linked to growth depression, poor palatability, decreased food efficiency, hypertrophy of the thyroid (goiter), and liver lesions and necrosis (Anilakumar et al., 2006, Hopkins et al., 2009). However, diets rich in cruciferous vegetables protect human beings specifically against cancers of the colon, rectum and thyroid (Mithen et al., 2000). This provides a strong motive for the manipulation of glucosinolate levels in human diets.

The p-hydroxybenzyl glucosinolates of yellow mustard seed are hydrolyzed by the endogenous enzyme “myrosinase” to release a range of breakdown products including p-hydroxybenzyl isothiocyanates (Mithen et al., 2000). Myrosinase is found in plant cells in a separate compartment from glucosinolates. When the plant cells are damaged, by cutting or chewing, the myrosinase comes in contact with the glucosinolates and hydrolysis occurs (Heaney and Fenwick, 1995).

The resulting p-hydroxybenzyl isothiocyanates are a major contributor to the hot flavour of mustard. This compound is wildly soluble in oil and it is therefore extracted with the oil, giving it hot flavour (Fenwick et al., 1983). The presence of isothiocyanates, the sulphur-containing compounds, in yellow mustard oil may affect the quality of the produced biodiesel, since the biodiesel specifications, ASTM D 6751, limit the total sulphur content in biodiesel to 0.05wt%.
2.4.2 Phenolics

Chemically, phenolic compounds can be defined as substances possessing an aromatic ring bearing one or more hydroxyl substituents, including their functional derivatives. Phenolic compounds are essential for growth and reproduction of plants and also act as antipathogens (Butler, 1992). While at low concentrations, phenolics may protect the food from oxidative deterioration, at high concentrations they may participate in discoloration of foods, interactions with proteins, carbohydrates and minerals. In addition, the astringency and bitterness of foods depends on their concentration of phenolic compounds (Shahidi and Naczk, 1995).

The dominant phenolic acids in yellow mustard are p-hydroxybenzoic and trans-sinapic acid at 56.2% and 36.1% of the total phenolic acids, respectively (Kozlowska et al., 1983). These compounds are chelating agents and reduce the bioavailability of minerals and proteins present in the mustard meal thus lowering mustard's nutritional quality. These compounds are also responsible for the dark colour and astringent taste present in mustard meal (Lorenzo, 2008).

2.4.3 Phytic Acid

Myo-inositol is considered to be the parent compound of phytic acid. Inositol is a simple hexacarbon carbohydrate, an essential nutrient, and a member of the B vitamins. When all of its six carbons are attached to phosphate groups, it is known as inositol hexaphosphate or phytic acid (IP6) (Vucenik and Shamsuddin, 2006). Phytic acid accumulates during seed development until the seed reaches maturity (Lott et al., 2000). It is the primary storage compound of phosphorus in seeds and accounting for up to 80% of the total seed phosphorus (Bohn et al., 2008). Yellow mustard seed contains 14mg/g phytic acid (Matthaus, 1997).

The negatively charged phosphate in phytic acid strongly binds to metallic cations of Ca, Fe, K, Mg, Mn and Zn, making them insoluble and thus unavailable as nutritional factors. The low bioavailability of the minerals bound to phytic acid can lead to deficiencies in human populations (Bohn et al., 2008). Phytic acid can also bind to proteins and decreases their solubility, thus altering their functionality properties (Lorenzo, 2008).
2.5 Recovery of Oil from Oilseeds

All oilseed crops serve dual purposes as sources of oil and of protein for human or animal food, and industrial products. The process of oil extraction can significantly affect the utility and quality of the protein products due to thermal or chemical denaturation (Lusas, 1983). Modern extraction processes, based on alternative solvents to hexane or aqueous processing, show potential in addressing protein utility and environmental concerns (Balke, 2006). Therefore, the conventional and modern extraction technologies have been reviewed in this section in order to identify a method for protein and oil extraction from yellow mustard seeds for human food and industrial applications.

2.5.1 Conventional Oil Extraction Processes

Although each oilseed crop has special requirements for recovery of oils and meals, the following generalized diagram (Figure 2-5) can be used to represent the process steps for the production of oils and meals by three different conventional extraction processes.

As shown in Figure 2-5, the seeds are first dried to 10-12% moisture content prior to storage. This is followed by cleaning the seeds to remove sticks and stones and dehulling the seeds to remove hulls. The dehulling operation is typically performed in two different steps: decortication by cracking of the hulls, and separation of the hulls from cotyledons by using aspiration or other techniques. The dehulled seeds are further processed to rupture the seed cells to facilitate the oil recovery by using pressing and/or solvent extraction processes (Lusas, 1983).

The three conventional oil extraction processes are hard pressing, direct solvent extraction, and prepress-solvent extraction (Lusas, 1983, Rosenthal et al., 1996, Wan and Wakelyn, 1997).

Hard pressing is the oldest and simplest oil extraction technology, for which the seeds are usually well-cooked to release the oil from oil bodies more easily, then continuously screw-pressed for oil recovery. Screw press is a continuous screw auger designed to accept seeds and subject them to gradually increasing pressure as they are conveyed through a barrel cage. In fact, the seeds fall into a rotating helical screw that introduces them into the pressing cage to extract oil (Lusas, 1983, Wan and Wakelyn, 1997). The residual oil content of hard-pressed meals is usually about 6-8 %. The oil cells are sealed when intense pressure is applied in this method; therefore, a
practical limit of 3-4% is usually considered for the lowest residual oil content of the meals obtained by hard pressing. The friction caused by screw pressing inside a barrel cage inflicts considerable heat damage to the protein. Consequently, in some hard pressing technologies the cooled pressed oil is recycled over the cage to remove excess heat, thus improving meal protein quality (Wan and Wakelyn, 1997).

Direct solvent extraction is typically used for low to medium oil content oilseeds (below 30%), including soybean and cottonseed. In direct solvent extraction, the seeds are first flaked by rolls. The next step involves a continuous counter-current extraction of oil using hexane. The residual oil contents in this method can be as low as 0.5%. Following the solvent extraction process, the mixture of oil and solvent (miscella) is separated by distillation to recover solvent for reuse. The mixture of meal and solvent (marc) is also passed through a desolventizer-toaster (DT) unit to dry and toast the meal to the desired level of protein solubility (Lusas, 1983).

The efficiency of the solvent extraction process depends on the following three factors: the solubility of the oil in the solvent, the degree of percolation of the solvent in the solid matrix, and
the degree of final drainage once the oil has been extracted (Lajara, 1989). These three factors were also found important when IPA was applied to solubilize the yellow mustard emulsion produced by aqueous extraction of dehulled mustard flour to recover oil (Ataya Pulido, 2010).

Prepress-solvent extraction method is primarily used for processing high oil content oilseeds (above 30%) including sunflower, canola, rapeseed and flaxseed. In prepress-solvent extraction, as shown in Figure 2-5, the conditioned seeds are first pressed to an oil content of 14-18%, followed by re-flaking of the intermediate meal for the subsequent solvent extraction. The prepressing step is required because the flakes of high oil content oilseeds tend to disintegrate into fines upon direct solvent extraction, thus plugging the extractor and reducing the oil extraction yields. Therefore, one of the main advantages of prepressing is that it allows solvent extraction to be applied to oilseeds that would be quite difficult to process by direct solvent extraction. Also, solvent requirements are lowered considerably. This extraction technology usually reduces the residual oil content to 0.5-1% (Lusas, 1983, Rosenthal et al., 1996, Wan and Wakelyn, 1997).

In modern solvent-based extraction technologies, the previously flaked oilseeds are introduced into the expanders (cooking extruders) in order to reduce the oil content of the oilseeds before extraction with hexane. The solvent extraction is then performed on “collets” which contain less residual oil than flakes since the expander process effectively ruptures the cell structures, increases the porosity and surface area to extract more oil. The lower oil content of collets reduces the load on the extractor and the energy required for separation of crude oil and solvent from miscellae (Lusas et al., 1989, Zhang et al., 2002).

The crude oil produced using all extraction technologies is then submitted to a refining process for removing oil-soluble and oil-insoluble impurities. The refining process includes several operations: the degumming step to remove phosphatides and mucilaginous gums, the alkali-refining step to remove FFAs, colour bodies, and metallic pro-oxidants, the bleaching step to separate more pigments and residual soaps, and the deodorization step to eliminate the remaining off-flavours using high-vacuum steam distillation (Rosenthal et al., 1996).

Solvent extraction is currently used in vegetable oil production as it is the most efficient process leading to a defatted meal with residual oil content as low as 0.5%. While n-hexane is broadly
used for oilseed processing, the concerns about its availability, fires and explosions in extraction plants, tighter emission restrictions, and its testing as a hazardous air pollutants have stimulated interest in alternative solvents (Gandhi et al., 2003, Johnson and Lusas, 1983, Rosenthal et al., 1996). Furthermore, the Environmental Protection Agency (EPA) has identified n-hexane emissions as a major source of air pollution, and has considered tighter emission standards to reduce hexane losses (Environmental Protection Agency, 2001). Therefore, the ultimate goal of this study is to simultaneously recover high-quality oil and protein from dehulled yellow mustard flour using an environmentally friendly technology in which the oil recovered could be directly used as a feedstock for biodiesel production without refining.

2.5.2 Alternative Solvents to Hexane

Many studies have been performed by the oil extraction industry with the aim of identifying ideal solvents which are being plentiful in supply, low in toxicity, non-flammable, and inexpensive. Having rapid penetration rate, high solvency power, ease of separation from extracted material, and desirable boiling point are also considered the desirable characteristics. While over 70 solvents have been considered practical for solvent extraction of oilseeds, the use of many of them has been banned in the USA by the Food and Drug Administration because of toxicity concerns. Research in alternative solvents has concentrated primarily on supercritical carbon dioxide (SC-CO₂), ethyl and isopropyl alcohols and water (aqueous extraction) (Lusas et al., 1989). The use of SC-CO₂, ethyl alcohol (ethanol), and isopropyl alcohol (IPA) in oilseeds extraction is briefly described in this section while aqueous extraction processes will be discussed in detail in the following section (2.5.3).

Supercritical extraction has introduced new methods to the food industry. Specific relations between temperature and pressure determine whether a chemical compound exists in a gas, solid, liquid, or supercritical state. Beyond the critical temperature of 31°C and critical pressure of 7.38 MPa (72.9 atm), carbon dioxide enters a “supercritical fluid” state (SC-CO₂). The oil was extracted from full-fat soy flakes with SC-CO₂ at pressures of 204-680 atm and 50 °C; the recovered oil had lighter colour and required less caustic refining (Lusas et al., 1989). The extraction of rice bran oil using SC-CO₂ at 45°C and 35 MPa could recover 0.222 kg oil per kg of rice bran. However, the results of the economic analysis showed that the SC-CO₂ extraction of rice bran is unprofitable (Sparks et al., 2006). Oil extraction using SC-CO₂ has not become a
reality, mainly because of inabilities to continuously load and discharge extractors operating at high pressures. While high operating pressures during SC-CO₂ extraction technology could result in high capital, operating and maintenance costs, a broad variety of high-value foods, including coffee and spices, were recently extracted using SC-CO₂ (Lusas and Gregory, 1996, Sparks et al., 2006).

Alcohols have been the most-studied alternatives for hexane. Ethanol and IPA are less flammable than hexane and exhibit lower toxicity. While hexane is totally miscible with oils, the solubility of oils in alcohols is dependent on the temperature and water content (Fig. 2-6). Alcohols have high oil solubility at elevated temperatures and low oil solubility at ambient temperature. As shown in figure 2-6, IPA is considered a more effective solvent than ethanol because of its greater solubility for oil. Lower costs of IPA and freedom from tight government regulations make it superior to ethanol. Both ethanol and IPA form azeotropes with water at 96 and 87.7 wt%, respectively. The formation of azeotropic mixtures significantly reduces oil extraction efficiencies (Lusas and Gregory, 1996, Wan and Wakelyn, 1997, Zhang et al., 2002). The major disadvantage of using IPA in oilseeds extraction includes the high energy costs for recovery of high concentration IPA from IPA/water azeotropes (IPAWA) for subsequent reuse. However, new developments in membrane technology such as pervaporation could effectively recover high concentration IPA by dehydration of IPAWA to concentrations above their azeotropic distillation levels (Lee et al., 1999, Lusas and Gregory, 1996, Lusas et al., 1989).

![Figure 2-6: Solubilities of cottonseed oil in absolute ethanol and isopropyl alcohol (IPA) and their azeotropes (Lusas and Gregory, 1996, Lusas et al., 1989, Wan and Wakelyn, 1997)](image-url)
Another drawback of using IPA includes the separation of oil from IPA-oil miscella since IPA boils at higher temperatures than hexane. Thus, IPA recovery by distillation is more energy-intensive than recovering hexane. However, the majority of the oil can be separated from miscella using chill separation. In this method the extracted miscella is cooled to ~ 5.5ºC to produce two phases, a solvent-rich upper phase and an oil-rich lower phase (Lusas and Gregory, 1996).

Since IPA is a less flammable and toxic solvent than hexane, many studies have been performed to extract oil from oilseeds using IPA (Gandhi et al., 2003, Lusas and Gregory, 1996, Turi, 2001, Zhang et al., 2002). Two novel practices in this regard are discussed below.

The oils from soybean and cottonseed collets were extracted using IPAWA (Lusas and Gregory, 1996). The maximum effective solubilities in IPAWA were reported about 15-18% for cottonseed oil and about 25-28% for soybean oil. However, infinite miscibility of cottonseed oil occurred at IPA concentrations of 93% or higher and at 91% or higher for soybean oil. In other words, at least 91% and 93% IPA must be used to achieve infinite miscibility for soybean oil and cottonseed oil, respectively. However, IPA solutions that are too concentrated (> 95 % IPA) restricted chill separation of the upper phase.

Another novel approach in the Food Engineering Group focused on the use of 100% IPA for the extraction of oil from ground yellow mustard seed. IPA extracted about 95% of the oil at the room temperature at a solvent to seed ratio of 10:1. About 91% of the oil in the resulting miscella was recovered by adjusting the polarity of the solvent through the addition of 30% water (Turi, 2001). More studies are still in progress in our group to extract oil from dehulled yellow mustard flour using pure IPA in the form of oil-IPA miscella for direct conversion to fatty acid isopropyl esters.

### 2.5.3 Aqueous or Enzyme-Assisted Aqueous Extraction (AEP/EAEP) from Oilseeds

Aqueous or enzyme-assisted aqueous extraction processes (AEP/EAEP) are technologies in which water is used as an extraction medium to extract protein and other soluble components present in oilseeds while separating the oil as an emulsion or free oil. This approach resembles the ancient practice of mixing ground dehulled oilseeds in vats of hot water, and skimming off
the oil rising to the surface (Lusas et al., 1989). It is an emerging alternative to traditional hexane extraction since it eliminates the potential hazards of explosion and fire, eliminates the negative environmental impacts due to emissions of organic solvents, and does not leave toxic or undesirable solvent residues in the resulting food products. It also allows the simultaneous production of high-quality protein and oil from different oilseeds (Chen and Diosady, 2003, De Moura et al., 2008, Rosenthal et al., 1996). Therefore, aqueous processing of dehulled yellow mustard flour was selected in this investigation as an environmentally friendly technology to simultaneously extract food-grade protein products free of solvent residues and high erucic acid oil/emulsion as biodiesel feedstock.

These environmentally clean technologies (AEP/EAEP), however, have significant challenges that limit their commercial applications. Each AEP/EAEP process results in production of three different fractions: an insoluble fiber-rich solid residual fraction, a protein-rich liquid extract fraction (skim), and an oil-rich emulsion fraction stabilized by proteins and phospholipids. Some of the main issues associated with AEP/EAEP processes are the low oil extraction yield due to the retention of coalesced oil droplets in the insoluble solid residual fraction and the dispersion of oil droplets in the skim fraction as well as production of stable oil-in-water emulsions that must be effectively demulsified to produce free oil (Campbell et al., 2011).

Resolving these problems has so far been the focus of most studies on aqueous processing. Many studies have been recently performed through chemical, mechanical and enzymatic treatments to maximize oil extraction yields and minimize the stability of the emulsions formed during AEP/EAEP processes. Factors that influence the efficiency of oil extraction include water-to-flour weight ratio, particle size, pH, temperature, degree of agitation, and the number of extraction stages. Optimum extraction conditions vary based on the composition and structure of oilseeds (Rosenthal et al., 1996). The mechanical oilseed preparation is important to rupture cotyledon cell walls to enhance oil recovery by subsequent AEP/EAEP. The grinding operation could not completely rupture cotyledon cell walls, and extensive grinding produced stable emulsion that was really difficult to demulsify (Rosenthal et al., 1996). A new form of mechanically treating oilseeds was tested by combining flaking and extruding (expanding) dehulled soybeans to achieve complete cellular disruption, thus improving oil extraction yields (Lamsal et al., 2006). The use of enzymes (cell-wall degrading and/or proteolytic enzymes)
through enzyme-assisted aqueous extraction process (EAEP) was found effective in increasing oil and protein extraction yields. Carbohydrases (cellulases, hemicellulases, and pectinases) are cell-wall degrading enzymes and are helpful for breaking down the structures of cotyledon cell walls. Besides carbohydrases, proteolytic enzymes can potentially break down the oil body membranes by hydrolysing the oleosin proteins, thus increasing the oil recovery (De Moura et al., 2008, Rosenthal et al., 1996, Wu et al., 2009). Moreover, protein and oil extraction yields were shown to be closely related, with both depending on the extent of cell wall disruption. Therefore, the conditions that favoured protein extraction including alkaline pH, small particle size, and temperature below the level that causes denaturation also favoured oil extraction (De Moura et al., 2008).

Much of the recent literature focused on enzymatic treatment during aqueous extraction of soybeans. Flaking and extruding dehulled soybeans into water followed by protease and phospholipase treatments recovered over 97% of the total available oil in the form of free oil and an unstable emulsion that was easily demulsified with enzymes (Jung et al., 2009). During aqueous processing of dehulled rapeseeds, the combined use of several carbohydrases and a protease (Alcalase) effectively recovered around 80% of the free oil; the total emulsions collected from this optimized process were unstable enough to demulsify by heating-centrifuging followed by freezing-thawing operations (Zhang et al., 2007). In another study, the free oil recoveries of around 99% were obtained during the aqueous carbohydrase treatment of brown mustard seeds in the presence of hexane (Sengupta and Bhattacharyya, 1996).

Unfortunately, the design of perfect AEP/EAEP technologies to overcome all of the involved challenges in processing is practically impossible since a solution to one challenge often adds new challenges to the existing process (Campbell et al., 2011). Considerable improvements have been recently achieved in aqueous extraction processing of extruded soybean flakes (De Moura et al., 2009, De Moura and Johnson, 2009, De Moura et al., 2011) and soybean flour (Chabrand and Glatz, 2009). While the most advanced EAEP process for extracting extruded soybean flakes with the aid of a protease enzyme in a countercurrent two-stage strategy resulted in high oil extraction yield of 99%, and the formation of an unstable emulsion that was easily demulsified by adjusting the pH of the emulsion to the isoelectric point of the soy proteins (4.5) (De Moura et al., 2008, Jung et al., 2009, Wu et al., 2009), it produced a soluble protein fraction (skim)
containing around 20% of the total oil that was very difficult to isolate due to the extensive hydrolysis used to free oil (Campbell et al., 2011). On the other hand, while the AEP of soybean flour resulted in lower oil extraction yield and the formation of stable emulsion, it produced a skim fraction containing native soluble proteins that could be used to produce high-quality protein products using low-cost processes such as isoelectric precipitation (Campbell et al., 2011). Although effective extraction of oil and protein from AEP/EAEP processes is desirable, the easy and economically viable recovery of free oil and isolate protein from emulsion and skim fractions, respectively, is essential. Therefore, a viable process should provide the best extraction conditions to modify the ease and efficiency of recovery of these marketable components.

The present study is intended to design an optimal AEP or EAEP process for dehulled yellow mustard flour leading to high oil and protein extractabilities, the formation of unstable emulsion easy to demulsify, and the formation of skim fraction containing native proteins easy to isolate. Although different types of proteases have been reported for the aqueous processing of oilseeds, none has been tested in the current stage of this study due to the consideration that hydrolysis reduced recovery of isoelectrically precipitated skim proteins by increasing the solubility of proteins at the isoelectric pH (Campbell et al., 2011). However, carbohydrase treatments were tested during the course of this investigation with the aim of increasing cellular disruption, thus releasing oil and protein.

In our laboratories, extensive work was done on non-enzymatic aqueous extraction (AEP) of dehulled yellow mustard flour by Balke (2006), who investigated the effect of primary extraction factors including pH, temperature, water-to-flour weight ratio, extraction time, and number of successive extraction stages on oil and protein extractability. These optimized aqueous extraction conditions are summarized in Table 2-3. This AEP of dehulled yellow mustard flour using two-stage alkaline extraction at pH 11 with the oil extraction yield of ~ 80% and protein extraction yield of ~ 90% recovered three separate fractions: a protein-rich skim fraction, an oil-rich emulsion fraction, and a solid residual fraction. The protein-rich skim fraction was further processed using membranes to recover food-grade protein isolates. The process involving ultrafiltration, followed by diafiltration, and protein precipitation has been developed in the Food Engineering Group to recover precipitated protein isolate (PPI) and soluble protein isolate (SPI) (Marnoch and Diosady, 2006, Soltero, 2013). The wiener and bologna prepared with 1-2% of
the PPI were comparable to those prepared with soy protein isolates, confirming the good binding and emulsifying properties of this product (Soltero, 2013). The oil-rich emulsion fraction is also processed in this investigation as high-quality biodiesel feedstock. The detailed information regarding the nature and destabilization procedures of the emulsions produced by aqueous processing of oilseeds will be discussed in the following section (2.6).

Table 2-3: Optimized aqueous extraction parameters for dehulled yellow mustard flour (Balke, 2006)

<table>
<thead>
<tr>
<th>Extraction Parameters</th>
<th>Optimal value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>11.0 ± 0.05</td>
</tr>
<tr>
<td>Temperature</td>
<td>Room temperature</td>
</tr>
<tr>
<td>Water-to-flour weight ratio</td>
<td>4:1</td>
</tr>
<tr>
<td>Extraction time</td>
<td>30 min</td>
</tr>
<tr>
<td>Number of extraction stages</td>
<td>2</td>
</tr>
<tr>
<td>Blending time</td>
<td>3 min</td>
</tr>
</tbody>
</table>

Sections 5.1 and 5.2 of this investigation focused on designing an optimal AEP or EAEP process for dehulled yellow mustard flour which have been previously published in the journal "Food Research International" (Tabtabaei and Diosady, 2013). In these sections, first, the carbohydrase treatments of yellow mustard flour were evaluated through the individual and/or combined use of cellulase, hemicellulase, and pectinase. Secondly, successive extractions without and with enzymes were investigated in which alkaline extraction (pH 11) was considered part of the process. Finally, the effect of extraction parameters including pH, enzyme, and alkaline treatment conditions on the stability of the emulsions and the quality of the skim proteins were determined.

2.6 Emulsions and Destabilization Procedures

An emulsion is a mixture of two immiscible liquids (usually oil and water), one of which is dispersed as small spherical droplets in the other. In emulsion terminology, the liquid phase that makes up the droplets in an emulsion is referred to as the dispersed phase, whereas the liquid phase that makes up the surrounding medium is called the continuous phase. Emulsions are classified to oil-in-water (O/W) or water-in-oil (W/O) type according to the relative spatial distribution of the oil and aqueous phases. Emulsions in which oil droplets dispersed in an
aqueous phase are called oil-in-water emulsions. Conversely, emulsions in which water droplets dispersed in an oil phase are called water-in-oil emulsions. The most common emulsions used in the food industry are oil-in-water emulsions that can be found in milk, mayonnaise, cream, soups and sauces. Water-in-oil emulsions are widely used in the production of margarine, butter and spreads. In most food emulsions, the diameter of the droplets usually lies between 0.1 and 100 µm (Dickinson, 1992, McClements, 2004, McClements et al., 2007).

Emulsions can be easily made by homogenizing pure oil and pure water together; however, they usually rapidly break down into two distinct phases of oil and water. This is related to the thermodynamic instability of emulsions due to the energetically unfavourable interactions between oil and water molecules. Thus, each emulsion tends to minimize the interfacial area between oil and water which ultimately leads to complete phase separation through a variety of physiochemical mechanisms, including gravitational separation, flocculation, coalescence, phase inversion, and Ostwald ripening. However, it is possible to form emulsions that are kinetically stable for a reasonable period of time by including emulsifiers or surfactants (surface acting agent) (Akoh and Min, 2002, Friberg et al., 2004, McClements, 2004, McClements et al., 2007).

The most commonly used emulsifiers in the food industry are small-molecule surfactants and amphiphilic biopolymers. Small molecule surfactants such as monoglycerides, diglycerides, fatty acids, or phospholipids contain both hydrophobic and hydrophilic groups that bind to the oil phase and water phase of the oil-water interface during homogenization. These emulsifiers can adsorb strongly to the oil-water interface and form a close-packed layer, thus improving the stability of the emulsions by generating low interfacial tensions. Amphiphilic biopolymers such as proteins act as emulsifiers, but behave differently from the small molecules. Proteins adsorb to an oil-water interface via their hydrophobic side chains of amino acids. When a protein is adsorbed, the 3D-structure of the protein itself will prevent close packing of the points of contact with the interface; therefore, adsorbed protein reduces the interfacial tension less than do small molecules (Friberg et al., 2004).

Most food emulsions are much more complex than the simple three-component (oil, water, and emulsifier) systems. The aqueous phase may contain a variety of water-soluble ingredients, including sugars, acids, bases, and proteins. The oil phase usually contains a complex mixture of
l lipid-soluble components, such as triglycerides, diglycerides, monoglycerides, free fatty acids (FFA), and vitamins. The interfacial region may contain a mixture of various emulsifiers, including proteins, phospholipids, and polysaccharides (Akoh and Min, 2002, McClements, 2004). Emulsions obtained during the aqueous extraction of oilseeds, including yellow mustard emulsion contain these complex structures.

As mentioned before, in yellow mustard seeds the triglyceride molecules are at the core of the oil bodies surrounded by a layer of phospholipids and oleosin proteins. Phospholipids belong to a group of small-molecule surfactants, and oleosin proteins belong to amphiphilic biopolymers group. Oleosins are strong emulsifiers as their hydrophobic middle section is partly embedded in the phospholipid layer and partly in direct contact with the triglyceride molecules of the core, while the two amphipathic N and C terminals are exposed to the aqueous phase (Nikiforidis and Kiosseoglou, 2009). During the aqueous extraction process of dehulled yellow mustard flour, the water soluble components disperse into the water; however, the released oil is emulsified since the PLs and oleosins surrounding the oil droplets form a very strong hydrophilic layer in aqueous medium, and prevent the coalescence of oil droplets. While the remarkable stability of the natural emulsions formed during AEP/EAEP of oilseeds was attributed to the complex phospholipid-oleosin layer surrounding the oil droplets (Tzen et al., 1993), the results of some recently published studies (Chabrand and Glatz, 2009, Chabrand et al., 2008, Nikiforidis and Kiosseoglou, 2009) indicated the presence of storage seed proteins adsorbed at the surface of oil droplets along with the mixed phospholipid-oleosin layer. Therefore, the emulsions obtained during AEP/EAEP technologies are stable multilayer protein-stabilized emulsions in which a multilayer film of proteins is adsorbed at the oil-water interface. AEP/EAEP technologies have not been proven feasible for extraction of oil from oilseeds due to the difficulties in breaking emulsions formed by the existence of natural emulsifiers. Therefore, new techniques for recovering the oil from the emulsion are desired.

Several demulsification treatments have been recently evaluated to break down these efficient emulsifiers, thereby promoting the coalescence of oil droplets (Chabrand and Glatz, 2009, Chabrand et al., 2008, De Moura et al., 2008, Jung et al., 2009, Lamsal and Johnson, 2007, Wu et al., 2009, Zhang et al., 2007).
Demulsification is the process whereby an emulsion is converted into separate oil and aqueous phases from which it was comprised, and it is achieved by causing the droplets to come into close contact to each other and then to coalesce. The selection of the most appropriate demulsification technique for a given emulsion depends on the type of emulsifier that stabilizes the system. The previous methods for breaking emulsions stabilized by small-molecule surfactants are heating, freeze-thaw cycling, and addition of medium chain alcohols. Heating an emulsion dehydrates the polar head groups of the surfactant molecules, reduces the hydration repulsion between the droplets, and allows them to come closer together. The addition of alcohols has also been found to be effective since the alcohol molecules are able to get between the tails of the surfactant molecules at the interface, thereby causing the optimum curvature of the interface to tend toward zero and increasing the likelihood of droplet coalescence. In all of these demulsification processes, the separation of the oil phase from the aqueous phase can be facilitated by centrifuging the emulsion after the coalescence process has been initiated because the centrifugation forces the droplets to one end of the container, which leads to separation (McClements, 2004, Menon and Wasan, 1985).

The previous demulsification methods identified in the literature, including heat treatment and freeze-thaw treatment were unable to break emulsions formed by AEP/EAEP processes. Heat treatment and freeze-thaw treatment have been explored with emulsions produced in conjunction with enzymatic and non-enzymatic aqueous extraction of various oilseeds (Chabrand et al., 2008, Lamsal and Johnson, 2007, Zhang et al., 2007). Heat treatment alone did not modify the free oil recovery from the emulsion produced from aqueous processing of soybean flour, and freeze-thaw treatment resulted in oil yields of only up to 22% (Chabrand et al., 2008). In the Food Engineering Group, some studies have been performed on demulsification of yellow mustard emulsion produced during two-stage alkaline aqueous extraction at pH 11. Heating at 90°C for 1 h followed by centrifuging resulted in no separation of the oil. Freezing at -20°C for 24h followed by thawing resulted in some separation of the oil into a clear upper phase and an oily solid phase (Ataya Pulido, 2010). However, energy requirements make this method unfeasible in industrial applications.

Acid treatment with hydrochloric acid has been also studied to destabilize emulsions. Adjusting the pH to the isoelectric point of soy proteins (pH 4.5) reduced the electrostatic repulsions
between the oil droplets, and resulted in recovery of 83-100% free oil from soy emulsions (Chabrand and Glatz, 2009, Jung et al., 2009, Wu et al., 2009). Enzymatic demulsification treatments with various proteases and phospholipases completely destabilized soy emulsions formed in the enzymatic aqueous processing of extruded soybean flakes and soybean flour (Chabrand and Glatz, 2009, De Moura et al., 2008, Jung et al., 2009, Wu et al., 2009). With effective enzyme recycling, the use of appropriate proteases and phospholipases during the extraction and demulsification steps could potentially achieve efficiencies approaching those of hexane extraction. However, due to the cost of enzyme treatment, we decided to concentrate on enzyme-free processes in this project.

Clearly, cost-effective techniques for recovering the oil from the emulsion are desired. Our group investigated the use of organic solvents with solubilities for both oil and water to recover free oil from yellow mustard emulsion. The use of organic solvents was reported to be effective in demulsification. One of the most successful chemical approaches in the literature is still the work of Li et al. (1977), who developed a process to break emulsions by adding a mixture of isopropyl alcohol and cyclohexane, one miscible with oil and one miscible with water, and separating the emulsion into two separate layers; however, this technique is too expensive and dangerous to be effectively used in industrial oil production. The addition of medium-chain alcohols has been reported to increase the likelihood of droplet coalescence, thereby promoting demulsification due to the presence of both polar and non-polar groups in the structure of these compounds. The alcohol molecules are able to destabilize the emulsions by either displacing some of the surfactants from the oil-water interface, or getting between the tails of the surfactants (McClements, 2004, Menon and Wasan, 1985). Isopropyl alcohol (IPA) was tested in our group for solubilizing the yellow mustard emulsion through single- and multiple-stage destabilization processes. The optimal conditions were four-stage extraction at 2:1 IPA:oil weight ratio, with 96.3% oil recovery from the emulsion to the IPA phase (Ataya Pulido, 2010). The use of other organic solvents with solubilities in both oil and water such as DMF, THF and 1,4-dioxane was evaluated in this project. In Section 5.3 of this investigation that was previously published in the journal of American Oil Chemists' Society (Tabtabaei and Diosady, 2012, Tabtabaei et al., 2013, Tabtabaei et al., 2014a), we report on the effectiveness of DMF, THF and 1,4-dioxane in solubilizing the emulsion to produce a single-phase oil-solvent-water miscella that can be used
directly in a transmethylation process to produce biodiesel after reducing the water content to the quality standards required for biodiesel feedstock.

### 2.7 Biodiesel Production

Interest in the use of renewable fuel started with the direct use of vegetable oils as a substitute for diesel in the early 1900s. However, the high viscosity of vegetable oils led to operational problems in the diesel engine such as deposits on various engine parts, oil ring sticking and thickening of the lubricating oil. To overcome this problem, various processes including pyrolysis, microemulsification, dilution and transesterification were investigated. One of the most viable process known so far to lower the viscosity is to transesterify the triglycerides. In the most commonly used process, biodiesel is made by the transesterification of triglycerides with methanol in the presence of basic catalysts to form fatty acid methyl esters (FAME) that have significantly lower viscosity than the original vegetable oil. Transesterification also produces glycerol as a by-product which usually separates from the ester phase by gravity. Figure 2-7 depicts the transesterification reaction.

![Transesterification Reaction](image)

Figure 2-7: The general transesterification reaction. $R_{1,2,3}$ is a mixture of various fatty acid chains

Biodiesel can be produced from any triglyceride feedstocks. The choice of feedstock depends largely on cost and availability; for example, canola oil is now considered the major Canadian source of biodiesel. Soybean oil and rapeseed oil are currently the dominant biodiesel feedstocks in United States and Europe, respectively. Palm and coconut oils are considered the major sources of biodiesel in Malaysia (Sharma et al., 2008). Alternative sources of biodiesel raw materials including inedible oils (linseed oil, castor oil, and tung oil), waste cooking oils, and animal fats (tallow, lard, and yellow grease) have been also investigated to reduce the cost of the
biodiesel. However, direct alkaline conversion of these feedstocks is hindered by their high FFA content. Biodiesel obtained from microalgae oil has been recently considered a promising option due to its higher photosynthetic efficiency, higher biomass production and faster growth as compared to other sources (Demirbas, 2009, Sharma et al., 2008). However, algal biodiesel production is still too expensive to be commercialized. As discussed before, yellow mustard oil can be a potential source of biodiesel production in Canada due to the high levels of erucic acid.

Generally, transmethylation can proceed by base or acid catalysis. The base-catalyzed transmethylation of triglycerides is a much more rapid process than the acid-catalyzed reaction. Basic catalysts are also less corrosive to industrial equipment than acid catalysts. Therefore, industrial processes usually favor basic catalysts including sodium (or potassium) methoxide or hydroxide. Sodium methoxide is currently the active catalyst for the production of fatty acid methyl esters since methoxide cannot form water upon reaction with methanol (Canakci and Van Gerpen, 2001, Knothe et al., 2005, Moser, 2009, Schuchardt et al., 1998). However, the use of sodium hydroxide is preferred because of its lower cost and the hazards and inconvenience of using sodium metal to produce sodium methoxide. In this case, the equilibrium between hydroxide ions and methanol is used to provide the necessary methoxide ions for the transesterification (Fig. 2-8a) (Boocock et al., 1998).

The mechanism of the base-catalyzed transesterification involves the attack of methoxide ion at the carbonyl group of the triglyceride. The resulting tetrahedral intermediate collapses to form methyl esters and an anion of diglyceride (Fig. 2-8b). The diglyceride anion rapidly abstract a proton from the bulk methanol to regenerate methoxide ion. This process is repeated on fatty acid chains on triglyceride to convert triglyceride into glycerol through a sequence of three reversible reactions where triglycerides are converted to diglycerides, which in turn are converted to monoglycerides, and finally to glycerol (Doell et al., 2008, Schuchardt et al., 1998, Sharma and Singh, 2008, Sharma et al., 2008). The formation of methyl esters stoichiometrically needs three moles of methanol for every mole of triglyceride. However, transmethylation is an equilibrium reaction that requires large additional amounts of methanol to proceed to completion. Fortunately, the equilibrium constant favours the formation of methyl esters such that only a 6:1 methanol:oil molar ratio is enough to drive the reaction close to completion (95-98% yield of methyl esters) (Boocock et al., 1996a, Freedman et al., 1984).
Two factors have a great impact on the yield of the base-catalyzed transmethylation process: moisture and FFA content. The absence of moisture in the system is really important because the presence of water results in irreversible consumption of catalyst through a side reaction of water with the catalyst to form hydroxide ions, which irreversibly attack all forms of esters (tri-, di-, monoglycerides and methyl esters) to produce soap. The level of FFA in the feedstock is also important since FFA can react with the alkaline catalyst to form sodium (or potassium) salts of FFAs (soaps) and water. Therefore, feedstocks with FFA levels between 0.5 and 4% are desirable during the base-catalyzed transesterification. However, multi-step protocols involving acid-catalysed esterification followed by base-catalyzed transesterification are employed with the feedstocks with high levels of FFA (Boocock, 2003, Chi, 1999, Knothe et al., 2005, Sharma et al., 2008).
Biodiesel offers great advantages compared with petrodiesel. It is biodegradable; it reduces most exhaust emissions such as particulate matter (PM), total hydrocarbon (THC), and carbon monoxide (CO) with the exception of nitrogen oxides (NO$_x$); it has a higher flash point which makes it safer to handle and store; it has excellent lubricating properties; and it is miscible with petrodiesel in all ratios (Knothe et al., 2005). Moreover, the high cetane number (CN) associated with methyl esters ensures that biodiesel fuels are suitable as diesel fuels. Global biodiesel production has increased significantly from over 5 billion litres (Bnl) in 2005 to around 27 Bnl in 2012. Global biodiesel production is expected to increase to above 42 Bnl by 2021. The European Union is expected to be the largest producer and user of biodiesel in the world. Other significant players are Argentina, the United States, Brazil, as well as Thailand and Indonesia (OECD-FAO Agricultural Outlook, 2012).

In order to increase the commercial viability of biodiesel, the ASTM (American Society for Testing and Materials) standard limits the level of certain components in the biodiesel including acids, free glycerol and bound glycerol to ensure the production of high-purity biodiesel as well as the optimum performance and durability of engine parts. Table 2-4 depicts the standard specification for biodiesel fuel (ASTM D6751, 2012). As shown, the total bound and unbound glycerol in the fuel is limited to 0.240 % since the combustion of glycerol moiety in monoglycerides, diglycerides, triglycerides, and free glycerol can lead to the formation of acrolein, a photochemical smog ingredient (Zhou and Boocock, 2006). This emphasizes the necessity for the transesterification reaction to be as complete as possible (>99% of the ester bonds) in order to decrease the concentrations of monoglycerides and diglycerides in the fuel to achieve the glycerol limit set by the ASTM standard. Therefore the main challenge for current biodiesel industry is to produce biodiesel that meets the ASTM standard.

The first experiments on the base-catalyzed transmethylation of vegetable oils were conducted by Freedman et al. (1984) to identify optimum reaction conditions. The optimal recommended conditions for transmethylation include molar ratio of methanol to oil of 6:1, 0.5% sodium methoxide or 1% sodium hydroxide as an effective catalyst, and the temperature of 60 °C. These conditions result in greater than 95% conversion to methyl esters in 1 h at ambient pressure and vigorous mixing; the reaction is also completed in 4 h at 32°C (Freedman et al., 1984). Clearly the necessary conversion was not achieved during this process to produce standard biodiesel.
Table 2-4: Biodiesel\(^{A}\) (B100) specification (ASTM D6751, 2012)

<table>
<thead>
<tr>
<th>Properties</th>
<th>Methods</th>
<th>Limits</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flash Point (closed cup)</td>
<td>D 93</td>
<td>93 min</td>
<td>°C</td>
</tr>
<tr>
<td>Water and sediment</td>
<td>D 2709</td>
<td>0.050 max</td>
<td>% volume</td>
</tr>
<tr>
<td>Kinematic viscosity, 40°C</td>
<td>D 445</td>
<td>1.9-6.0</td>
<td>mm(^2)/s</td>
</tr>
<tr>
<td>Sulfated ash</td>
<td>D 874</td>
<td>0.020 max</td>
<td>% mass</td>
</tr>
<tr>
<td>Sulfur</td>
<td>D 5453</td>
<td>15 max</td>
<td>ppm</td>
</tr>
<tr>
<td>Copper strip corrosion</td>
<td>D 130</td>
<td>No. 3 max</td>
<td></td>
</tr>
<tr>
<td>Cetane number</td>
<td>D 613</td>
<td>47 min</td>
<td></td>
</tr>
<tr>
<td>Cloud point(^{B})</td>
<td>D 2500</td>
<td>Report</td>
<td>°C</td>
</tr>
<tr>
<td>Carbon residue</td>
<td>D 4530</td>
<td>0.05 max</td>
<td>% mass</td>
</tr>
<tr>
<td>Acid number</td>
<td>D 664</td>
<td>0.5 max</td>
<td>mg KOH/g</td>
</tr>
<tr>
<td>Free glycerin</td>
<td>D 6584</td>
<td>0.02 max</td>
<td>% mass</td>
</tr>
<tr>
<td>Total glycerin</td>
<td>D 6584</td>
<td>0.24 max</td>
<td>% mass</td>
</tr>
<tr>
<td>Phosphorus content</td>
<td>D 4951</td>
<td>0.001 max</td>
<td>% mass</td>
</tr>
<tr>
<td>Distillation temperature</td>
<td>D1160</td>
<td>360 max, T-90</td>
<td>°C</td>
</tr>
<tr>
<td>(Atmospheric equivalent temperature)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium and potassium (combined)</td>
<td>EN 14538</td>
<td>5 max</td>
<td>ppm</td>
</tr>
<tr>
<td>Oxidation Stability</td>
<td>EN 15751</td>
<td>3 min</td>
<td>hours</td>
</tr>
</tbody>
</table>

\(^{A}\) Biodiesel (B100) Grade No. 2-B S15 (A general purpose biodiesel blendstock intended for use in middle distillate fuel applications that require a fuel blend component with 15 ppm sulfur (maximum)).

\(^{B}\) The cloud point of biodiesel is generally higher than petroleum based diesel fuel and should be taken into consideration when blending.

In 1986, Freedman et al. studied the base-catalyzed kinetics of both methanolysis and butanolysis of soybean oil using 0.5% sodium methoxide and 1% sodium butoxide, respectively. It was found that butanolysis followed second-order kinetics, whereas methanolysis did not. This deviation was first explained by introduction of a shunt reaction, a reaction in which all three positions of the triglyceride react simultaneously to give three alkyl esters and glycerol, which was inaccurate (Freedman et al., 1986). It was demonstrated later that miscibility phenomena play a significant role in determining the kinetics of methanolysis and butanolysis (Boocock et al., 1996a).
The solubility of oil in methanol is low at ambient temperature and the reaction is mass transfer limited, taking place only in the methanol phase where the catalyst is located. Therefore, the reaction is limited by the oil concentration in that phase. Triglycerides are first converted to diglycerides and then to monoglycerides, and because they are formed in the methanol phase they have a greater chance of reacting there rather than moving back to the oil phase. Therefore, their concentrations do not increase to those predicted by second-order kinetics (Boocock et al., 1996a). Such an explanation helped the scientists to find new methods to increase the rate of methanolysis considerably, thus producing standard biodiesel.

One approach to facilitate the transmethylation of a two-phase system involves conducting the reaction in supercritical methanol. Although reported conversions are high, more studies must be performed on the economical aspect of this process (Saka and Kusdiana, 2001).

Another approach involves the use of simple ethers, such as tetrahydrofuran (THF) and methyl tertiary butyl ether (MTBE) to enhance the methanolysis by producing a single phase containing both triglyceride and methanol. THF is preferred because its boiling point of 67°C is only two degrees higher than that of methanol. A volume ratio of THF to methanol of 1.25 is enough for miscibility at the methanol/oil molar ratio of 6:1 and a temperature of 23°C. Methanolysis occurs considerably faster in the single phase system than in the two-phase system, with 95% methyl ester being formed at 23°C in 20 min. The formation of methyl esters in this process is initially fast, but decelerates rapidly due to a polarity effect caused by the mixing of the methanol with the nonpolar oil. This problem can be improved by addition of excess methanol. Therefore, extensive studies have been performed at 23 °C to produce high-purity FAME that met the ASTM standard by utilizing two different single-phase and pseudo-single-phase transesterification reactions (Boocock et al., 1998, Boocock et al., 1996a, Mahajan et al., 2006, Mao et al., 2004, Zhou and Boocock, 2006). About 99.4% methyl esters were formed within only 7 min by using single-phase transestrification at the methanol/oil molar ratio of 27:1 and methanol/THF volumetric ratio of 1.15 (Boocock et al., 1998). The optimal pseudo-single-phase transesterification used lower methanol/oil molar ratio of 14:1 resulting in the formation of 99.1% of methyl esters within 10 min since the reaction started as a single phase with the aid of THF, but divided into two phases as the glycerol layer separated during the reaction (Mahajan et al., 2006). Therefore, the use of both single-phase and pseudo-single-phase transmethylation
reactions at the methanol/oil molar ratio of 27:1 and 14:1, respectively, resulted in the production of standard biodiesel in terms of total glycerol content and acid number (Boocock et al., 1998, Mahajan et al., 2006).

In this investigation, organic solvents including DMF, THF, or 1,4-dioxane were used to solubilize the emulsion produced during aqueous extraction of yellow mustard flour to produce oil-solvent miscella. This miscella can be mixed with methanol and used as the medium for transmethylation to produce fatty acid methyl esters (FAME) after reducing the water content. In this case, the presence of DMF, THF or 1,4-dioxane accelerates the transmethylation process by making a single phase between yellow mustard oil and methanol. The quality of the biodiesel produced by this method is expected to meet the standard specification for biodiesel fuel (Table 2-4).

Some problems associated with biodiesel are its inherent higher price, slightly increased NO\textsubscript{x} exhaust emissions, oxidative stability when exposed to air, and cold flow properties that are especially relevant in North America (Knothe et al., c2005).

One of the major problems of biodiesel fuels is associated with crystallisation at low temperatures which can be predicted by high cloud points (CPs) and pour points (PPs). Once biodiesel fuels crystallize, the formed solids and crystals grow and agglomerate rapidly, clogging fuel lines and filters and causing major operability problems. The CP is the temperature at which a fatty material becomes cloudy due to the formation of crystals, and the PP is the lowest temperature at which the crystal agglomeration is extensive enough to prevent free pouring of fuel. Several approaches to low-temperature problems of alkyl esters have been investigated, including blending with petrodiesel, winterization, using additives, and production of branched-chain esters (Knothe, 2005). As discussed before, the use of yellow mustard oil as biodiesel feedstock can improve the cold flow properties of the resulting biodiesel due to its high levels of erucic acid. The reason is attributed to the presence of unsaturated fatty acids in the feedstock that yields good low-temperature properties but poor oxidation stabilities (Sharma et al., 2008).

### 2.8 Adsorptive Dehydration of Oil-Water-Solvent Miscella

As mentioned previously, the existence of water in transmethylation represents great challenge because even a small amount of water can hydrolyze the resulting FAME to free fatty acids to
produce soap (Atadashi et al., 2012, Sharma et al., 2008). In this investigation, the oil-solvent-water miscellae produced by emulsion treatment with DMF, THF, or 1,4-dioxane contains high amounts of water that must be reduced to less than 300 ppm (0.3 wt%) prior to methanolysis.

Adsorption can be a useful method for water removal due to the progress made in adsorbent and cyclic process developments (Yang, 2003). Adsorption is an energy-efficient and economically feasible purification tool that involves the separation of an adsorbate from the solution phase to the adsorbent surface (Proctor and Toro-Vazquez, 1996). The heart of an adsorption process is a porous solid medium called adsorbent that provides a very high surface area or high micropore volume into which adsorbate molecules can penetrate (Do, 1998). The most commonly used industrial adsorbents are alumina, silica gel, activated carbon, and molecular sieves (zeolites).

The use of silica and bleaching clays has been evaluated for adsorptive refining of vegetable oils and miscella vegetable oils to improve edible oil quality and stability by removing pigments and other contaminants including free fatty acids, peroxides, phospholipids, and carbonyl compounds (Feuge and Janssen, 1951, Toro-Vazquez and Mendez-Montealvo, 1995, Toro-Vazquez and Rocha-UrIBE, 1993). The use of other adsorbents including molecular sieves has been also evaluated for dehydrating low-water content solutions of organic solvents including isopropyl alcohol (Jain and Gupta, 1994), tetrahydrofuran (Liang et al., 2006, Liang et al., 2007), and ethanol (Teo and Ruthven, 1986).

A molecular sieve is defined as a material capable of separating molecules based on the basis of molecular size and shape, and zeolites are one type of molecular sieves. Zeolites are porous crystalline aluminosilicates of alkali or alkali earth elements, such as sodium, potassium, and calcium. These crystals are characterised by a three-dimensional pore system, with pores of precisely defined diameter (Yang, 2003).

The zeolite framework structurally consists of an assemblage of SiO₄ and AlO₄ tetrahedra, joined together in various regular arrangements through shared oxygen atoms, to form an open crystal lattice containing pores of molecular dimensions. Some of the more important commercial zeolite adsorbents are zeolite A, X, and Y (Ruthven, 1984). The A-type zeolites are widely used as desiccants to dry organic solvents or control the level of water in gas streams.
The framework structure of zeolite A (Fig. 2-9) consists of eight sodalite cages connected by double four-rings (D4Rs). This creates a large $\alpha$-cage of free diameter about 11.4 Å in the center of the unit cell (Ruthven, 1984, Wakahara et al., 2005). Due to the presence of alumina, zeolites exhibit a negatively charged framework, which is counter-balanced by positive cations. These cations can be exchanged to determine the pore size or the adsorption characteristic. For example, sodium is the cation in the 4A molecular sieve with the pore opening of approximately 4Å. If the sodium ion is exchanged with the larger potassium ion, the pore opening is reduced to approximately 3Å or 3A molecular sieve. Moreover, if the sodium ion is exchanged with calcium ion, the pore opening increases to approximately 5Å since calcium ion replaces two sodium ions (Ruthven, 1984, Yang, 2003).

![Figure 2-9: Schematic framework structure of Zeolite A (Wakahara et al., 2005)](image)

Zeolites 3A and 4A can be used to adsorb water and other compounds with molecular size below 3Å or 4Å, such as ethanol, methanol, carbon dioxide, and ethylene. Jain and Gupta (1994) studied the absorption of water from aqueous solution of isopropyl alcohol on 4A molecular sieve, and defined the adsorption capacity of 0.25 g of water/g of molecular sieve equal to 0.29 g water/cm$^3$ of molecular sieve pellets. The adsorption of water from aqueous tetrahydrofuran (THF) solution was carried out over zeolite 4A in both batch and fixed-bed systems. The equilibrium behaviour was shown to fit the Langmuir isotherm with maximum adsorption capacity of 0.246 g of water/g of molecular sieve (Liang et al., 2006, Liang et al., 2007).
As zeolite 4A was found to be an effective adsorbent for adsorption of water from organic solutions due to its energetically uniform adsorption sites, its use was evaluated in this investigation to separate water from the oil-solvent miscellae based on the fact that the adsorption sites are too small to be penetrated by the oil and solvent molecules. The adsorptive dehydration of miscellae was performed in both batch and continuous fixed-bed systems, and the results were analyzed through (1) equilibrium isotherms which indicate adsorption capacity of a zeolite 4A in equilibrium with water; and (2) breakthrough curves which represent the time dependence of adsorption for a given set of parameters including adsorbent weight, flow rate, and bed length. The following sections (2.8.1 and 2.8.2) describe the fundamentals of equilibrium isotherms and breakthrough curves.

2.8.1 Adsorption Isotherms

Adsorption equilibrium isotherm is the fundamental property of the adsorbate-adsorbent interaction (Proctor and Toro-Vazquez, 1996). It describes the relation between the amount of adsorbate on the solid and its concentration in the solution. This is accomplished at constant temperature by placing a known concentration of adsorbate in the presence of an adsorbent and then allowing the adsorbate concentration to come to equilibrium (Vera et al., 2011). Once the thermodynamic equilibrium of the adsorbate concentration is established between solution and adsorbent, no further net adsorption occurs (Proctor and Toro-Vazquez, 1996). Different theoretical and empirical models were developed to describe this equilibrium by measuring the adsorption capacity based on the amount of adsorbate adsorbed per unit mass of adsorbent (Vera et al., 2011, Watson, 1999).

The basic equilibrium adsorption isotherms including Langmuir, linear and Freundlich models are illustrated in Fig. 2-10 and their equations are summarized in Table 2-5.
Table 2-5: Common adsorption isotherm models describing adsorbate-adsorbent interaction at equilibrium (Proctor and Toro-Vazquez, 1996, Volesky, 2003)

<table>
<thead>
<tr>
<th>Adsorption Isotherm</th>
<th>Equation</th>
<th>Equation (Linear)</th>
<th>Constants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Langmuir</td>
<td>[ q = \frac{q_m K_L C_e}{1 + K_L C_e} ]</td>
<td>[ \frac{1}{q} = \frac{1}{q_m K_L C_e} + \frac{1}{q_m} ]</td>
<td>( q_m, K_L )</td>
</tr>
<tr>
<td>Linear</td>
<td>[ bq = q_m K_L C_e = H C_e ]</td>
<td>-</td>
<td>( H )</td>
</tr>
<tr>
<td>Freundlich</td>
<td>[ c q = K_f C_e^{1/n_f} ]</td>
<td>[ \log q = \log K_f + \frac{1}{n_f} \log C_e ]</td>
<td>( K_f, 1/n_f (= n) )</td>
</tr>
</tbody>
</table>

\[ a \] Legend: \( q \), the amount of substance adsorbed per mass of adsorbent at equilibrium; \( C_e \), residual adsorbate concentration in solution at equilibrium; \( q_m \), maximum adsorption capacity; \( K_L \), energy of adsorption (\( q_m \), intercept and \( K_L \), slope of Langmuir isotherm when expressed as linear format).

\[ b \] Legend: \( H \), Henry's constant

\[ c \] Legend: \( K_f \), adsorption capacity; \( n = 1/n_f \), energy of adsorption (\( K_f \), intercept and \( 1/n_f \), slope of Freundlich isotherm when expressed as linear format).

The theoretical Langmuir model is of a hyperbolic form (Table 2-5) and remains the most widely used isotherm (Vera et al., 2011, Volesky, 2003). This equation assumes that all adsorption sites are identical, that the probability of a molecule adsorbing is proportional to the concentration of the adsorbate in the solution and the number of vacant adsorption sites (Watson, 1999). At extreme dilution, the Langmuir isotherm reduces to a linear form, or Henry's law form (Table 2-5), indicating that the adsorption of an adsorbate molecule is independent of the concentration of the adsorbate (Vera et al., 2011, Watson, 1999).
The empirical Freundlich isotherm equation is exponential (Table 2-5). It is the two-parameter isotherm and the parameters of $K_f$ and $1/n_f$ are relative indicators of adsorption capacity and energy of adsorption, respectively. The adsorption parameter $1/n_f$ gives an indication of the favorability of the adsorption in which values of $1/n_f < 1.0$ represent favorable adsorption conditions (Proctor and Toro-Vazquez, 1996). The shape of the Freundlich isotherm is quite similar to the Langmuir isotherm when the exponent $1/n_f$ is less than unity (Fig. 2-10) (Watson, 1999).

2.8.2 Analysis of Breakthrough Curves

In continuous fixed-bed adsorption systems, the influent stream with initial adsorbate concentration of $C_0$ passes through a column packed with adsorbent. As the adsorbate comes in contact with the adsorbent, a concentration profile known as the mass transfer zone (MTZ) develops at the entrance of the bed in which the mass transfer process of adsorption occurs. As the region near the entrance of the bed becomes saturated, the MTZ moves through the column in the direction of the flow at a certain velocity. The column is operational until this zone reaches the column end which is known as the "breakthrough point" at which the adsorbate concentration in the effluent starts to gradually increase and, for all practical purposes, the working life of the column is over. After breakthrough point, the effluent concentration rises quickly to the inlet concentration ($C_0$) which is the exhaustion point of the column ($C/C_0=1$). The loading behavior of the fixed-bed adsorption system and the schematic of a typical breakthrough curve are illustrated in Figure 2-11 (Barros et al., 2013, Volesky, 2003, Watson, 1999). For experimental purposes, as it is difficult to determine the start and end points of the MTZ, the breakthrough time ($t_b$) and exhaustion time ($t_e$) are usually defined as the time of adsorption when the effluent adsorbate concentration, $C$, from the column are about 5 and 95% of the initial adsorbate concentration, $C_0$, respectively.

Individual breakthrough curves (S-shaped curves) can be used to calculate a number of different properties of the packed-bed adsorption systems including the capacity of the bed at the points of breakthrough and exhaustion. These properties can be easily calculated by dividing up any breakthrough curve into two distinct areas (A and B), as illustrated in Figure 2-11 (Pure Water Lab, 2014). The breakthrough capacity ($q_b$) that is represented in Table 2-6 corresponds to the area A which expresses the total amount of adsorbate trapped over the solid adsorbent until the
time of breakthrough. The exhaustion or maximum capacity of the bed \((q_e)\) (Table 2-6) corresponds to the sum of the A and B areas which represent the amount of adsorbate trapped over the solid adsorbent until complete saturation.

Figure 2-11: The progress of Mass Transfer Zone (MTZ) through a fixed-bed column and the corresponding breakthrough curve; \(C_0\), concentration of the adsorbate in the influent; \(C_b\), breakthrough concentration of the adsorbate; \(C\), concentration of the adsorbate in the effluent; \(t_b\), breakthrough time; \(t_e\), exhaustion/saturation time (Barros et al., 2013, slightly modified).

Table 2-6: The properties of fixed-bed adsorption systems calculated by utilizing breakthrough curves (S-shaped curves)

<table>
<thead>
<tr>
<th>Properties of Fixed-bed Adsorption System</th>
<th>Equations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breakthrough capacity ((q_b)^a)</td>
<td>(q_b = \frac{\text{area } A \times Q}{W} = \frac{Q}{W} \int_{t=0}^{t=t_b} (C_0 - C)dt)</td>
</tr>
<tr>
<td>Exhaustion capacity ((q_e)^a)</td>
<td>(q_e = \frac{\text{area } (A + B) \times Q}{W} = \frac{Q}{W} \int_{t=0}^{t=t_e} (C_0 - C)dt)</td>
</tr>
</tbody>
</table>

\(^a\text{Legend: } Q, \text{ volumetric flow rate to adsorber; } W, \text{ mass of the adsorbent; } t_b, \text{ breakthrough time; } t_e, \text{ exhaustion time.}\)
The discussed fundamentals of equilibrium isotherms and breakthrough curves are applied in this investigation to design the adsorptive dehydration technology for miscellae produced by emulsion destabilization. The objectives of Section 5.4 of this investigation that was previously submitted to the Journal of American Oil Chemists' Society (Tabtabaei et al., 2014b) were: (1) to explore the possibility of utilizing zeolite 4A for the adsorptive removal of water from miscellae through the design of both batch and fixed-bed systems by investigating the effect of various factors including adsorbent dose, contact time, initial water content, miscella oil concentrations, and flow rates; (2) to regenerate zeolite 4A to ensure that the amount of water retained after regeneration is negligible and the surface of the adsorbent remains clean enough for recycling; (3) to investigate the suitability of the dried oil-solvent miscella as a feedstock for producing FAME that meets the ASTM standards.
CHAPTER 3

3 Research Motivations and Objectives

The overall goal of the program is to develop an integrated process for dehulled yellow mustard flour consisting of an aqueous extraction step (AEP/EAEP) to recover high-quality food protein, free of solvent contact/residues, as the main product, followed by destabilization of the resulting oil-in-water emulsion for direct conversion to biodiesel. To achieve this goal, first, an industrially feasible AEP/EAEP technology for dehulled yellow mustard flour should be designed. Then, an appropriate solvent for extracting the emulsion should be developed and the conditions for obtaining the maximum recovery of oil into the single-phase miscella system have to be defined. After this, an effective technique for converting the dissolved oil directly into methyl esters (biodiesel) should be developed. This requires the reduction of the water content in the system.

The objectives of the work are to design an optimal AEP/EAEP technology for dehulled yellow mustard flour and to investigate the use of dimethylformamide (DMF), tetrahydrofuran (THF), and 1,4-dioxane to destabilize the resulting emulsion for biodiesel production. The operational objectives of the experimental program are as follows:

1. Development of an optimal aqueous extraction process for dehulled yellow mustard flour with improved oil and protein extractabilities while addressing the quality of the protein extracted and the stability of the emulsion released. In an attempt to achieve this goal, the detailed objectives of this process step were:

1a. To examine the effect of process variables (pH, temperature, number of extraction stages, and use of various carbohydrases) on the distribution of oil, protein, and solids between protein-rich skim fraction, oil-rich emulsion fraction and solid residual fraction.

1b. To identify the factors minimizing emulsion stability during successive non-enzymatic and enzymatic aqueous extraction processes.

1c. To assess the quality of the protein-rich skim fraction with emphasis on its use in high-quality food-grade protein isolates.
2. Development of a novel chemical approach to destabilize yellow mustard emulsion to produce single-phase oil-solvent-water miscella for conversion to biodiesel. In an attempt to achieve this goal the detailed objectives of this step were:

2a. To destabilize the oil-in-water emulsion produced during the aqueous process by dissolving it in DMF, which has a high boiling point, allowing subsequent removal of water by distillation.

2b. Determination of the oil/water/DMF ternary phase diagram to fully understand the oil recovery mechanism from the emulsion.

2c. To destabilize the emulsion produced during the aqueous process by dissolving it in cyclic ethers including THF and 1,4-dioxane which are soluble in both oil and water.

2d. Determination of the oil/water/THF and oil/water/dioxane ternary phase diagrams to fully understand the oil recovery mechanism from the emulsion.

2e. To optimize the emulsion destabilization process for recovering low-water content-miscellae by utilizing the synthesized ternary phase diagrams.

2f. To evaluate the suitability of the resulting oil-water-solvent miscella in terms of water, free fatty acid (FFA) and phospholipids for conversion to standard biodiesel.

3. Dehydration of oil-water-solvent miscella by adsorption over 4A molecular sieves (zeolite 4A) to reduce their water contents to the quality standards required for biodiesel production. In an attempt to achieve this goal the detailed objectives of this step were:

3a. To evaluate the favorability of adsorptive dehydration of miscella by constructing the adsorption equilibrium isotherms.

3b. To evaluate the use of both batch and continuous fixed-bed adsorption systems for miscella dehydration.

4. To examine the use of dehydrated oil-solvent miscella as the medium for a single-phase transmethylation to produce standard fatty acid methyl esters (FAME).

5. To conduct a preliminary economic analysis of the integrated process.
CHAPTER 4

4 Experimental Materials and Methods

4.1 Materials

Dehulled yellow mustard flour (Product Code:106) purchased from G.S. Dunn & Co. Ltd. (Hamilton, Ontario) was used as the starting material for the aqueous extraction process. The flour was received in HDPE-lined, multi-wall paper bags, and stored at room temperature. It contained 32.2±1.5 wt% oil (as-is basis), 32.9±0.08 wt% protein (as-is basis), 3.5±0.1 wt% ash (as-is basis), and 6.2±0.04 wt% moisture. The remaining 25.2 wt% was attributed to the carbohydrates including fibres and other polysaccharides.

Alcalase 2.4L (E.C. 3.4.21.14, Protease Bacillus licheniformis, optimal pH 6.5-8.5, optimal temperature 45-65 °C), Viscozyme® L (cell wall degrading enzyme complex from Aspergillus sp., optimal pH 3.3-5.5, optimal temperature 25-55 °C), Celluclast 1.5L (E.C. 3.2.1.4, cellulase from Trichoderma reesei ATCC 26921, optimal pH 4.5-6.0, optimal temperature 50-60 °C), and Pectinex Ultra SP-L (pectinase from Aspergillus aculeatus, optimal pH 4.5, optimal temperature 50 °C) were purchased from Sigma Aldrich (Oakville, ON, Canada). Protex 6L (alkaline serine endopeptidase from Bacillus licheniformis, optimal pH 9.5, optimal temperature 60 °C), Protex 51FP (endo/exo-peptidase complex from Aspergillus oryzae, optimal pH 8.0, optimal temperature 50 °C), Protex 89L (endopeptidase from Bacillus subtilis, optimal pH 8.0, optimal temperature 50 °C), and G-ZYME® G999 (E.C.3.1.1.32, lysophospholipase, optimal pH 4.5, optimal temperature 50 °C) were kindly provided by Genencor Division of Danisco (Rochester, NY, USA). Lipomod 699L (phospholipase A2, optimal pH 8.0, optimal temperature 40 °C) was kindly provided by Biocatalysts, Ltd (Wales, UK). The activities of all enzyme solutions were specified by the manufacturers. All enzyme solutions were stored at 4°C. Enzyme use was expressed as weight percentages (wt%) based on the actual weight of the enzyme solution.

Anhydrous grade solvents used for oil content determination including diethyl ether and petroleum ether were purchased from Sigma Aldrich (Oakville, ON, Canada). RO water was used in all experiments.
ACS grade solvents were used for emulsion destabilization. DMF was purchased from Caledon Laboratories Ltd. (Georgetown, ON, Canada). Cyclic ethers including THF and 1,4-dioxane were provided by Sigma Aldrich (Oakville, ON, Canada).

A Type 4A (8-12 mesh beads) molecular sieve (zeolite 4A) was purchased from Sigma Aldrich (Oakville, ON, Canada) and used for both batch and fixed-bed adsorption processes. Food-grade canola oil (100% Canadian canola oil; Unico, Concord, ON, Canada) was used instead of yellow mustard oil for designing adsorption experiments to determine the optimum conditions for the adsorption of water as it was commercially available, and its solubility properties at room temperature were expected to be similar to mustard oil. Methanol (anhydrous, 99+%), THF (anhydrous, 99+%), hexane (mixture of isomers, anhydrous, 99+%), heptane (anhydrous, 99%), potassium hydroxide solution (0.1 M KOH in isopropanol, volumetric standard), sodium chloride (99+%), sodium hydroxide pellets (reagent-grade, 98+%, anhydrous), and the Karl Fischer moisture determination reagents including HYDRANAL®-LipoSolver CM and HYDRANAL®-Composite 2 were supplied by Sigma-Aldrich (Oakville, ON, Canada). ACS grade sodium sulphate (anhydrous) was supplied by VWR International (Toronto, Ontario, Canada). Oxalic acid dihydrate (ACS reagent, 99.5+%), ASTM D 974 titration solvent (toluene/water/anhydrous 2-propanol in the ratio of 100:1:99) and ASTM D 974 p-naphtholbenzein indicator solution (1% (w/v) in titration solvent) were purchased from Fisher Scientific Company (Ottawa, ON, Canada). Kimble-Chase Kontes™ borosilicate glass threaded column (2.5-cm diameter × 30.0-cm height) with PTFE end fittings and tubing was also purchased from Fisher Scientific Company and used for designing the fixed-bed adsorption process.

The standard solutions of ASTM D 6584, packaged in 1-mL ampoules in pyridine, were purchased from Ultra Scientific (N. Kingstown, RI, USA). The following products were purchased through Chromatographic Specialties Inc. (Brockville, ON, Canada): N-methyl-N-trimethylsilyl trifluoroacetamide (silylation reagents); 1,2,4-butanetriol internal standard solution (1000µg/mL pyridine); tricaprin internal standard solution (8000µg/mL pyridine); MXT-Biodiesel TG column (15 m × 0.32 mm inside diameter, 0.10 um film thickness) with a high-temperature guard column (2 m × 0.53 mm).
4.2 Aqueous Extraction and Enzyme-Assisted Aqueous Extraction Processes (AEPs and EAEPs) from Dehulled Yellow Mustard Flour

The methods and procedures described in Sections 4.2 and 4.3 of this investigation have been previously published in the journal "Food Research International" (Tabtabaei and Diosady, 2013).

Dehulled yellow mustard flour was selected for this study since the mucilage from the whole seed complicates the extraction of the oil during AEP/EAEP due to its excellent emulsifying properties (Balke and Diosady, 2000). Different AEPs and EAEPs were designed based on the following two steps to determine the best physical, chemical, and enzymatic conditions leading to an optimal AEP/EAEP technology. The first step (a) focused on the individual and combined usage of the three selected carbohydrases (Viscozyme L, Celluclast 1.5L, and Pectinex Ultra SP-L) to evaluate the role of these cell wall-degrading enzymes on enhancing oil and protein extractability. In the second step (b), the role of alkaline extraction (pH 11) was evaluated by completing successive extractions in which alkaline extraction was considered the main, second, or third stage of the process.

(a) We first designed simple processes in order to select suitable carbohydrases to aid subsequent extractions. These carbohydrase treatments were performed through a series of six experiments including one control run examining the extractability of oil and protein from the flour. In each experiment, 350 g of dehulled yellow mustard flour was added to 1400 g water (4:1 water to flour weight ratio) and blended on low speed using a Waring Commercial Blender for 3 min to reduce the mean size of the particles. The smooth slurry was then transferred into a 2-L beaker. The beaker was placed in a water bath, and the temperature of the slurry was adjusted to 40.0-42.0 °C while stirred using a Caframo mixer with a four-bladed propeller stirrer (Caframo, Wiarton, ON) at 500 rpm. The mixture was then treated with the selected carbohydrases: Viscozyme L, Pectinex Ultra SP-L, or Celluclast 1.5L. Enzymes were introduced individually or in combination at a total level of 3 wt% of the flour weight. The enzymatic reaction was allowed to continue for 3 h while oil and protein were extracted. The native pH of the slurry was 4.8-5.0, and it was unchanged throughout the entire process, remaining within the optimal pH range of 4.5-5.0 for these enzymes. The separation of the slurry into a solid residual fraction, a skim fraction, and an oily emulsion was accomplished by splitting the slurry into two 1-L centrifuge
bottles and centrifuging at 9000 g (6500 rpm) for 20 min at 25 °C in an Avanti J-20 XP centrifuge (Beckman Coulter, Inc., Palo Alto, CA). These three fractions were separated after centrifugation. The solid residual fraction was then re-blended for 30 sec with enough water to reconstitute the original mass of 1400 g. After stirring for another 30 min and re-centrifuging, the emulsion and skim were removed and combined with the respective first-stage fractions. Since the goal of this step is to study the effect of carbohydrases on total oil and protein extraction yields, the insoluble solid residual fraction was the only phase analyzed for the oil and protein content; therefore, it was first freeze-dried using a Labconco FreeZone Freeze Dry System (Labconco Corp., Kansas City, MO) under 1.8 Pa vacuum for 48 hours at a freeze chamber temperature of -85°C, before determining the oil and protein extraction yields.

The total oil and protein extraction yields were determined indirectly from the oil and protein content of the solid residual fraction and subtracted from the initial oil and protein content of the flour using Eq. 1.

\[
\text{Extraction Yield (\%)} = 100 \times \frac{(\text{oil or protein (g) in the flour}) - (\text{oil or protein (g) in the insoluble fraction})}{\text{oil or protein (g) in the flour}}
\] (1)

For comparison one control run was performed without the use of carbohydrases using the above procedure.

(b) The successive extractions of yellow mustard flour were developed over the course of five series of experiments (Figs. 4-1 and 4-2). The first and second successive extractions, as shown in Fig. 4-1, are: (i) two-stage AEP: pH 4.8, 11, in which the mustard slurry (4:1 water to flour weight ratio) was first extracted at native pH (4.8-5.0) and 25°C for 30 min followed by second-stage alkaline extraction (pH 11) for another 30 min at room temperature; and (ii) EAEP: pH 4.8, 11, in which the slurry was first extracted at pH 4.8 and 40°C in the presence of 3 wt% carbohydrate enzymes as of the flour weight (1 wt% Viscozyme L, 1 wt% Pectinex Ultra SP-L, and 1 wt% Celluclast 1.5L) for 3 h, followed by second-stage alkaline extraction (pH 11) at room temperature for 30 min. The third successive extraction is (iii) two-stage AEP: pH 11 (Fig. 4-1), in which the extraction of the slurry was conducted at 25°C for 30 min using two stages of alkaline extraction at pH 11. In each process, the slurry obtained after the first extraction stage was centrifuged to produce the first skim, solid residue, and emulsion fractions. The first skim
fraction was then re-centrifuged to recover more emulsion (3rd emulsion). The first solid residual fraction was fed to the second extraction stage, stirred with water at pH 11 for 30 min and centrifuged to produce the second skim, solid residue, and emulsion fractions. The second emulsion and skim fractions were skimmed off and poured off, and combined with their respective first-stage fractions. The final emulsion fraction was analyzed directly for oil and protein content while the final skim and insoluble residual fractions were freeze-dried prior to oil and protein analysis.

Figure 4-1: The experimental procedure of the successive aqueous and enzyme-assisted aqueous extraction processes of dehulled yellow mustard flour including (i) AEP: pH 4.8, 11; (ii) EAEP: pH 4.8, 11; and (iii) AEP: pH 11.
Figure 4-2: The experimental procedure of the successive AEP: pH 4.8, 11, and EAEP: pH 4.8, 11, followed by extracting the emulsion at pH 11.
In the fourth and fifth successive extractions (Fig. 4-2), the mustard flour was extracted using the same protocol as the first (AEP: pH 4.8, 11) and second (EAEP: pH 4.8, 11) processes, respectively, followed by the third extraction stage in which the collected emulsion was extracted using 3:1 water to emulsion weight ratio at pH 11 and 25°C for 30 min. Each process was repeated three times; therefore, the reported oil, protein, and solid yields were the averages of 9 determinations.

These successive AEP/EAEP processes were identified by Roman numerals throughout the following sections (4.3.1 - 4.3.5), as summarized in Table 4-1.

<table>
<thead>
<tr>
<th>Successive AEP/EAEP</th>
<th>Process Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two-stage AEP: pH 4.8, 11</td>
<td>AEP (i)</td>
</tr>
<tr>
<td>Two-stage EAEP: pH 4.8, 11</td>
<td>EAEP (ii)</td>
</tr>
<tr>
<td>Two-stage AEP: pH 11</td>
<td>AEP (iii)</td>
</tr>
<tr>
<td>Three-stage AEP: pH 4.8, 11, followed by</td>
<td></td>
</tr>
<tr>
<td>emulsion extraction at pH 11</td>
<td>AEP (iv)</td>
</tr>
<tr>
<td>Three-stage EAEP: pH 4.8, 11, followed by</td>
<td></td>
</tr>
<tr>
<td>emulsion extraction at pH 11</td>
<td>EAEP (v)</td>
</tr>
</tbody>
</table>

4.2.1 Analytical Methods

The oil content of dehulled yellow mustard flour and freeze-dried skim and insoluble solid residual fractions was determined in triplicate using the Mojonnier method (AOAC Method 922.06 for solid samples), and crude protein content (N×6.25) was determined in triplicate by the Kjeldahl method, according to AOCS Method Ba4d-90. The ash content of dehulled yellow mustard flour was determined based on AOCS Method Ba5a-49. The methods used to determine the compositions of the emulsions produced during successive AEPs and EAEPs will be discussed in section 4.3.

4.2.2 Statistical Analysis

Least Significant Difference (LSD) method was used to compare means (n = 3) at 5% significance level by using the SAS system (version 9.2., SAS Institute Inc., Cary, NC, USA).
4.3 Characterization of the Emulsions Produced During Successive AEPs and EAEPs

4.3.1 Emulsion Compositions

Five different emulsions produced through successive extractions including AEP (i), EAEP (ii), AEP (iii), AEP (iv) and EAEP (v) were analyzed for oil, moisture, protein, and phospholipids content. The oil content of the emulsions was determined in triplicate using the Mojonnier method (AOAC Method 995.19). Crude protein content (N×6.25) was determined in triplicate by the Kjeldahl method, according to AOCS Method Ba4d-90. Moisture content of the emulsion was determined gravimetrically by AACC Method 44-15A, and the phospholipids content was determined by ashing the sample and measuring the phosphorus spectrophotometrically (AOCS Method Ca 12-55). To convert the percentage of total phosphorous to the equivalent phospholipids, a multiplication factor of 30 was applied.

4.3.2 Oil Droplet Size Analysis

The freshly prepared emulsions were analyzed for droplet size distribution by laser diffraction (Mastersizer S, Malvern Instruments Ltd., Montreal, QC, Canada). Emulsion samples were diluted with distilled water as the dispersant to obtain an obscuration of about 11-14%. The refractive index (RI) used for the mustard oil droplets was 1.47 and the RI for the dispersant was 1.333. The area-weighted mean diameters (d_{3,2}) and the volume-weighted mean diameters (d_{4,3}) of the oil droplets in the original emulsions were measured. The samples were analyzed in duplicate at 25°C.

4.3.3 Measurement of Surface Protein Concentration (\(\Gamma\))

Surface protein concentration, \(\Gamma\), of the emulsions was determined according to (Agboola et al., 1998, Chabrand and Glatz, 2009, Morales Chabrand et al., 2008). The emulsions were first washed by dispersion into distilled water using 4:1 water to emulsion weight ratio, and then recovered by centrifugation at 12,000 g and 25°C for 20 min. The washed emulsions were then skimmed off and analyzed for oil and protein content. The surface protein concentration was then measured using Eq. 2.

\[
\Gamma = \frac{mg \text{ of protein/g of oil}}{SSA (m^2/g \text{ of oil})}
\]
where SSA, the specific surface area, of the oil droplets was calculated using Eq. 3.

$$SSA = \frac{6/D_{3,2}}{1/P_{oil}}$$

(3)

where $D_{3,2}$, area-weighted mean diameter, was determined from oil droplet size analysis using Mastersizer data, and the density of crude yellow mustard oil measured at 19°C was 0.909 g/cm$^3$.

### 4.3.4 Analysis of the Proteins Adsorbed at the Emulsions Interface by SDS-PAGE Electrophoresis

SDS-PAGE was performed to determine the type of proteins adsorbed at the oil-water interface of the emulsions. The emulsions were first washed as described by (Chabrand and Glatz, 2009, Morales Chabrand et al., 2008). The washed emulsions were then analyzed for protein content, and precipitated by adding cold (-20°C) acetone to the emulsions in a ratio of 4:1 (v/w). The solutions were mixed, incubated at -20°C for 60 min, and then centrifuged for 10 min at 12,000 g. The supernatants were separated and the precipitates were washed several times with cold acetone until no oil was observed in the solvent. The protein pellets were dried at room temperature to remove residual acetone (Pierce Technical Resource).

The dried protein pellets precipitated from each emulsion (2-10 mg) were then dispersed in 1 ml of sample buffer containing 50 mM Tris-HCl pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol in a way to provide the same concentration of protein in each well of the gel. The solutions were then heated at 95°C for 10 min before being loaded to a ready gel Tris-HCl 4-20% polyacrylamide linear gradient gel (Bio-Rad, Cat# 161-1105, Hercules, CA). SDS-PAGE was performed on these protein fractions and run at 135 V for 45 min on a Mini-PROTEAN® II Electrophoresis Cell (BioRad, Hercules, CA). The loading amount of protein into the gel was 15 μl. The gels were stained in a 0.33% Coomassie blue solution, and destained in a solution containing 10% methanol and 20% acetic acid. Prestained protein marker (Fermentas, Cat# SM1811, Burlington, ON, Canada) was used to identify the molecular mass region between 10-250 kDa.
4.3.5 Demulsification Treatments

To evaluate the effectiveness of successive aqueous or enzyme-assisted aqueous extraction processes on the stability of the emulsions produced, two different demulsification treatments including pH adjustment and enzymatic treatments were developed. These demulsification treatments were carried out on the emulsions produced during successive extraction processes denoted as AEP (i), EAEP (ii), AEP (iii), AEP (iv), and EAEP (v).

To study the effect of pH on emulsion destabilization, the emulsion (10 g) was transferred into a 50-ml plastic centrifuge tube and diluted with an equal mass of water, the pH of the diluted emulsion was then adjusted to the appropriate pH (2.0 to 10.0) by addition of 1N NaOH or 1N HCl, and the pH-adjusted cream was incubated at 60°C for 3 h with constant stirring. The treated emulsions were centrifuged at 9000 g for 20 min at 25°C to release oil. The free oil recovery was determined using Eq. 4.

\[
\text{Free Oil Recovery} = 100 \frac{\text{free oil recovered (g)}}{\text{original oil in yellow mustard emulsion (g)}}
\]  
(4)

Enzymatic demulsifications were performed through the use of different proteases (Alcalase 2.4L, Protex 6L, Protex 51FP, and Protex 89L) and phospholipases (G-ZYME® G999 and Lipomod 699L). The emulsion (15 g) was first transferred into a 50-ml centrifuge tube, and adjusted to the optimum pH and temperature of the enzyme tested. The enzyme was then added at 2.5 wt% as of the emulsion weight, and the reaction was performed with constant stirring for 3 h. The control was performed without enzyme addition using the same pH, temperature, and incubation time. The tubes of the treated emulsions were centrifuged to free oil. The free oil recovery was calculated using Eq. 4.

4.4 Destabilization of Yellow Mustard Emulsion with Organic Solvents

The two-stage alkaline extraction of dehulled yellow mustard flour, (iii) AEP: pH 11, was selected as a potentially viable process; therefore, the destabilization of its emulsion using organic solvents was further studied to produce oil-solvent-water miscella for subsequent biodiesel production. Consequently, the "yellow mustard emulsion" referred to in the following sections (4.4.1 - 4.4.4), is the emulsion produced during the two-stage AEP: pH 11.
The methods and procedures described in Section 4.4 of this investigation have been previously published in the journal of the American Oil Chemists’ Society (Tabtabaei and Diosady, 2012, Tabtabaei et al., 2013, Tabtabaei et al., 2014a).

This aqueous extraction of dehulled yellow mustard flour, as described previously (Section 4.2 and Fig 4-1), was conducted at room temperature using two stages of alkaline extraction at pH 11. About 700 g of dehulled yellow mustard flour (as is) was added to 2800 g water (4:1 water-to-flour weight ratio) and blended on low setting in a Waring blender for 3 min. The smooth slurry was then transferred into a 4-L beaker, and the pH was adjusted to 11 by the addition of sodium hydroxide (50% w/w). The slurry was then stirred for 30 min using a Caframo mixer (Wiarton, ON, Canada) with a three-blade propeller stirrer at 500 rpm. The slurry obtained in the first extraction stage was centrifuged at 9000 g (6500 rpm) for 20 min at 25 °C to produce a solid residual fraction, a skim fraction, and an oily emulsion. The solid residual fraction obtained in the first extraction stage was diluted with 2800 g water. After stirring for another 30 min at pH 11 and re-centrifuging, the emulsion and skim were removed and combined with the respective first-stage fractions. In the following sections only the emulsion was used for further experimentation. The other fractions had well-documented properties and were discarded. Before any treatment, the oil content of the collected emulsion was determined using the Moissonier method described in emulsion compositions section (4.3.1).

### 4.4.1 Single-Stage Treatment of Yellow Mustard Emulsion with DMF

A 30g sample of emulsion was transferred into 250-mL centrifuge bottles, and DMF was added to the centrifuge bottle until the desired DMF:oil weight ratio was reached. The mixture was agitated using a wrist action shaker (Burrell Scientific, Pittsburgh, PA) for 30 min followed by centrifugation for 20 min at 6,000 rpm in an Avanti J-20 XP centrifuge (Beckman Coulter, Inc., Palo Alto, CA). After centrifugation, two phases were observed: the miscella phase at the bottom of the container composed of oil, water, and DMF; and the upper creamy phase, i.e. the emulsion residue composed of oil, water, DMF, and protein. These two phases were individually collected for analysis. The miscella phase was analyzed for oil, water, and DMF content. The oil extracted from the original emulsion to the miscella phase was calculated based on the oil content in the miscella phase that was determined by measuring the weight loss after evaporation of the DMF and water in a vacuum oven at 120°C and ~20 mmHg for 3 h; the water content of the miscella
was determined by BIOX Corporation (Hamilton, ON) using a Karl Fischer titrimeter. The percentage of DMF was then determined by subtracting the percentage of oil and water from 100. The creamy emulsion residue phase was first dried in a vacuum oven under the above conditions to determine the solvent holdup; it was then analyzed for protein content using the Kjeldahl method. The residual oil and moisture content were calculated by mass balance on the oil and water in the system. The experimental procedure of the treatment of yellow mustard emulsion with DMF, and the schematic representation of the two phases separated after centrifugation are presented in Fig. 4-3.

![Flow diagram for treatment of yellow mustard emulsion with dimethylformamide (DMF)](image)

**Figure 4-3: Flow diagram for treatment of yellow mustard emulsion with dimethylformamide (DMF)**

### 4.4.1.1 Determination of Oil/Water/DMF Ternary Phase Diagram

The usual method for constructing ternary phase diagrams is to first measure the compositions of the phases that are in equilibrium in the two-phase region; the compositional pairs are then connected by tie lines to construct the solubility curve and phase diagram. In the oil/DMF/H₂O system, at room temperature, both oil-DMF and oil-H₂O are partially miscible, while DMF dissolves in any proportion in water; therefore, two pairs of partially miscible liquids are formed. In this system, DMF is the solvent, water is considered the solute since it is more soluble in DMF, and oil is considered the diluent. Commercial canola oil (100% Canadian canola oil; Unico, Concord, ON, Canada) was used as canola belongs to the same family as yellow mustard (cruciferae family) (Appelqvist, 1971), and it was readily available. The first step involved the determination of the solubility of oil in DMF and the solubility of DMF in oil. The solubility of oil in DMF was measured by adding pure canola oil from a burette to the specified amount of stirred DMF until turbidity was observed. The same procedure was applied to determine the solubility of DMF in oil. The solubility of oil in water and water in oil was considered negligible.
since cloud point method is not a practical way to measure the very limited solubility of oil in water and water in oil. In the second step, nine different mixtures composed of canola oil, water, and DMF were prepared with compositions presented in Table 4-2. The mixtures were stirred vigorously for 30 min at room temperature, and immediately transferred into separatory funnels. The separated solvent-rich and oil-rich phases were collected, and their weights were recorded. The next step focused on the measurement of the composition of the coexisting phases as described by Mehta and Fraser (1985). The oil contents of the two phases were determined by calculating the weight loss after evaporating all water and DMF, and the water content was determined by Karl Fischer titration (Volumetric Karl Fischer ASTM standard method E203 and Coulometric Karl Fischer ASTM standard method D6304).

Table 4-2: Composition of the solutions used for preparation of oil/water/dimethylformamide ternary phase diagram

<table>
<thead>
<tr>
<th>Bulk Solution No.</th>
<th>Composition (mass fraction)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMF</td>
</tr>
<tr>
<td>1</td>
<td>0.10</td>
</tr>
<tr>
<td>2</td>
<td>0.20</td>
</tr>
<tr>
<td>3</td>
<td>0.30</td>
</tr>
<tr>
<td>4</td>
<td>0.40</td>
</tr>
<tr>
<td>5</td>
<td>0.60</td>
</tr>
<tr>
<td>6</td>
<td>0.50</td>
</tr>
<tr>
<td>7</td>
<td>0.70</td>
</tr>
<tr>
<td>8</td>
<td>0.80</td>
</tr>
<tr>
<td>9</td>
<td>0.80</td>
</tr>
</tbody>
</table>

4.4.2 Single-Stage Treatment of Yellow Mustard Emulsion with THF and 1,4-Dioxane

The emulsion (20 g) was transferred into two 50-mL Teflon centrifuge tubes, and the required amount of THF or dioxane was added to each tube to reach the desired THF:oil or dioxane:oil weight ratio, respectively. The mixture was then agitated for 30 min using a wrist action shaker to accelerate the process, and it was centrifuged for 20 min at 4500 rpm. After centrifugation, three phases were observed in the centrifuge tube: emulsion residue in the form of solid phase at the bottom of the centrifuge tube composed of water, protein, oil, and solvent; the polar phase mostly composed of water and solvent in the middle; and the miscella or the upper phase mostly composed of oil and solvent. These three phases were separated from each other, and their
weights were recorded. The oil extracted from the original emulsion to the miscella and polar phases was calculated based on their oil content that was determined by measuring the weight loss after evaporation of the solvent and water initially at room temperature in a fume hood and then at 110°C for 1.5 h using a forced air oven. The extraction yields were calculated using Eqs. 5 and 6.

\[
\text{Oil extraction yield (\%) to miscella phase} = \frac{\text{oil (g) in miscella phase}}{\text{oil (g) in starting emulsion}} \times 100
\]  

(5)

\[
\text{Oil extraction yield (\%) to polar phase} = \frac{\text{oil (g) in polar phase}}{\text{oil (g) in starting emulsion}} \times 100
\]  

(6)

The water content of the miscella and polar phases was determined by Karl Fischer titration (Volumetric Karl Fischer ASTM standard method E203 and Coulometric Karl Fischer ASTM standard method D6304), and was performed for us by BIOX Corporation Company (Hamilton, ON). The percentage of THF or dioxane was then determined by subtracting the percentage of oil and water from 100.

4.4.2.1 Determination of Oil/Water/THF and Oil/Water/Dioxane Ternary Phase Diagrams

Commercial canola oil (100% Canadian canola oil; Unico, Concord, ON, Canada) was used for the preparation of phase diagrams as it was readily available, and its solubility properties at room temperature are similar to mustard oil. In the oil/water/THF and oil/water/dioxane systems, at room temperature, both oil-solvent and water-solvent are completely miscible, while the solubility of oil and water is partial; therefore, a pair of partially miscible liquids is formed. For each system, first, the solubility curve was determined using the cloud point method. Based on this method, to determine the water-rich side of the solubility curve, different water/solvent mixtures of known composition were prepared; for each water/solvent mixture, the canola oil was added from a burette to a stirred mixture until turbidity appeared. The weight of oil required to just achieve turbidity was then recorded. This procedure was also repeated for the oil-rich side of the solubility curve in which water was added to stirred mixtures of oil/solvent of known composition until turbidity appeared. The compositions of the resulting mixtures obtained in this way for all original mixtures were finally plotted to form a mutual solubility curve. In the second step, the solutions containing a mixture of canola oil, water, and solvent were prepared in which the composition of oil and water was equivalent to the composition of mustard oil and
water in the original emulsion; and the composition of solvent was similar to the amount of THF or dioxane added to solubilize the emulsion at the specified THF:oil or dioxane:oil weight ratios. This selected approach would help us to analyze the behaviour of the system during emulsion treatment at different weight ratios. The prepared mixtures were then stirred for 30 min using magnetic stirrer at room temperature, and transferred into separatory funnels. After 6 h the two phases were assumed to be in equilibrium, and the water-rich phase and oil-rich phase, were separated and their weights were recorded. The next step involved the determination of the composition of the coexisting phases based on the method found in the literature (Mehta and Fraser, 1985). The oil content of the both phases was measured by calculating the weight loss after evaporating all water and solvent, and the water content was determined using the Karl Fischer titration method.

4.4.3 Multi-Stage Treatment of Yellow Mustard Emulsion with THF and 1,4-Dioxane

Single-stage treatment of yellow mustard emulsion, as described previously (section 4.4.2), was conducted at THF:oil weight ratios of 1:1 to 5:1, and dioxane:oil weight ratios of 1:1 to 10:1. Three-stage treatments of the emulsion using lower THF:oil weight ratios of 0.25:1, 0.5:1, and 0.75:1, and lower dioxane:oil weight ratios of 0.25:1, 0.5:1, 1:1, 1.5:1, and 2:1 were evaluated for the purpose of destabilization as shown in Figure 4-4. Aliquots of 10 g of emulsion were poured into 50-mL Teflon centrifuge tubes. Once the desired amounts of THF or dioxane were added to the tubes, they were shaken for 30 min by using a wrist action shaker. Following the first-stage centrifugation at 4,500 rpm for 20 min, the mixtures of emulsion and solvent were separated into three different phases: emulsion precipitate in the form of solid phase along with some unextracted emulsion (residual emulsion) at the bottom of the centrifuge tube, and the miscella phase on top (Fig. 4-5a). The first-miscella phase was separated, and analyzed for oil, water, and solvent contents while the remaining phases were subjected to the second-stage extraction by mixing with the same volume of solvent used for the first extraction stage. The resulting mixture was then centrifuged and separated into four different phases including emulsion precipitate in the form of a compact solid phase at the bottom of the centrifuge tube; the water-rich polar phase along with some residual emulsion in the middle; and the miscella on top (Fig. 4-5b). The second-miscella phase was removed and analyzed for oil, water, and solvent contents while the remaining phases were fed to the third extraction stage, mixed with the same
volume of solvent and centrifuged. The third-stage extraction destabilized the emulsion and resulted in the formation of three distinct phases: emulsion precipitate phase at the bottom of the centrifuge tube, water-rich polar phase in the middle, and miscella phase on top (Fig. 4-5c). The third-miscella was separated from the final polar and emulsion precipitate phases; however, a very thin layer of residual emulsion, which was dispersed into the polar phase during phase separation was observed on top of the polar phase at some of the tested THF:oil and dioxane:oil weight ratios.

The next step involved determining the composition of the third-miscella, final polar, and emulsion precipitate phases. The oil contents of the miscellae (first-, second-, third-, and combined-miscella phases) and final polar phases were measured by evaporating all water and solvent initially in a fume hood at room temperature and then in a forced-air oven at 110 °C for 1.5 h. The water content was determined by Karl Fischer titration (Hanna Instruments Canada Inc-Laval, QC, Canada). Accurate estimates of the percentage of THF or dioxane in the miscellae was obtained based on the oil and water content by difference. The final polar and emulsion precipitate phases were dried in a forced-air oven and analyzed for protein content by the Kjeldahl method. The oil and moisture contents of the final emulsion precipitate phase were calculated by mass balance of the oil and water in the system.

The oil, water, and protein extracted from the original emulsion to the miscella, final polar, and emulsion precipitate phases were calculated based on the composition of each specific phase and the original emulsion. Equations 7, 8, and 9 represent the oil, water, and solvent extraction yields to the combined miscella phase.

\[
\text{Oil Extracted (\% to combined miscella phase) = \frac{\text{oil (g) in combined miscella}}{\text{oil (g) in starting emulsion}} \times 100} \quad (7)
\]

\[
\text{Water Extracted (\% to combined miscella phase) = \frac{\text{water (g) in combined miscella}}{\text{water (g) in starting emulsion}} \times 100} \quad (8)
\]

\[
\text{Solvent Extracted (\% to combined miscella phase) = \frac{\text{solvent (g) in combined miscella}}{\text{total solvent (g) used for 3-stage treatment}} \times 100} \quad (9)
\]
Figure 4-4: Three-stage treatment of yellow mustard emulsion with tetrahydrofuran or dioxane.
Figure 4-5: The distribution of the oil-rich miscella, water-rich polar, and emulsion precipitate phases after centrifugation at 4,500 rpm for 20 min. The three-stage treatment of yellow mustard emulsion was conducted at room temperature using 0.75:1 tetrahydrofuran:oil weight ratio. a First-stage extraction, b Second-stage extraction, c Third-stage extraction (the emulsion was completely destabilized in the third stage).

4.4.3.1 Finalizing Oil/Water/THF and Oil/Water/Dioxane Ternary Phase Diagrams

The preparation of oil/water/THF and oil/water/dioxane ternary phase diagrams were described in section 4.4.2.1 by first determining the solubility curve using the cloud point method and then determining the tie lines that matched the THF:oil or dioxane:oil weight ratios used during the single-stage destabilization of the emulsions. As this approach was essential to understand the behaviour of the single-stage destabilization process, the oil/water/THF and oil/water/dioxane ternary phase diagrams were completed in this section by determining the tie lines related to the lower THF:oil and dioxane:oil weight ratios including 0.25:1, 0.5:1, and 0.75:1 THF:oil; and 0.5:1 and 1.5:1 dioxane:oil weight ratios. This was performed by preparing solutions of oil, water, and solvent for each specific THF:oil or dioxane:oil weight ratio, followed by stirring for 30 min before transferring into separatory funnels. After 6 h, the two coexisting phases (water-rich phase and oil-rich phase) were separated and their compositions were determined by the method of Mehta and Fraser (1985) as described above (section 4.4.2.1).
4.4.3.2 Quality of Oil Recovered Through Three-Stage THF Treatment of the Emulsion

The emulsion (200 g) was destabilized by using three-stage treatment at 0.5:1 and 0.75:1 THF:oil weigh ratios following the procedures described in section 4.4.3 (Fig. 4-4). The first-, second-, and third-miscella phases were collected and combined. The resulting oil-water-THF miscella was transferred into a round-bottomed flask and the majority of the water and THF was evaporated in a rotary evaporator under approximately 36 mm of Hg vacuum (~4.79 KPa) produced by a water aspirator and a water bath at 85 °C. The water and THF removal was considered to be complete after 1 h when no further condensate was observed. The recovered oil was then cooled to room temperature and analyzed for FFA and phospholipids. The phospholipid content of the recovered yellow mustard oil was determined according to AOCS Method Ca 12-55. FFA was determined by alkaline titration using AOCS Official Method Ca 5a-40, and the results were presented as percent oleic acid.

4.4.4 Statistical Analysis

Single-stage treatments of yellow mustard emulsion were performed at ten different DMF:oil weight ratios between 2.5:1 and 30:1, seven different THF:oil weight ratios between 1:1 and 5:1, and ten different dioxane:oil weight ratios between 1:1 and 10:1. Each treatment was repeated three times. The means from the miscella and polar phases composition and from the oil extraction yields were reported as means ± STD (n=3×3). Least Significant Difference (LSD) method was used to compare the means of the oil yields at 5% significance level by using the SAS system (version 9.2., SAS Institute Inc., Cary, NC, USA).

Multi-stage treatments of yellow mustard emulsion were performed at three different THF:oil weight ratios of 0.25:1, 0.5:1, and 0.75:1; and five different dioxane:oil weight ratios of 0.25:1, 0.5:1, 1:1, 1.5:1, and 2:1. The means of the oil extraction yields (n = 3) from each treatment were also compared by using Least Significant Difference (LSD) method at 5% significance level in the SAS system (version 9.0, SAS Institute Inc., Cary, NC, USA).

4.5 Adsorptive Dehydration of Miscellae and Biodiesel Production

The miscellae produced during single-stage emulsion destabilization at 4:1 THF:oil weight ratio and three-stage destabilization at 0.5:1 and 0.75:1 THF:oil weight ratios were dehydrated by
adsorption over zeolite 4A to produce oil-THF miscellae for direct conversion to biodiesel. The compositions of these miscellae and their equivalent destabilization processes are summarized in Table 4-3. The detailed discussions about the compositions and behavior of destabilization processes will be discussed in sections 5.3.2 and 5.3.4. These miscellae were identified by Roman numerals throughout the following sections (4.5.1 - 4.5.4), as represented in Table 4-3. The methods and procedures described in Section 4.5 have been previously submitted to the Journal of the American Oil Chemists’ Society for publication (Tabtabaei et al., 2014b).

Table 4-3: Compositions of the miscellae produced through single-stage and three-stage treatments of the emulsion with tetrahydrofuran (THF)

<table>
<thead>
<tr>
<th>Miscella Number</th>
<th>Characterization of Destabilization Processes</th>
<th>Miscella Compositions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>THF:Oil Weight Ratios</td>
<td>Number of Stages</td>
</tr>
<tr>
<td>I</td>
<td>0.5:1</td>
<td>3</td>
</tr>
<tr>
<td>II</td>
<td>0.75:1</td>
<td>3</td>
</tr>
<tr>
<td>III</td>
<td>4:1</td>
<td>1</td>
</tr>
</tbody>
</table>

All values represent the composition means (n=3) ± standard deviation. All the measurements were performed in triplicate.

4.5.1 Adsorption Equilibrium Study

Equilibrium studies were carried out to compare the favorability of adsorptive dehydration of miscellae (I, II, and III) based on their oil and THF concentrations (dilute miscella vs. concentrated miscella). In this part of the study, food-grade canola oil, anhydrous THF and reverse osmosis (RO) water were used to directly synthesize miscellae (I), (II), and (III) with compositions listed in Table 4-3 as it would be tedious and expensive to produce sufficient yellow mustard emulsion and miscellae at all THF:oil weight ratios in the laboratory. For adsorption isotherms, experiments were conducted in duplicate in 100-mL flasks by contacting a fixed amount (50.0 g) of miscellae (I, II, and III) with various amounts of zeolite 4A ranging from 1.0 to 22.5 g. The samples were agitated vigorously at 125 cycles/min and room temperature for 24 h to reach equilibrium. The supernatants were then analyzed for water contents using Karl Fischer titration (Hanna Instruments Canada Inc-Laval, QC, Canada) to determine the amount of water adsorbed per unit mass of adsorbent (q) using Eq. 10.
\[ q = \frac{(C_i - C_e)V}{W} \]  

where \( q \) is the equilibrium adsorption capacity (g g\(^{-1}\)); \( C_i \) and \( C_e \) are the initial and equilibrium concentrations (g g\(^{-1}\)) of water in the miscella, respectively; \( V \) is the amount of the miscella solution (g); and \( W \) is the mass (g) of the adsorbent.

### 4.5.2 Batch Adsorption Experiments

Batch experiments were carried out at room temperature to investigate the effects of adsorbent dose and contact time on uptake of water from the combined miscella (I and II) prepared from canola oil, RO water and THF based on the compositions represented in Table 4-3. To investigate the effect of adsorbent dose on uptake of water from miscella, a series of experiments were performed where zeolite 4A masses of 1.0 to 20.0 g were transferred into different 100-mL round-bottomed flasks containing 50 g of miscella (I) (1 wt% initial water content). The flasks were incubated at room temperature and continuously agitated at 125 cycles/min for 24 h to reach equilibrium. The supernatants were then analyzed for water content using Karl Fischer titration, and the percentages of water removal were calculated using Eq. 11.

\[
\text{Water Removal (\%)} = \frac{C_i - C_e}{C_i} \times 100
\]

where \( C_i \) and \( C_e \) are the initial and final concentrations (wt\%) of water in the miscella, respectively. The same procedure was repeated for miscella (II) containing 1.5 wt% water initially. These experiments were performed in duplicate.

Experiments to determine the effect of contact time were also performed at room temperature where 50 g of miscella (I) was transferred into a round-bottomed flask holding 7.5 g of adsorbent. The flask was agitated at a constant shaking rate of 125 cycles/min and aliquots of supernatant (0.2 - 0.5 ml) were withdrawn at different time intervals (from 1 to 240 min), and directly analyzed for water by Karl Fischer titration. The percentage of water removed from miscella (I) was determined based on Eq. 11. The same procedure was also repeated for miscella (II). All experiments for the effect of contact time on water removal were performed in triplicate.

Since the regeneration of adsorbents is necessary for maximum process profitability, the thermal regeneration of zeolite 4A was evaluated with respect to contact time. The first set of
regeneration experiments was carried out by contacting 7.5 g of fresh adsorbent with 50.0 g of miscella (I) using the batch procedure described above for determining the effect of contact time on water removal. After 240 min the adsorbents were recovered by sieving. The saturated adsorbents were dried in a fume hood at room temperature for 24 h and then regenerated by heating in nitrogen under approximately 30 mmHg vacuum (~ 4 kPa) for 6 h at 275 °C. The regenerated adsorbents were cooled in a desiccator before reuse. To determine the capacity of the regenerated adsorbents for water removal from miscella (I), the adsorption experiment was repeated four additional times. All of the regeneration experiments were performed in duplicate.

4.5.3 Fixed-bed Adsorption Experiments

Fixed-bed experiments were carried out at room temperature using glass columns (diameter, 2.5 cm; length, 30 cm) that were packed with fresh zeolite 4A (111.1 g). Miscella (I) or miscella (II) were continuously introduced at the bottom of the columns at flow rates (Q) of 1.6 and 2.0 mL/min, respectively. The column effluent samples were collected at selected time intervals and the column was operated until the concentration of water in the effluents exceeded 97 % of its initial concentration. The water contents of effluent samples were analyzed using a Karl Fischer titrimeter to construct the breakthrough curves in terms of the normalized concentration defined as the ratio of effluent water concentration to initial water concentration (C/Ci) as a function of time. The breakthrough time (tb) and exhaustion time (te) were defined as the time of adsorption when the effluent water concentration, C, from the column was about 10 and 97 % of the initial water concentration, Ci, respectively. As previously described in Fig. 2-11 and Table 2-6, the area above the breakthrough curves was obtained by integrating the adsorbed concentration (Cad = Ci − C; g/g) versus time (min) and used to find the breakthrough capacity (qb, g g⁻¹) and maximum capacity (qe, g g⁻¹) of the column using Equations 12 and 13, respectively.

\[ q_b = \frac{Q \rho}{W} \int_{t=0}^{t=t_b} (C_i - C) \, dt \]  \hspace{1cm} (12)

\[ q_e = \frac{Q \rho}{W} \int_{t=0}^{t=t_e} (C_i - C) \, dt \]  \hspace{1cm} (13)

where qb represents the adsorption capacity of the column (g g⁻¹) at breakthrough time (tb, min); qe represents the maximum adsorption capacity of the column (g g⁻¹) at exhaustion time (te, min); Q is the volumetric flow rate (mL min⁻¹); W is the mass (g) of the adsorbent; and \( \rho \) is the density
of miscellae at room temperature, measured as 0.904 and 0.902 g mL\(^{-1}\) for miscellae (I) and (II), respectively.

Total water removal percentage \((S_e)\) was calculated using Eq. 14 from the ratio of total adsorbed quantity of water \((i.e., q_e \times W)\) to the total amount of water delivered to the column until complete saturation was achieved. The water removal percentage at breakthrough \((S_b)\) was also determined using Eq. 15 from the ratio of adsorbed quantity of water at the point of breakthrough \((i.e., q_b \times W)\) to the amount of water delivered to the column until the appearance of breakthrough.

\[
\begin{align*}
\text{Total water removal } \% &= S_e = \frac{q_e \times W}{\rho Q C_{i_e}} \times 100 \\
\text{Water removal at breakthrough } \% &= S_b = \frac{q_b \times W}{\rho Q C_{i_b}} \times 100
\end{align*}
\]

4.5.4 Methylation of Dehydrated Oil-THF Miscella

Upon determination of the optimum conditions for the adsorption of water from miscellae in a batch system, yellow mustard emulsion (200 g) was made and then destabilized through three-stage treatment at 0.5:1 THF:oil weight ratio based on the procedure described in section 4.4.3. The resulting combined oil-THF-water miscella (100.0 g) was placed in a flask over zeolite 4A (15.0 g) and agitated for 40 min to reduce the water content to 0.3%. The dehydrated miscella was weighted after separation from the saturated adsorbents and analyzed for oil, water and THF contents. The dehydrated miscella was used as a feedstock for FAME production based on the pseudo-single-phase base-catalyzed transmethylation described by Mahajan et al. (2006). Reaction conditions were methanol:oil molar ratio of 14:1, THF/methanol volume ratio of 1.0, and sodium hydroxide concentration of 1.2 wt% (based on the oil).

The dried oil-THF miscella (83.5 g) consisting of 0.3% water, 30.1% THF, and 69.6% oil was transferred into a 200-mL two-necked round-bottomed flask equipped with a magnetic stirrer, a reflux condenser, and a calcium chloride guard tube. Sodium hydroxide [0.699 g, 1.2 wt% with respect to mustard oil] solution in methanol [7.4 mL, 3:1 methanol-to-oil molar ratio] was made separately in a 20-mL vial. The rest of the methanol (27.2 ml) was added to the flask followed by the addition of a small amount of anhydrous THF (6.4 ml) required to reach the desired
THF/methanol volume ratio. The catalyst solution was added immediately to the flask with continuous stirring and the timer was started. Samples (~5-ml aliquots) of the reaction mixture were taken at 2, 4, 8, 10, 12, 16, 20, 30, and 60 min and quenched by addition into 15-mL vials containing oxalic acid solutions (~1 mL) to neutralize the catalyst. The amount of oxalic acid solution was determined by calculating the molar equivalent of catalyst that is present in the 5 mL aliquots. The 5-mL samples were washed with 10 wt% brine solution (4 × 5 mL) and then with water (3 × 5 mL). This washing step removed free glycerol (FG), methanol, THF, sodium oxalate, and excess oxalic acid. The washed products were stored over sodium bicarbonate in the fume hood to evaporate all the solvent and finally analyzed for total monoglycerides (MG), diglycerides (DG), and triglycerides (TG) in accordance with (ASTM D 6584, 2010). The percentage of FAME was calculated based on the MG, DG, and TG contents by difference, and the total bound and unbound glycerol content (GT) in the fuel was determined by Eq. 16.

\[ GT = FG + (0.25)MG + (0.15)DG + (0.1)TG \]  

(16)

where FG, MG, DG, and TG are the weight percentages of the corresponding free glycerol and glycerides in the product. The same procedure was repeated using the above conditions, but the reaction stopped after exactly 10 min, and the resulting FAME was analyzed for acid number in accordance with (ASTM D 974, 2008).
CHAPTER 5

5 Results and Discussion

5.1 Aqueous and Enzymatic Extraction Processes for Simultaneous Production of Oil and Protein from Dehulled Yellow Mustard Flour

To design an optimal AEP or EAEP process for dehulled yellow mustard flour leading to high oil and protein extractabilities, the formation of unstable emulsion and high-quality skim fraction, carbohydrase treatments were initially evaluated through the individual and/or combined use of cellulase, hemicellulase, and pectinase (section 5.1.1). Secondly, successive extractions without and with enzymes were investigated in which alkaline extraction was considered part of the process (section 5.1.2). Finally, the effect of extraction parameters including pH, enzyme, and alkaline treatment conditions on the stability of the emulsions and the quality of the skim proteins were determined (section 5.2). The results of Sections 5.1 and 5.2 have been previously published in the journal "Food Research International" (Tabtabaei and Diosady, 2013).

5.1.1 Carbohydrase Treatments

Table 5-1 summarizes the effectiveness of various carbohydrases on the total oil and protein extraction yields, defined here as the oil and protein recovered from initial dehulled yellow mustard flour during carbohydrase-assisted aqueous extraction processes. The aqueous processing of dehulled yellow mustard flour without the assistance of carbohydrase (control) resulted in the oil and protein extraction yields of 55.5% and 60.7%, respectively. Celluclast 1.5L is a cellulase enzyme, Pectinex Ultra SP-L is a pectinase enzyme with limited cellulase and hemicellulytic activities, and Viscozyme L is a multi-enzyme complex containing a wide range of carbohydrases, including arabanase, cellulase, Q-glucanase, hemicellulase, xylanase, and pectinase (Novozymes, product data sheet). Although, the individual use of all of the three commercial carbohydrases represented significant improvement (P < 0.05) in the oil extraction yields, Celluclast 1.5L and Viscozyme L were more effective by increasing the oil extraction to around 67%, indicating that the breakdown of cellulose was certainly beneficial to the release of oil into the aqueous medium. While Celluclast 1.5L was able to facilitate the oil yield, it was a relatively inefficient way to improve protein extraction yield, compared with Viscozyme L and
Pectinex Ultra SP-L. Therefore, these commercial carboxydrases were combined to evaluate their collaborative effects on the oil and protein extractabilities. The outcome was encouraging as all the yields were further increased, especially with a combination of three enzymes in Run 6, where 76.3% of the oil in the flour was extracted along with 74.9% of the proteins, indicating more efficient hydrolysis of the cell wall polysaccharides by the combination of carboxydrases.

Table 5-1: Effects of different carboxydrases on total oil and protein extraction yields

<table>
<thead>
<tr>
<th>Run No.</th>
<th>Enzyme %w/w (w/w, enzyme/yellow mustard flour)</th>
<th>Oil Extraction Yield (wt%)</th>
<th>Protein Extraction Yield (wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Celluclast 1.5L</td>
<td>Pectinex Ultra SP-L</td>
<td>Viscozyme L</td>
</tr>
<tr>
<td>1 (control)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>1.5</td>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Means (n=2) sharing the same capital letters in each column are not significantly different.

Our oil extraction yields are slightly higher than the values reported by (Sengupta and Bhattacharyya, 1996) who achieved oil extraction yield of 61.2% during the aqueous carboxydrase treatment of pulverized brown mustard seeds (Brassica juncea) using 2% pectinase (Pectinex Ultra SPL) and 2% cellulase (Celluclast R). Their lower yield was likely due to the presence of hulls and mucilage that reduce the release of oil into the aqueous medium (Balke and Diosady, 2000).

The combination of 1 wt% Celluclast 1.5L, 1 wt% Viscozyme L, and 1 wt% Pectinex Ultra SP-L was eventually selected as the best enzymatic treatment for the subsequent successive extractions.

**5.1.2 Successive AEP/EAEP Extractions from Dehulled Yellow Mustard Flour**

Table 5-2 summarizes the oil, protein, and solids distribution in the emulsion, skim, and solid residual fractions from two-stage successive AEP/EAEP processes of dehulled yellow mustard flour including AEP (i), EAEP (ii), and AEP (iii).
From Table 5-2, it is clear that the AEP (i) had slightly higher oil extraction yield than the AEP (iii) (17.5 vs. 20.6% oil remaining in the solid residue, respectively). The EAEP (ii) had the highest oil extraction yield of 86.5% (13.5% oil remaining in the solid residue), around 10.2% higher than the oil extraction yield obtained by the equivalent carbohyrdrase treatment (Run 6 in Table 5-1). Clearly the successive alkaline extraction enhanced oil recovery. The high oil extraction yield obtained during the EAEP (ii) compared to AEP (i) and AEP (iii) is also shown by the higher proportion of oil extracted to the emulsion fraction (78.7 vs. 71.4 and 64.6%, respectively), and less oil dispersed in the skim fraction (10.0 vs. 10.3 and 14.8%, respectively).

As shown in Table 5-2, the AEP (iii) and AEP (i) had similar protein extraction yields of just under 90% and EAEP (ii) had the highest protein extraction yield of 91.8%, some 17% higher than the protein extraction yield obtained by the equivalent carbohyrdrase treatment (Run 6 in Table 5-1). Contacting the partially extracted solid residue remaining after the first-stage carbohyrdrase treatment with alkaline extraction media at pH 11 increased protein and oil extraction yields significantly. This improvement is consistent with results reported in the literature (De Moura et al., 2008, Rosenthal et al., 1996, Rosenthal et al., 1998), as higher oil extraction generally occurs at basic pH due to enhanced protein solubility.

However, this successive extraction (EAEP (ii)) was not superior to AEP (iii) in terms of protein distribution between emulsion and skim fractions, as only 67.2% of the total protein was extracted to the skim fraction while 20.3% was recovered with the emulsion fraction. Although, the first-stage carbohyrdrase treatment during the EAEP (ii) improved the release of protein into the aqueous medium, it was not able to extract proteins attached to the emulsion droplets, thereby forming a viscous emulsion with high protein content. This undesirable protein distribution and formation of a viscous emulsion was also observed with the AEP (i). Therefore, alkaline extraction is vital in the production of a fluid emulsion containing little protein, and a skim fraction containing most of the extracted protein; however, as indicated in Table 5-2, alkaline extraction increased the disruption of oil droplets in the skim fraction by around 5% which would result in reduced purity of proteins recoverable from the skim fraction by isoelectric precipitation (Campbell et al., 2011).
Table 5-2: The distribution of oil, protein, and solids (% of total in original flour) between the various fractions produced during successive extractions including AEP (i), EAEP (ii), and AEP (iii).

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Oil (wt% of total)</th>
<th>Protein (wt% of total)</th>
<th>Solids (wt% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AEP (i)</td>
<td>EAEP (ii)</td>
<td>AEP (iii)</td>
</tr>
<tr>
<td>Emulsion</td>
<td>71.4 ± 0.3^B</td>
<td>78.7 ± 3.2^A</td>
<td>64.6 ± 1.8^C</td>
</tr>
<tr>
<td>Skim</td>
<td>10.3 ± 0.6^C</td>
<td>10.0 ± 3.0^C</td>
<td>14.8 ± 2.6^B</td>
</tr>
<tr>
<td>Solid Residue</td>
<td>17.5 ± 1.6^B</td>
<td>13.5 ± 1.0^C</td>
<td>20.6 ± 0.8^A</td>
</tr>
</tbody>
</table>

Means (n=3) for each fraction (Tables 5-2 and 5-3) sharing the same capital letters are not significantly different (P<0.05).

Total solids extraction yields of 85.4%, 78%, and 75.4% were achieved when using EAEP (ii), AEP (i), and AEP (iii), respectively. Total solid contents were significantly different (P < 0.05) in the skim and emulsion fractions. The higher amounts of solids in the skim fraction, produced during AEP (iii), was consistent with the higher protein extraction yield associated with alkaline extraction as the main step.

Although the EAEP (ii) had the highest oil and protein extraction yields, and resulted in desirable oil distribution between emulsion and skim fractions, it produced a skim fraction containing only 67.2% of the total protein, and a viscous emulsion with high protein content. To overcome this problem, we tested three-stage successive extraction processes (Fig. 4-2) in which the third stage was performed by extracting the emulsions at pH 11 to transfer the proteins attached to the emulsion droplets to the skim fraction, thus improving protein distribution and producing fluid emulsions with low protein content. Table 5-3 summarizes the oil, protein, and solids distribution in the emulsion, skim, and solid residual fractions from three-stage AEP/EAEP extractions.

Table 5-3: The distribution of oil, protein, and solids (% of total in original flour) between the various fractions produced during successive extractions including AEP (iv) and EAEP (v).

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Oil (wt% of total)</th>
<th>Protein (wt% of total)</th>
<th>Solids (wt% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AEP (iv)</td>
<td>EAEP (v)</td>
<td>AEP (iv)</td>
</tr>
<tr>
<td>Emulsion</td>
<td>54.9 ± 1.9^D</td>
<td>60.5 ± 3.7^C</td>
<td>0.4 ± 0.1^C</td>
</tr>
<tr>
<td>Skim</td>
<td>25.4 ± 0.4^A</td>
<td>26.0 ± 1.2^A</td>
<td>79.5 ± 2.6^B</td>
</tr>
<tr>
<td>Solid Residue</td>
<td>18.1 ± 0.7^B</td>
<td>13.7 ± 0.8^C</td>
<td>11.4 ± 0.7^A</td>
</tr>
</tbody>
</table>

Means (n=3) for each fraction (Tables 5-2 and 5-3) sharing the same capital letters are not significantly different (P < 0.05).
Fortunately, the addition of the third stage increased the extraction of protein to the skim fraction from 61.3% and 67.2% to 79.5% and 83.4%, respectively, and resulted in the formation of very fluid emulsions containing < 1% of the total protein. However, the recovery of the proteins attached to the emulsion droplets was not possible without increasing the dispersion of oil droplets into the skim fraction to 26% (Table 5-3), some 11% more than that obtained in AEP (iii). Accordingly, the two-stage alkaline extraction (AEP (iii)) was the optimal process tested in terms of its desirable oil and protein distribution.

Figure 5-1 represents the composition of the solid residue, skim, and emulsion fractions produced during this optimal two-stage alkaline extraction process (AEP (iii)); it also summarizes the oil, protein, and solid distribution between these fractions based on the results of Table 5-2. The skim represented 79.8±0.3% of the total weight of the three phases, the solid residue 16.1±0.3%, and the emulsion 4.1±0.1%. Around 64.6% of the oil in the original flour was extracted into the emulsion phase, 14.8% extracted into the skim, and 20.6% remained in the solid residue. Over 83% of the total protein present in the flour was extracted into the skim and 3.2% to the emulsion, while 11.1% remained in the solid residue.

Figure 5-1: The composition of the various fractions produced during AEP (iii) and the distribution of oil, protein, and solid (% of total in original flour) between them (all values represent the means (n=3) ± standard deviation)
5.2 Emulsions Characterization

Emulsions generated by successive AEP/EAEP processes were analyzed for oil, moisture, protein, and phospholipids content (Table 5-4).

The alkaline emulsion produced through AEP (iii) consisted of less water (39.8%), protein (3.2%), and phospholipids (3.3%) than the acidic emulsions from AEP (i) (58.7% water, 8.3% protein, and 5.2% phospholipids) and EAEP (ii) (52.2% water, 9.1% protein, and 5.9% phospholipids). Since more of the yellow mustard seed proteins is soluble under alkaline conditions than at the native pH (4.5-5.0) (Xu et al., 2003), the emulsion recovered at high pH through AEP (iii) had lower protein content due to the improved protein recovery to the skim fraction (Table 5-2). The lower phospholipids content of this alkaline emulsion can be attributed to the extraction of some of the phospholipids surrounding the oil droplets into the skim fraction along with the proteins due to the strong interaction between proteins and phospholipids (Yao and Jung, 2010). This might also explain the higher crude oil recovery (14.8%) to the skim fraction in AEP (iii) compared to AEP (i) and EAEP (ii) (10.3 and 10.0%, respectively). Yao and Jung (2010) reported over 50% phospholipid recovery to the protein-rich skim fraction produced during AEP of soybean flour.

While alkaline emulsion from AEP (iii) exhibited lower amounts of natural emulsifiers (proteins and phospholipids) with approximately 92% of the dry emulsion being oil, dry acidic (native pH) emulsions from AEP (i) and EAEP (ii) had approximately 74% oil, indicating higher amounts of natural emulsifiers in the emulsions.

The mean oil droplet diameter (D_{4,3}) of the emulsions recovered from successive AEP/EAEP processes are presented in Table 5-5 and Fig. 5-2. The oil droplet size analysis in Fig. 5-2 showed the bimodal size distribution for the emulsions recovered from two-stage processes of AEP (iii), AEP (i), and EAEP (ii) in which the largest proportion of the droplets were of a small size. The mean oil droplet diameter of the alkaline emulsion was 38.6 μm, significantly (P < 0.05) higher than the mean oil droplet diameter of the acidic emulsions (11.0 and 15.2 μm, respectively), indicating that AEP (iii) was more effective in promoting the coalescence of original oil bodies in the flour. Yellow mustard seed oil bodies have the average diameter of 0.73 μm (Tzen et al., 1993) as described in section 2.2.1.
Table 5-4: The composition of the yellow mustard emulsions formed during successive aqueous extraction and enzyme-assisted aqueous extraction processes (AEP/EAEP)

<table>
<thead>
<tr>
<th>Components</th>
<th>Emulsion AEP (i)(^a)</th>
<th>Emulsion EAEP (ii)(^a)</th>
<th>Emulsion AEP (iii)(^b)</th>
<th>Extracted Emulsion AEP (iv)(^a)</th>
<th>Extracted Emulsion EAEP (v)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil (wt%)</td>
<td>30.0 ± 2.5(^D)</td>
<td>35.3 ± 2.1(^C)</td>
<td>55.5 ± 4.6(^B)</td>
<td>80.0 ± 3.4(^A)</td>
<td>80.1 ± 2.2(^A)</td>
</tr>
<tr>
<td>Water (wt%)</td>
<td>58.7 ± 2.2(^A)</td>
<td>52.2 ± 1.0(^B)</td>
<td>39.8 ± 3.9(^C)</td>
<td>20.6 ± 3.3(^D)</td>
<td>20.8 ± 3.8(^D)</td>
</tr>
<tr>
<td>Protein (wt%)</td>
<td>8.3 ± 0.1(^B)</td>
<td>9.1 ± 0.8(^A)</td>
<td>3.2 ± 0.4(^C)</td>
<td>0.6 ± 0.1(^D)</td>
<td>0.7 ± 0.1(^D)</td>
</tr>
<tr>
<td>Phospholipids (wt%)</td>
<td>5.2 ± 0.1(^B)</td>
<td>5.9 ± 0.5(^A)</td>
<td>3.3 ± 0.3(^C)</td>
<td>0.6 ± 0.0(^D)</td>
<td>0.8 ± 0.0(^D)</td>
</tr>
</tbody>
</table>

All measurements were performed in triplicate.

Means sharing the same capital letters in each row are not significantly different (P < 0.05).

\(^a\) All values represent the composition means (n=3) ± standard deviation.

\(^b\) For the emulsion of AEP (iii), the values of oil, water, and protein represent the means of 33 determinations (n=33)± SD. The value of phospholipids represent the mean of three determinations (n=3) ± SD.

\(^c\) Crude oil content.

The emulsions recovered from three-stage processes of AEP (iv) and EAEP (v) represented the highest oil content of ~ 80%, and the lowest protein and phospholipids content (< 1%) compared to the emulsions from two-stage processes (Table 5-4). Therefore, the alkaline extraction of the acidic emulsions produced through AEP (i) and EAEP (ii) purified the emulsions to approximately 100% oil on a dry basis by releasing most of the proteins and phospholipids attached to the oil droplets to the skim fractions. However, this purification increased the dispersion of oil droplets to the skim (from ~10% to ~26%), and reduced the oil recoveries to the emulsion fractions (from ~71 to 55% and 78 to 60%, respectively) (Tables 5-2 and 5-3). The purification of these acidic emulsions using alkaline solutions promoted the coalescence of oil droplets, thereby reducing the interfacial curvature, resulting in the formation of unstable emulsions having large mean droplet diameters (D\(_{4,3}\)) of 73.3 and 96.4 μm, respectively (Table 5-5). Oil droplet size distributions of these emulsions were still bimodal, but a significant increase in the proportion of the large oil droplets was observed (Fig. 5-2).
Table 5-5: Mean droplet diameters ($D_{4,3}$) along with peaks locations and surface protein concentrations ($\Gamma$) of the emulsions formed during successive aqueous extraction and enzyme-assisted aqueous extraction processes (AEP/EAEP)

<table>
<thead>
<tr>
<th>Emulsion Characterization</th>
<th>Emulsion AEP (i)</th>
<th>Emulsion EAEP (ii)</th>
<th>Emulsion AEP (iii)</th>
<th>Extracted Emulsion AEP (iv)</th>
<th>Extracted Emulsion EAEP (v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_{4,3}$ ($\mu$m)$^a$</td>
<td>11.0 ± 0.4$^D$</td>
<td>15.2 ± 0.1$^D$</td>
<td>38.6 ± 4.3$^C$</td>
<td>73.3 ± 0.2$^B$</td>
<td>96.4 ± 3.5$^A$</td>
</tr>
<tr>
<td>1st Peak Location ($\mu$m)</td>
<td>0.4</td>
<td>0.4</td>
<td>3.1</td>
<td>1.9</td>
<td>3.6</td>
</tr>
<tr>
<td>2nd Peak Location ($\mu$m)</td>
<td>12.2</td>
<td>9.0</td>
<td>258.9</td>
<td>190.8</td>
<td>222.3</td>
</tr>
<tr>
<td>$\Gamma$ (mg/m$^2$)</td>
<td>22.03</td>
<td>19.38</td>
<td>7.64</td>
<td>1.01</td>
<td>1.44</td>
</tr>
</tbody>
</table>

$^a$ All values represent the means (n=2) ± SD. Means sharing the same capital letters in each row are not significantly different (P < 0.05).

Figure 5-2: Oil droplet size analysis of the emulsions formed during successive aqueous extraction and enzyme-assisted aqueous extraction processes (AEP/EAEP)
The SDS-PAGE gel of the surface proteins adsorbed at the interface of the emulsions produced during two-stage processes of AEP (iii), AEP (i), and EAEP (ii) (Fig. 5-3) revealed the presence of oleosin proteins (low molecular weight alkaline proteins, ranging from 15 to 26 kD) and high molecular weight proteins. Oleosins are amphipathic proteins consisting of amphipathic N- and C-terminal domains, and a long central hydrophobic stretch of 72 amino acids (Tzen and Huang, 1992). The surface protein concentration (Γ) of these three emulsions (Table 5-5) was 7.64, 22.03, and 19.38 mg/m², respectively, indicating stable multilayer emulsions since their surface protein concentration was much higher than 1-2 mg/m² reported for a protein-stabilized emulsion in which a protein monolayer adsorbed at the oil-water interface (Nikiforidis and Kiosseoglou, 2009). Therefore, during aqueous processing of yellow mustard, the oil bodies consisting of a matrix of triacylglycerols surrounded by phospholipids and oleosins coalesced and formed larger oil droplets with diameters ranging from 0.06 to 480 μm, and were further stabilized by extracted storage proteins. The formation of stable multilayer emulsions was also reported during the aqueous and enzyme-assisted aqueous extraction of soybean flour (Chabrand and Glatz, 2009, Morales Chabrand et al., 2008).

![Figure 5-3: SDS-PAGE profile of proteins adsorbed at the interface of the emulsions produced during successive aqueous extraction and enzyme-assisted aqueous extraction processes (AEP/EAEP). Lane 1, emulsion for AEP (i); 2, emulsion for EAEP (ii); 3, emulsion for AEP (iii); 4, M.W. marker (10-250 kDa range); 5, alkaline extracted emulsion for AEP (iv); 6, alkaline extracted emulsion for EAEP (v).](image-url)
SDS-PAGE results showed that the proteins adsorbed at the oil-water interface of the emulsions ranged in size from ~10 to ~100 kDa (Fig. 5-3, lanes 1, 2, 3, 5, and 6). Tzen et al. (1993) studied the SDS-PAGE of proteins of oil bodies isolated from mustard seeds (Brassica juncea L.) and found two distinct bands ~20 kDa for oleosins. Therefore, as it is shown in Fig. 5-3, the two major bands that correspond to protein constituents of molecular weight of 20 kDa could be attributed to oleosin proteins, and the rest of the bands should probably belong to the yellow mustard storage proteins extracted from the flour under different conditions applied during successive AEP/EAEP. The SDS-PAGE of the surface proteins from the acidic emulsions (Fig 5-3, lanes 1 and 2) revealed the presence of two bands between 10 and 15 kDa, three bands between 15 and 27 kDa with the last two (~20 kDa) attributed to oleosins, four bands between 27 and 35 kDa, two distinct bands around 45 kDa, one band around 65 kDa, and one band around 100 kDa. However, the SDS-PAGE of the surface proteins from the alkaline emulsion (lane 3) showed the presence of fewer high molecular weight proteins compared to the surface proteins identified for the acidic emulsions. This indicates that alkaline extraction (AEP (iii)) dissolved most of the non-oleosin proteins extracted from the flour, and released the emulsion stabilized by few non-oleosin proteins compared to the acidic emulsions from AEP (i) and EAEP (ii).

The emulsions recovered from three-stage AEP (iv) and EAEP (v) processes contained < 1% protein since the alkaline extraction of the acidic emulsions dissolved most of the proteins adsorbed at the oil-water interface, resulting in the formation of unstable emulsions with larger oil droplets and lower surface protein concentration of 1.01 and 1.44 mg/m², respectively. This did not change the SDS-PAGE profile of proteins adsorbed at the oil-water interface since the bands identified in acidic emulsions (Fig. 5-3, lanes 1 and 2) were also observed for the alkaline-extracted emulsions (Fig. 5-3, lanes 5 and 6).

5.2.1 Effect of pH Adjustment on Emulsions Destabilization

The effect of pH adjustment on emulsion destabilization is presented in Fig. 5-4. I adjusted the pH of the emulsions to the isoelectric point of yellow mustard proteins to minimize the electrostatic repulsions between the oil droplets, and thus recover free oil. The pH adjustment to the isoelectric point of soy proteins (pH 4.5) of the emulsions produced during enzyme-assisted aqueous extraction of extruded soybean flakes and soybean flour recovered up to 100% free oil.
(Chabrand and Glatz, 2009, Jung et al., 2009, Wu et al., 2009). Unfortunately, yellow mustard has a wide variety of proteins with a broad spectrum of isoelectric points (Xu et al., 2003) that results in a complex relationship between recovery of free oil and pH.

Adjustment the pH of the native emulsion (AEP (i)) to 2 and 3 slightly increased the free oil recovery to around 17%, while adjusting to pH 4 to 6 recovered around 30% of the oil in the emulsion, indicating that several proteins have isoelectric points in this region. Further increases in the pH decreased the free oil recovery until no further oil was released above pH 8. The pH adjustment for EAEP (ii) was more dramatic, as adjusting the pH to 3 recovered over 51% of the oil in the emulsion. Further increases in the pH slightly decreased the free oil recovery until no free oil was observed above pH 8.

Figure 5-4: Effects of pH on free oil recovery from emulsions produced during successive aqueous extraction and enzyme-assisted aqueous extraction processes (AEP/EAEP) of dehulled yellow mustard flour. Results are expressed as means of two independently prepared samples with error bars representing standard deviations of the means. If not visible, it means the STD was < 0.5 wt%.

Adjusting the pH of the alkaline emulsion (AEP (iii)) did not improve the free oil recovery. Unfortunately, while the alkaline emulsion had a desirable composition, its destabilization by pH adjustment was inefficient. This can be attributed to the hydrophobic nature of the proteins
adsorbed at the oil-water interface of the oil droplets. As shown in Fig. 5-3 (lane 3), few high molecular weight proteins were adsorbed at the interface since most of the non-oleosin proteins were extracted to the skim fraction, resulting in a hydrophobic multilayer film of proteins surrounding the oil droplets. The hydrophobic emulsifiers around the oil droplets resulted in a very stable emulsion, as the recovery of free oil must not only overcome the charge repulsion, but also to desorb protein molecules from the interface.

Adjusting the pH to 4.5 of the alkaline-extracted emulsions from three-stage AEP (iv) and EAEP (v) processes recovered 96.5 and 91.1% of the oil, respectively, implying less stability of these emulsions compared to their equivalent acidic emulsions.

5.2.2 Effect of Enzymatic Treatments on Emulsions Destabilization

Enzymatic demulsification treatments were performed using different proteases and phospholipases in order to investigate the effect of enzymatic hydrolysis of interfacial proteins and phospholipids on free oil extraction. The free oil yield from the control emulsions treated without enzyme addition was used as a baseline to evaluate the efficiency of the selected proteases and phospholipases in releasing oil.

The protease treatments were performed using 2.5% Protex 6L, 2.5% Alcalase 2.4L, 2.5% Protex 51FP, and 2.5% Protex 89L for 3 h at their optimum pH and temperature. The results are summarized in Table 5-6. The free oil recoveries obtained by treating the emulsions with Protex 51FP and Protex 89L were not presented in Table 5-6, as they were similar to those obtained with Alcalase 2.4L. Phospholipase treatments were performed using 2.5% Lipomode 699 L and 2.5% G-ZYME G999 for 3 h at their optimum pH and temperature, and the results are presented in Table 5-7.

Among the proteolytic enzymes tested for destabilizing the emulsions produced during two-stage processes (AEP (i), EAEP (ii), and AEP(iii)), the best oil extraction yields were obtained with endoprotease Protex 6L, with over 91% free oil recovery (Table 5-6). When this enzyme was replaced with Alcalase 2.4L, Protex 51FP, and Protex 89L, free oil extraction yields decreased significantly to less than 65%. This indicates that Protex 6L was more efficient in totally hydrolyzing the proteins surrounding the oil droplets, thereby decreasing the molecular size of
the polypeptides, limiting the formation of rigid barriers around oil droplets. Protex 6L was also reported as an efficient enzyme in demulsification of the emulsions produced during enzyme-assisted aqueous extraction of extruded soybean flakes (Campbell et al., 2011, De Moura et al., 2008) and soybean flour (Chabrand and Glatz, 2009).

The effectiveness of protease demulsification was related to the concentration of proteins in the emulsions. The highest free oil extraction yield was achieved for alkaline emulsion (AEP (iii)) where less protein was available for hydrolysis (Tables 5-4 and 5-5). The alkaline-extracted emulsions produced by three-stage AEP/EAEP processes (AEP (iv) and EAEP (v)) were unstable enough without the addition of proteases. All of the tested proteases were found effective in totally destabilizing these emulsions at a concentration of 2.5 wt% (Table 5-6).

Table 5-6: Effects of protease treatments (Protex 6L and Alcalase 2.4L) on free oil recovery from the emulsions produced during successive aqueous extraction and enzyme-assisted aqueous extraction processes (AEP/EAEP)

<table>
<thead>
<tr>
<th>Emulsions</th>
<th>Protex 6L (2.5 wt%)</th>
<th>Alcalase 2.4L (2.5 wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Free Oil Recovery (wt%)</td>
</tr>
<tr>
<td>Emulsion Two-Stage AEP (i)</td>
<td>4.5</td>
<td>92.7 ± 2.6B</td>
</tr>
<tr>
<td>Emulsion Two-Stage EAEP (ii)</td>
<td>4.1</td>
<td>91.3 ± 2.4B</td>
</tr>
<tr>
<td>Emulsion Two-Stage AEP (iii)</td>
<td>4.8</td>
<td>94.7 ± 4.2AB</td>
</tr>
<tr>
<td>Extracted Emulsion Three-Stage AEP (iv)</td>
<td>76.1</td>
<td>97.8 ± 0.9A</td>
</tr>
<tr>
<td>Extracted Emulsion Three-Stage EAEP (v)</td>
<td>66.5</td>
<td>94.6 ± 0.1AB</td>
</tr>
</tbody>
</table>

Enzymatic demulsifications were performed using 2.5wt% enzymes as of the emulsion weight at optimum temperature and pH for 3h. All values represent the means (n=3) ± SD. Means sharing the same capital letters in each column are not significantly different (P < 0.05).

The Lipomode treatment at pH 7.5 and 40°C not only decreased free oil recoveries from all of the emulsions (Table 5-7), but also increased the stability of the emulsions. This surprising observation can be probably attributed to the role of Lipomode (Phospholipase A2) in cleaving the ester bond at the sn-2 position in the acylglycerophosphatide, to produce lysophospholipids that are reported to be more effective emulsifiers than the intact diacylglycerophosphatide (Chabrand and Glatz, 2009).
Table 5-7: Effects of phospholipase treatments (Lipomode 699 L and G-ZYME G999) on free oil recovery from the emulsions produced during successive aqueous extraction and enzyme-assisted aqueous extraction processes (AEP/EAEP)

<table>
<thead>
<tr>
<th>Emulsions</th>
<th>Lipomode 699 L (2.5 wt%)</th>
<th>G-ZYME G999 (2.5 wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Free Oil Recovery (wt%)</td>
</tr>
<tr>
<td>Emulsion Two-Stage AEP (i)</td>
<td>8.0</td>
<td>1.9&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
<tr>
<td>Emulsion Two-Stage EAEP (ii)</td>
<td>3.4</td>
<td>1.3 ± 0.0&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
<tr>
<td>Emulsion Two-Stage AEP (iii)</td>
<td>1.2</td>
<td>1.5 ± 0.4&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
<tr>
<td>Extracted Emulsion Three-Stage AEP (iv)</td>
<td>86.0</td>
<td>86.2 ± 1.0&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>Extracted Emulsion Three-Stage EAEP (v)</td>
<td>72.5</td>
<td>72.9 ± 4.9&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Enzymatic demulsifications were performed using 2.5wt% enzymes as of the emulsion weight at optimum temperature and pH for 3h. All values represent the means (n=3) ± SD. Means sharing the same capital letters in each column are not significantly different (P < 0.05).

G-ZYME (lysophospholipase A1) is the enzyme that hydrolyzes the ester bond at the sn-1 position only if position sn-2 is absent (Genecore international, product data sheet). The G-ZYME treatment at pH 4.5 and 60°C did slightly improve the oil extraction yields from the emulsions produced through two-stage processes, but it did not improve the oil extraction yields from the alkaline-extracted emulsions formed during three-stage AEP/EAEP processes. While Chabrand and Glatz (2009) recovered 100% of the oil in the emulsion produced during enzyme-assisted aqueous extraction of soybean flour by simultaneously acidification to the isoelectric point of soy proteins (pH 4.5) and addition of G-ZYME, this was not the case in this study. The reason might be either attributed to the presence of small amounts of lysophospholipids to affect the stability of the emulsions or to the presence of the multilayer protein interface that would reduce the access of G-ZYME to the lysophospholipids surrounding the oil droplets in the emulsions. However, since G-ZYME treatment was not effective in increasing oil extraction yields from the unstable alkaline-extracted emulsions containing much lower surface protein concentrations (Table 5-5), the first hypothesis is more likely.
5.3 Destabilization of Yellow Mustard Emulsions with Organic Solvents

The results indicated that the enzymatic aqueous extraction of dehulled yellow mustard flour did not offer sufficient improvement in usable protein recovery to warrant the extra effort and cost. Therefore, the two-stage alkaline extraction of dehulled yellow mustard flour, (iii) AEP: pH 11, was further investigated as a potentially viable process. The destabilization of its emulsion using DMF, THF, and dioxane was studied (5.3.1 - 5.3.5). In the following sections "yellow mustard emulsion" refers to the emulsion produced during the two-stage AEP: pH 11. The results of Section 5.3 have been previously published in the Journal of the American Oil Chemists' Society (Tabtabaei and Diosady, 2012, Tabtabaei et al., 2013, Tabtabaei et al., 2014a).

5.3.1 Single-Stage Treatment of Yellow Mustard Emulsion with DMF

DMF is often referred to as the “universal solvent” as it dissolves both polar and non-polar compounds. The purpose of using DMF was to solubilize the emulsion and recover all of the oil and water from the emulsion in a single-phase oil-water-DMF miscella, which could be dried later to remove water. DMF:oil weight ratios of 2.5:1 to 30:1 were tested. Table 5-8 presents the oil extracted from yellow mustard emulsion to miscella. The compositions of the miscella and emulsion residue phases are presented in Figs. 5-5a and b.

Table 5-8: Oil extracted from the emulsion to the miscella and the mass yields at different dimethylformamide:o oil weight ratios

<table>
<thead>
<tr>
<th>DMF:Oil Weight Ratios</th>
<th>2.5:1</th>
<th>5:1</th>
<th>7.5:1</th>
<th>10:1</th>
<th>12.5:1</th>
<th>15:1</th>
<th>17.5:1</th>
<th>20:1</th>
<th>25:1</th>
<th>30:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil Extracted to Miscella (wt%)</td>
<td>7±3D</td>
<td>11±5D</td>
<td>23±4C</td>
<td>28±9BC</td>
<td>32±7ABC</td>
<td>34±7AB</td>
<td>35±3AB</td>
<td>35±3AB</td>
<td>39±3A</td>
<td>38±3A</td>
</tr>
<tr>
<td>Mass Yields a</td>
<td>1.1±0.2</td>
<td>3±0.2</td>
<td>6±0.1</td>
<td>8.1±0.4</td>
<td>9.7±0.8</td>
<td>11.6±1.4</td>
<td>12.9±1.3</td>
<td>14.7±1.6</td>
<td>16.9±1.8</td>
<td>23±4.7</td>
</tr>
</tbody>
</table>

Means sharing the same capital letters are not significantly different (P<0.05).

Results are expressed as means of three independently prepared samples ± standard deviations of the means.

a Mass yield values represent the weight of the miscella phases to the emulsion residue phases produced after centrifugation.
Figure 5-5: **a** Composition of the miscellae (wt%) at different dimethylformamide:oil weight ratios. **b** Composition of the emulsion residues (wt%) at different dimethylformamide:oil weight ratios. Compositions are expressed as means of three independently prepared samples with error bars representing standard deviations of the means. If not visible, it means the SD was < 0.5 wt%.

For weight ratios below 2.5:1, no separation of oil from the emulsion was observed since the amount of added DMF was not enough to solubilize the emulsion. The oil extraction yield increased from 7% at 2.5:1 to ~38% at 25:1 and 30:1 weight ratios. Increasing the amount of DMF from 12.5:1 to 30:1 did not significantly (P<0.05) improve the oil extraction yields; therefore, weight ratios higher than 30:1 were not examined because the potential increase in oil recovery would not be significant enough to justify the use of larger quantities of DMF (Table 5-8).
The low efficiency of oil extraction from the emulsion to miscella phases is associated with the solubility of water and oil in DMF. The high affinity of DMF towards water and its low affinity towards oil represent a major disadvantage. Low concentrations of the oil in the miscella phases at all weight ratios (Fig. 5-5a) reflects the poor solubility of the oil in DMF. As can be seen in Fig. 5-5b, the amount of DMF at 2.5:1 and 5:1 weight ratios was not enough to move all the water to the miscellae since the water content in the emulsion residue at these two weight ratios were 14% and 3%, respectively. The small amount of the DMF added to the system was bound with water and there was not enough solvent in the system to attack the oil, thereby depressing the oil extraction yields to 7% and 11%, respectively (Table 5-8).

At DMF:oil weight ratios of 7.5:1, 10:1, and 12.5:1, no water was observed in the emulsion residues as all the water moved to the miscella phases. Therefore, the amount of the solvent in the system was enough to dissolve all the water, and this amount of solvent was also sufficient to dissolve some of the oil in the emulsion. Therefore, at these weight ratios, the oil extraction yields increased significantly to 23%, 28%, and 32%, respectively (Table 5-8). The maximum solubility of the oil in DMF was achieved at 12.5:1 weight ratio. At DMF:oil weight ratios greater than 12.5:1, the oil extraction yields did not improve significantly due to the limited solubility of the oil in DMF; this limitation can also be seen in the composition of the miscella phases in which oil concentration started to decrease from 2.5% to 1.3% by increasing the ratio of DMF from 12.5:1 to 30:1 (Fig. 5-5a).

The oil/water/DMF ternary phase diagram was developed to explain the low oil extraction yields from the emulsion to the miscellae. Figure 5-6 shows the phase diagram including the mutual solubility curves. The points on the right solubility curve have more oil in comparison with the amount of DMF; conversely, the points on the left solubility curve have more DMF. According to this diagram, the shaded area in which oil, water, and DMF exist in a single phase is very small. However, a larger area of solubility exists at high DMF content. The intersection of the left solubility curve and the horizontal axis (point K, composed of 94.3wt% DMF and 5.7 wt% oil) shows the maximum solubility of oil in DMF when no water is present in the system, and the solubility curve represents the decreasing solubility of the oil in DMF in the presence of water.
Figure 5-6: Experimental ternary phase diagram of dimethylformamide (A) + Oil (B) + Water (C) at room temperature: ●, overall composition for the tie lines; ■, tie line values; ▬■▬, Solubility curves; △, composition of the miscella phases produced during emulsion destabilization. The overall compositions of the tie lines (bulk solutions compositions) are presented in Table 4-2.

The creamy emulsion residue phase floating on top of the miscella phase (Fig. 4-3) at all of the discussed DMF:oil weight ratios was mostly composed of oil (49±5 wt%) and DMF (47±8 wt%), with only 4 wt% protein (Fig. 5-5b). Therefore, the limited solubility of the oil in DMF, especially in the presence of water resulted in partial destabilization of the emulsion and released an oil-rich emulsion residue phase in the form of thin creamy layer on top of the miscella phase.

5.3.2 Single-Stage Treatment of Yellow Mustard Emulsion with THF

THF is a volatile solvent (bp, 66°C) that can dissolve a wide range of non-polar and polar compounds. In this regard, THF:oil weight ratios of 1:1 to 5:1 were used to recover oil from the
emulsion. Table 5-9 presents the results for oil extraction from yellow mustard emulsion to the miscella and polar phases formed after centrifugation.

Table 5-9: Oil extracted from yellow mustard emulsion to the miscella and polar phases at various tetrahydrofuran:oil weight ratios

<table>
<thead>
<tr>
<th>THF:Oil Weight Ratios</th>
<th>1:1</th>
<th>1.5:1</th>
<th>2:1</th>
<th>2.5:1</th>
<th>3:1</th>
<th>4:1</th>
<th>5:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil Extracted to: (wt%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Miscella Phase</td>
<td>87.0±1.6&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>87.0±2.0&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>83.2±5.2&lt;sup&gt;C&lt;/sup&gt;</td>
<td>88.7±4.4&lt;sup&gt;D&lt;/sup&gt;</td>
<td>94.7±1.9&lt;sup&gt;A&lt;/sup&gt;</td>
<td>97.2±0.9&lt;sup&gt;A&lt;/sup&gt;</td>
<td>98.3±1.9&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>Polar Phase</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>5.1±0.7&lt;sup&gt;B&lt;/sup&gt;</td>
<td>3.5±0.6&lt;sup&gt;B&lt;/sup&gt;</td>
<td>3.6±0.7&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means sharing the same capital letters in each row are not significantly different (P<0.05).
Results are expressed as means of three independently prepared samples ± standard deviations of the means.

The oil extraction yield to the miscella phases using THF was 87.0% at both 1:1 and 1.5:1 weight ratios; it decreased to 83.2% at 2:1, before increasing to 98.3% at 5:1 weight ratio (Table 5-9). The oil extraction to miscella phases were significantly different (P<0.05) when using high weight ratios of 3:1, 4:1, and 5:1 than when using lower weight ratios of 1:1 to 2.5:1. Although the quantities of oil extracted to the miscellae were not significantly different (P<0.05) at lower weight ratios (i.e. 1:1 to 2.5:1), the unexpected yield at 2:1 THF:oil weight ratio is more complicated to explain. At low weight ratios the polar phase observed after centrifugation was tied up in the emulsion residue phase at the bottom of the centrifuge tubes, which made the separation and analysis of that phase very difficult. Therefore, the polar phases at higher weight ratios (i.e. 3:1 to 5:1) were analyzed for their composition and oil recoveries. As can be seen in Table 5-9, 5.1% of the oil was extracted to the polar phase at 3:1. Oil losses were smaller at 4:1 and 5:1 weight ratios (3.5% and 3.6%, respectively); therefore, 4:1 is the best weight ratio where ~97% of the oil can be successfully recovered in the miscella phase.

The purpose of using THF was to solubilize all of the oil and water from the emulsion to the single-miscella phase and reduce the water content to less than 1% for industrial applications. The composition of miscella and polar phases at different weight ratios are presented in Figs. 5-7 and 5-8, respectively. Surprisingly, the results presented in Fig. 5-7 showed high concentration of oil and low concentration of water. The water content was less than 7% in all of the miscellae, and at 1:1 and 1.5:1 weight ratios, most of the water in the emulsion was extracted to the polar
phase or remained in the emulsion residue phase leaving only ~ 1% in the miscella phases. This simplifies subsequent water removal steps and also facilitates the dissolution of the oil in the miscella phase. From results in Figs. 5-7 and 5-8, it is clear that the miscella phases were high in oil content and low in water content whereas the polar phases were low in oil content and high in water content. Although the oil extraction in miscella phases increased moderately from 87% to 98.3% as the THF:oil weight ratio was increased from 1:1 to 5:1 (Table 5-9), the concentration of the oil in miscellae decreased from 58% to around 18% (Fig. 5-7). In order to fully understand the behaviour of the extraction process, we prepared the oil/water/THF ternary phase diagram (Fig. 5-9).

Figure 5-7: Composition of the miscellae (wt%) at different tetrahydrofuran:oil weight ratios. Compositions are expressed as means of three independently prepared samples with error bars representing standard deviations of the means. If not visible, it means the STD was < 0.5 wt%.
Figure 5-8: Composition of the polar phases (wt%) at different tetrahydrofuran:oil weight ratios. Compositions are expressed as means of three independently prepared samples with error bars representing standard deviations of the means. If not visible, it means the STD was < 0.5 wt%.

Figure 5-9 shows the phase diagram of the oil/water/THF system including the mutual solubility curve. The different solutions of oil, water, and THF selected for the determination of tie lines were equivalent to the amount of THF added to the emulsion at different weight ratios. Intermediate points on any tie line are in the two-phase region and represent a mixture with two phases having as their respective compositions the points at the terminals of the tie line (Othmer et al., 1941). The points on the right branch of the solubility curve have more oil in comparison with the amount of water; conversely, the points on the left branch of the curve have more water (Table 5-10). This clarifies the formation of two different phases, miscella and polar, one with high concentration of oil and the other with high concentration of water during the destabilization process (Figs. 5-7 and 5-8). Moreover, at each specific weight ratio, the composition of the oil-rich phase on the right branch of the solubility curve and the composition of the miscella formed during the emulsion destabilization were similar (Table 5-10).
Figure 5-9: Experimental ternary phase diagram of Water (A) + Canola Oil (B) + tetrahydrofuran (C) at room temperature: ×, solubility curve results; ●, overall composition for the tie lines; ■, tie line values. The overall compositions of the tie lines were ascertained based on the examined weight ratios in the extraction process.

Table 5-10: Tie lines for water (A) + canola oil (B) + tetrahydrofuran (C)

<table>
<thead>
<tr>
<th>WR</th>
<th>Water-rich phase</th>
<th>Oil-rich phase</th>
<th>Miscella phase</th>
<th>Oil-rich phase (g)</th>
<th>Miscella phase (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a     b     c</td>
<td>a     b     c</td>
<td>a     b     c</td>
<td>Water-rich phase (g)</td>
<td>Polar phase (g)</td>
</tr>
<tr>
<td>1:1</td>
<td>0.67  0.00  0.32</td>
<td>0.02  0.56  0.42</td>
<td>0.01  0.58  0.41</td>
<td>2.4</td>
<td>-</td>
</tr>
<tr>
<td>1.5:1</td>
<td>0.59  0.00  0.41</td>
<td>0.02  0.47  0.51</td>
<td>0.01  0.47  0.51</td>
<td>2.3</td>
<td>-</td>
</tr>
<tr>
<td>2:1</td>
<td>0.53  0.00  0.47</td>
<td>0.02  0.40  0.58</td>
<td>0.02  0.40  0.58</td>
<td>2.6</td>
<td>-</td>
</tr>
<tr>
<td>2.5:1</td>
<td>0.45  0.00  0.55</td>
<td>0.03  0.34  0.63</td>
<td>0.03  0.34  0.63</td>
<td>2.6</td>
<td>-</td>
</tr>
<tr>
<td>3:1</td>
<td>0.37  0.00  0.63</td>
<td>0.04  0.29  0.67</td>
<td>0.04  0.30  0.66</td>
<td>3.0</td>
<td>3.2</td>
</tr>
<tr>
<td>4:1</td>
<td>0.29  0.01  0.70</td>
<td>0.05  0.22  0.73</td>
<td>0.05  0.23  0.72</td>
<td>3.8</td>
<td>4.0</td>
</tr>
<tr>
<td>5:1</td>
<td>0.25  0.02  0.74</td>
<td>0.06  0.18  0.76</td>
<td>0.07  0.18  0.75</td>
<td>5.3</td>
<td>5.7</td>
</tr>
</tbody>
</table>

a, mass fraction of water; b, mass fraction of oil; c, mass fraction of tetrahydrofuran (THF).
The key factor that affects the oil extraction yield from the emulsion to the miscella phase is the weight ratio of the miscella to the polar phase formed during the emulsion destabilization. Higher miscella to polar weight ratio results in higher oil extraction yield. The ratios of the miscella phase to the polar phase obtained during the emulsion treatment were high enough to extract over 97% of the oil in the emulsion to the miscellae at THF:oil weight ratios of 4:1 and 5:1. This improvement could be easily interpreted using the tie-lines in the oil-water-THF ternary phase diagram. The weights of the two coexisting phases in the phase diagram are in the reverse ratio of the length of the line segments from the point on the tie line to the intersections of the tie line with the mutual solubility curve (Othmer and Tobias, 1942). In this system the weight of oil-rich phase to the weight of water-rich phase was more than one, and grew from 2.4 at 1:1 THF:oil weight ratio to 3.8 and 5.3 at 4:1 and 5:1, respectively. Table 5-10 compares the weight ratio of the coexisting phases with the weight ratio of the miscella and polar phases formed during the emulsion treatment.

### 5.3.3 Single-Stage Treatment of Yellow Mustard Emulsion with Dioxane

Although dioxane (bp, 101°C) is relatively non-polar, its special structure allows solvation with water molecules. It is sometimes used in place of THF due to its lower toxicity. Therefore, different dioxane:oil weight ratios of 1:1 to 10:1 were examined in this study to solubilize the emulsion and to recover all of the oil in a single-phase miscella. The composition of the miscella and polar phases produced after centrifugation are presented in Figs. 5-10 and 5-11. The results for oil extraction from yellow mustard emulsion are summarized in Table 5-11.

#### Table 5-11: Oil extracted from yellow mustard emulsion to the miscella and polar phases at various dioxane:oil weight ratios

<table>
<thead>
<tr>
<th>dioxane:oil Weight Ratios</th>
<th>Missella Phase</th>
<th>Polar Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>71±3&lt;sup&gt;C&lt;/sup&gt;</td>
<td>7±2&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
<tr>
<td>2:1</td>
<td>71±3&lt;sup&gt;C&lt;/sup&gt;</td>
<td>5±2&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
<tr>
<td>3:1</td>
<td>73±1&lt;sup&gt;RC&lt;/sup&gt;</td>
<td>5±2&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
<tr>
<td>4:1</td>
<td>78±1&lt;sup&gt;RC&lt;/sup&gt;</td>
<td>4±0&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
<tr>
<td>5:1</td>
<td>79±1&lt;sup&gt;B&lt;/sup&gt;</td>
<td>6±0&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
<tr>
<td>6:1</td>
<td>71±2&lt;sup&gt;C&lt;/sup&gt;</td>
<td>9±1&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>7:1</td>
<td>63±10&lt;sup&gt;D&lt;/sup&gt;</td>
<td>30±10&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>8:1</td>
<td>53±8&lt;sup&gt;B&lt;/sup&gt;</td>
<td>43±9&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>9:1</td>
<td>95±3&lt;sup&gt;A&lt;/sup&gt;</td>
<td>–</td>
</tr>
<tr>
<td>10:1</td>
<td>96±3&lt;sup&gt;A&lt;/sup&gt;</td>
<td>–</td>
</tr>
</tbody>
</table>

Means sharing the same capital letters in each row are not significantly different (P<0.05). Results are expressed as means of three independently prepared samples ± standard deviations of the means.

Single-phase miscellae were observed after centrifugation at 9:1 and 10:1 dioxane:oil weight ratios.
The oil extraction to miscella was 71% at 1:1 weight ratio; it slightly increased to 79% at 5:1 weight ratio, and decreased significantly to 63% at 7:1 and to 53% at 8:1, before recovering all of the oil from the emulsion at dioxane:oil weight ratios of 9:1 and 10:1. Although the miscellae produced at weight ratios of 1:1 to 8:1 were high in oil content and considerably low in water content (less that 2%), the low oil recoveries (Table 5-11) indicated the partially destabilization of the emulsion using dioxane. Therefore, it was necessary to increase the dioxane:oil weight ratios to 9:1 and 10:1 to successfully solubilize the emulsion. At these weight ratios only a single-phase miscella of oil-water-dioxane was observed in the centrifuge tubes that contained over 95% (Table 5-11) and 99% of the oil and water in the emulsion, respectively. To analyze the behaviour of this extraction process, the phase diagram of oil-water-dioxane was determined (Fig. 5-12).

Figure 5-10: Composition of the miscellae (wt%) at different dioxane:oil weight ratios. Compositions are expressed as means of three independently prepared samples with error bars representing standard deviations of the means. If not visible, it means the STD was < 0.5 wt%.
Figure 5-11: Composition of the polar phases (wt%) at different dioxane:oil weight ratios. Compositions are expressed as means of three independently prepared samples with error bars representing standard deviations of the means. If not visible, it means the STD was < 0.5 wt%.

Figure 5-12 indicates the behaviour of dioxane in destabilizing the emulsion. The low oil extraction yields at dioxane:oil weight ratios below 8:1 could be interpreted using the tie line results presented in Table 5-12. In this system, according to the negative slopes of the tie lines, the ratio of oil-rich layer to water-rich layer decreased moderately from 0.96 at 1:1 to 0.21 at 8:1. The descending trend was also observed regarding the ratio of the miscella phase to polar phase during the emulsion treatment (Table 5-12). Thereby, there was no significant increase (P<0.05) in the amount of the oil recovered at weight ratios below 5:1, and there was significant decrease (P<0.05) in the amount of the oil recovered at weight ratios of 6:1, 7:1, and 8:1 (from 71% at 6:1 to 53% at 8:1, Table 5-11) due to the considerable reduction in the amount of miscella phases released after centrifugation (Table 5-12). Dioxane:oil weight ratios of 9:1 and 10:1 were located on and outside of the mutual solubility curve, respectively (Fig. 5-12). Therefore, this amount of dioxane was enough to completely solubilize the emulsion to form a single-phase miscella.
Figure 5-12: Experimental ternary phase diagram of Water (A) + Canola Oil (B) + Dioxane (C) at room temperature: ×, solubility curve results; *, overall composition for the tie lines; ■, tie line values. The overall compositions of the tie lines were ascertained based on the examined weight ratios in the extraction process.

Table 5-12: Tie lines for water (A) + canola oil (B) + dioxane (C)

<table>
<thead>
<tr>
<th>WR</th>
<th>Water-rich phase</th>
<th>Oil-rich phase</th>
<th>Miscella phase</th>
<th>Oil-rich phase (g)</th>
<th>Miscella phase (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a  b  c</td>
<td>a  b  c</td>
<td>a  b  c</td>
<td>Water-rich phase (g)</td>
<td>Polar phase (g)</td>
</tr>
<tr>
<td>1:1</td>
<td>0.47 0.00 0.52</td>
<td>0.00 0.77 0.22</td>
<td>0.00 0.79 0.20</td>
<td>0.96</td>
<td>0.81</td>
</tr>
<tr>
<td>2:1</td>
<td>0.28 0.00 0.72</td>
<td>0.01 0.61 0.38</td>
<td>0.01 0.60 0.39</td>
<td>0.88</td>
<td>0.71</td>
</tr>
<tr>
<td>3:1</td>
<td>0.22 0.00 0.78</td>
<td>0.01 0.56 0.43</td>
<td>0.01 0.57 0.43</td>
<td>0.63</td>
<td>0.48</td>
</tr>
<tr>
<td>4:1</td>
<td>0.17 0.00 0.83</td>
<td>0.01 0.50 0.49</td>
<td>0.01 0.53 0.46</td>
<td>0.55</td>
<td>0.42</td>
</tr>
<tr>
<td>5:1</td>
<td>0.14 0.01 0.86</td>
<td>0.01 0.45 0.53</td>
<td>0.01 0.49 0.50</td>
<td>0.48</td>
<td>0.37</td>
</tr>
<tr>
<td>6:1</td>
<td>0.11 0.02 0.87</td>
<td>0.02 0.41 0.58</td>
<td>0.01 0.48 0.51</td>
<td>0.41</td>
<td>0.28</td>
</tr>
<tr>
<td>7:1</td>
<td>0.08 0.04 0.88</td>
<td>0.02 0.33 0.65</td>
<td>0.02 0.39 0.60</td>
<td>0.35</td>
<td>0.25</td>
</tr>
<tr>
<td>8:1</td>
<td>0.06 0.07 0.87</td>
<td>0.03 0.29 0.69</td>
<td>0.02 0.35 0.63</td>
<td>0.21</td>
<td>0.19</td>
</tr>
</tbody>
</table>

a, mass fraction of water; b, mass fraction of oil; c, mass fraction of dioxane.
The reliability of experimentally measured tie line compositions in oil-water-THF and oil-water-dioxane systems was ascertained by applying Othmer-Tobias correlation method (Othmer and Tobias, 1942) by plotting $\log \left\{ \frac{(1-b_2)}{b_2} \right\}$ against $\log \left\{ \frac{(1-a_1)}{a_1} \right\}$ (Fig. 5-13). The Othmer-Tobias correlation equation is:

$$\left( \frac{1-b_2}{b_2} \right) = k \left( \frac{1-a_1}{a_1} \right)^n$$  \hspace{1cm} (17)

Where, $a_1 =$ mass fraction of water (A) in water-rich phase, $b_2 =$ mass fraction of oil (B) in oil-rich phase, and $k, n =$ Othmer-Tobias constants. The linearity of the plot, for both systems, indicates the degree of consistency of the related data.

Figure 5-13: Othmer-Tobias correlation for Water (A) + Canola Oil (B) + Solvent (C) at room temperature: •, 1,4-dioxane; ■, tetrahydrofuran (THF).
5.3.4 Three-Stage Treatment of Yellow Mustard Emulsion with THF

Single-stage treatment of yellow mustard emulsion with THF was performed previously (section 5.3.2) at THF:oil weight ratios of 1:1, 1.5:1, 2:1, 2.5:1, 3:1, 4:1, and 5:1. This approach separated the emulsion into oil-rich miscella and water-rich polar phases. To accurately understand the behaviour of the destabilization process, each specific THF:oil weight ratio was analyzed using the oil/water/THF ternary diagram (Fig. 5-9). All of the tested THF:oil weight ratios were located within the two-phase region (under the solubility curve), thus separating into oil-rich and water-rich phases (Fig. 5-14) in which the composition of the oil-rich phase on the right-branch of the solubility curve was similar to the composition of the miscella released during emulsion destabilization. The quantities of oil extracted to the miscella increased moderately as the THF:oil weight ratio was increased from 1:1 to 5:1 leading to complete destabilization at 4:1 and 5:1 since the highest ratios of the weight of the miscella phase to polar phase were obtained at these two weight ratios due to the positive slopes of the tie lines (Fig. 5-14). However, as the THF:oil weight ratio increased from 1:1 to 5:1, the water contents of the resulting miscella increased from 1 to 7% equal to 2 and 50% of the water in the emulsion, respectively. Based on these observations, the synthesized ternary phase diagram was first used in this section to select weight ratios so that their corresponding miscella phases would contain less than 0.7% water (Fig. 5-14). As THF:oil weight ratios of 0.25:1, 0.5:1, and 0.75:1 were not high enough to completely destabilize the emulsion, a three stage process was evaluated. The quantities of oil extracted to miscella are shown in Figure 5-15.

The amount of THF at 0.25:1 weight ratio was not sufficient to break the emulsion; however, the emulsion was completely destabilized by three-stage extraction at 0.5:1 and 0.75:1 THF:oil weight ratios. As can be seen in Figure 5-15, 97 and 100% of the oil in the emulsion were recovered into miscella phases by using three stages at 0.5:1 and 0.75:1 THF:oil weight ratios, respectively. For these two weight ratios, the same oil recoveries (around 85-87%) were achieved in the first-stage extraction. For the second stage, the highest oil extraction yield, 95%, was achieved at 0.75:1 followed by 92% at 0.5:1 THF:oil weight ratios even though no significant differences (P<0.05) were observed between the oil extraction yields at this stage. For the third stage, the oil extraction yield achieved at 0.75:1 THF:oil weight ratio was significantly higher than that obtained at 0.5:1 (100% at 0.75:1 vs. 97% at 0.5:1).
From results in Figure 5-15, it is also clear that each extraction stage performed as part of the three-stage treatment of emulsion using either 0.5:1 or 0.75:1 weight ratios significantly (P<0.05) increased the oil extraction yields into the miscella phases. The three-stage treatment of the emulsion at 0.5:1 THF:oil weight ratio significantly improved the initial oil extraction yield from 87 to 92%, and 97% through the second and third steps of the treatment process, respectively. The same ascending trend can be also observed for 0.75:1 weight ratio.

The total amounts of THF utilized during the three-stage destabilization of the emulsion at 0.5:1 and 0.75:1 weight ratios were identical to those consumed during the single-stage processes at 1.5:1 and 2.25:1, respectively. While the quantities of oil extracted to miscellae by a single-stage treatment at these weight ratios were 87-89% (Table 5-9), three-stage treatments using the same
amount of solvent resulted in over 97% oil recoveries, thereby providing satisfactory destabilization.

Figure 5-15: Oil extracted from the emulsion into the miscellae produced in each stage of the 3-stage extraction process using tetrahydrofuran:oil weight ratios of 0.5:1(●) and 0.75:1(▲). Results are expressed as means of three independently prepared samples ± standard deviations of the means.

**ABC** Oil extraction yields in the same tetrahydrofuran:oil weight ratios with different upper case letters are significantly different (P<0.05).

**abc** Oil extraction yields in each stage with different lower case letters are significantly different (P<0.05).

The phase behaviour in the three-stage treatment at 0.75:1 THF:oil weight ratio was analyzed by the oil/water/THF ternary phase diagram (Fig. 5-16). The weight fractions of miscella phases in the mixtures after each extraction stage along with their compositions are summarized in Table 5-13. The composition of the combined miscella created by mixing all of the miscellae produced during the three-stage treatment is also presented in Table 5-13.
The first extraction stage was started by mixing the original emulsion with pure THF (first pre-separated mixture, Fig. 5-16), and completed by extracting the majority of the oil (85%) to the first-miscella while leaving rest of the oil in the residual emulsion and emulsion precipitate phases (Fig. 5-16). The composition of the mixtures, before and after separation, and the first-miscella were all located on the same tie line closer to the water-oil (horizontal) axis. In this stage, the weight fraction of first-miscella in the mixture was 0.48 w/w, indicating that approximately one-half of the pre-separated mixture was extracted in the form of homogeneous oil-rich miscella composed of 65.3% oil, 0.6% water, and 34.1% THF (Table 5-13). The addition of THF to the remaining phases from the first extraction stage shifted the location of the second pre-separated mixture towards the left side of the solubility curve far from the water-oil axis of the phase diagram (Fig. 5-16), thereby decreasing the weight fraction of the second-miscella in the mixture to 0.13 w/w as indicated by the reduction of the length of its tie line segment, since the weights of the two coexisting phases in the phase diagram are inversely proportional to the length of the tie line segments (Othmer et al., 1941). This stage increased the oil extraction yield by 10% by producing the second homogeneous miscella composed of 34.5% oil, 2.5% water, and 63.0% THF while leaving rest of the oil in the remaining mixture located on the same tie line as the second-miscella (Fig. 5-16). The addition of THF to the second remaining mixture shifted the location of the third pre-separated mixture to the top left corner of the solubility curve, thereby extracting all of the oil to the third homogenous miscella composed of 22.8% oil, 5.4% water, and 71.8% THF in which the weight fraction of the third-miscella in the mixture was very low ~0.08 w/w, inversely proportional to its tie line segment. The remaining polar and emulsion precipitate phases were free of oil, and located on the left side of the solubility curve roughly on the same tie line as the third-miscella.

Mixing the first-, second-, and third-miscella phases resulted in the formation of single-phase oil-solvent-water combined miscella composed of 55.9% oil, 1.5% water, and 42.6% THF (Table 5-13 and Fig. 5-16). Although the resulting second- and third-miscella phases contained high concentrations of water (2.5 and 5.4%, respectively), they weighed less than the first-miscella, resulting in low water concentration in the combined miscella.
Figure 5-16: Three-stage treatment of yellow mustard emulsion at 0.75:1 tetrahydrofuran:oil weight ratio on oil/water/tetrahydrofuran ternary phase diagram: (■), first-stage extraction; (▲), second-stage extraction; (▽), third-stage extraction; and (◆), combined miscella composition. The protein contents of the remaining mixtures after first and second extraction stages and the final polar and emulsion precipitate phases were considered too small to represent them on the ternary phase diagram.

The same phase behaviour was also observed for the destabilization of the emulsion using 0.5:1 THF:oil weight ratio; however, the resulting combined miscella at this point contained higher concentration of oil (68.3 vs. 55.9%) and lower concentration of water (1 vs. 1.5%), making it more suitable for direct conversion to biodiesel through single-phase or pseudo-single-phase transesterification. On the other hand, using 0.5:1 THF:oil weight ratio recovered 97% of the oil to the combined miscella (Fig. 5-15), leaving around 3% in the polar and emulsion precipitate phases.
Table 5-13: Compositions of the miscella and their weight fractions at each stage of the 3-stage treatment of yellow mustard emulsion using 0.5:1 and 0.75:1 tetrahydrofuran:oil weight ratios

<table>
<thead>
<tr>
<th>Components</th>
<th>Weight Ratios</th>
<th>Stage 1</th>
<th>Stage 2</th>
<th>Stage 3</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil Content (wt%)</td>
<td>0.5:1</td>
<td>75.1±0.5A</td>
<td>44.8±1.6C</td>
<td>31.4±0.8D</td>
<td>68.3±2.5B</td>
</tr>
<tr>
<td></td>
<td>0.75:1</td>
<td>65.3±0.4A</td>
<td>34.5±0.4C</td>
<td>22.8±0.4D</td>
<td>55.9±1.5B</td>
</tr>
<tr>
<td>Water Content (wt%)</td>
<td>0.5:1</td>
<td>0.5±0.1C</td>
<td>2.4±0.6B</td>
<td>4.2±0.8A</td>
<td>1.0±0.2C</td>
</tr>
<tr>
<td></td>
<td>0.75:1</td>
<td>0.6±0.1D</td>
<td>2.5±0.2B</td>
<td>5.4±0.7A</td>
<td>1.5±0.1C</td>
</tr>
<tr>
<td>THF Content (wt%)</td>
<td>0.5:1</td>
<td>24.5±0.5D</td>
<td>52.9±1.2B</td>
<td>64.4±1.3A</td>
<td>30.7±2.3C</td>
</tr>
<tr>
<td></td>
<td>0.75:1</td>
<td>34.1±0.4D</td>
<td>63.0±0.5B</td>
<td>71.8±1.0A</td>
<td>42.6±1.6C</td>
</tr>
<tr>
<td>Miscella</td>
<td>0.5:1</td>
<td>0.48A</td>
<td>0.07C</td>
<td>0.07K</td>
<td>0.42B</td>
</tr>
<tr>
<td></td>
<td>0.75:1</td>
<td>0.48A</td>
<td>0.13C</td>
<td>0.08D</td>
<td>0.43B</td>
</tr>
</tbody>
</table>

All the measurements were performed in triplicate.

All values represent the composition means (n=3) ± standard deviation.

Means sharing the same capital letters in each row are not significantly different (P<0.05).

*Miscella weight fraction values represent the weight ratio of the miscella phase to the total mixture after centrifugation during each stage of emulsion destabilization [miscella (g)/(miscella + polar + emulsion precipitate + residual emulsion) (g)].

Figures 5-17a, b, c, and d show the distributions of oil, water, THF, and protein between combined miscella, final polar and emulsion precipitate phases produced after the third stage of the 3-stage extraction of the emulsion using 0.5:1 and 0.75:1 THF:oil weight ratios. The results obtained during single-stage treatment at 4:1 THF:oil weight ratio are also provided for comparison. From the results of the 3-stage treatment of the emulsion, it is clear that the majority of oil (>97%) in original emulsion was extracted into the combined miscellae (Fig. 5-17a), the majority of water (>79%) into the final polar phases (Fig. 5-17b), and the majority of protein (>69%) into the final emulsion precipitate phases (Fig. 5-17d).

The effects of multi- and single-stage destabilization processes on oil extraction yield are shown in Figure 5-17a. The oil extracted during the 3-stage process at 0.5:1 was distributed as 97.2% in the combined miscella and 4.2% in the final polar phase. Since the total amount of THF used during this process was not high enough to completely destabilize the emulsion, a very thin layer of residual emulsion was observed on top of the polar phase after the third extraction stage which was dispersed into the polar phase during phase separation, thereby increasing the oil content of the resulting final polar phase. However, the use of the 3-stage process at 0.75:1 THF:oil weight
ratio destabilized the emulsion and enabled 100% of the oil in the emulsion to be extracted to the combined miscella, leaving no oil in the final polar and emulsion precipitate phases.

The water extraction yields achieved during three- and single-stage destabilization processes are compared in Figure 5-17b. The majority of the water (79-82%) was extracted into the final polar phase when using the 3-stage processes while only 54% of the water was extracted into the polar phase when using the single-stage process. No statistical difference in water extraction into the emulsion precipitate phases was observed at P<0.05. However, as it is shown in Figure 5-17b, only 1.9 and 3.5% of the water in the original emulsion was extracted into the combined miscella when using 3-stage processes at 0.5:1 and 0.75:1, respectively, significantly lower than 30.1% reported for the single-stage treatment at 4:1. This simplifies subsequent water removal step for biodiesel production from the combined miscella.

As shown in Figure 5-17c, 29.6 and 33.5% of the THF used during the 3-stage treatment of the emulsion at 0.5:1 and 0.75:1 was extracted into the combined miscella that would be directly used for conversion to biodiesel, whereas 75.2% of the THF used during the single-stage treatment of the emulsion at 4:1 was extracted into the miscella phase that needs to be reduced to the desired level before transesterification. Therefore, 3-stage treatment of the emulsion at THF:oil weight ratios of 0.5:1 and 0.75:1 was the best tested process based on the desired distribution of oil, water, and THF between combined miscella, final polar, and emulsion precipitate phases and low consumption of THF.
Figure 5-17: a Oil extraction yields obtained by 3-stage extraction of emulsion at 0.5:1 and 0.75:1 and single-stage extraction at 4:1 tetrahydrofuran:oil weight ratios. b Water extraction yields obtained by 3-stage extraction of emulsion at 0.5:1 and 0.75:1 and single-stage extraction at 4:1 tetrahydrofuran:oil weight ratios. c Tetrahydrofuran yields obtained by 3-stage extraction of emulsion at 0.5:1 and 0.75:1 and single-stage extraction at 4:1 tetrahydrofuran:oil weight ratios. d Protein extraction yields obtained by 3-stage extraction of emulsion at 0.5:1 and 0.75:1 and single-stage extraction at 4:1 tetrahydrofuran:oil weight ratios. Extraction yields (n=3) for each phase sharing the same lower case letters are not significantly different (P < 0.05).

5.3.5 Suitability of the Recovered Oil-Water-THF Miscella for Biodiesel Production

The presence of free fatty acids (FFA) and water during base-catalyzed transesterification results in irreversible consumption of catalyst and soap formation. The resulting soaps inhibit separating glycerol, thereby significantly reducing the yield of methyl esters. Therefore, all reaction materials should have FFA and water contents below 0.5 and 0.3%, respectively (Freedman et al., 1984). The presence of other impurities including phospholipids also reduce methyl ester yields (Du et al., 2004).

While the 3-stage destabilization of yellow mustard emulsion at 0.5:1 and 0.75:1 THF:oil weight ratios significantly decreased the water contents of the combined miscella to ~1 and 1.5%,
respectively, their water contents exceeded the desired level (0.3%) for direct conversion to biodiesel. Consequently, an additional dehydration step is required to reduce the water content to <0.3%. Other important steps required to validate the suitability of the resulting combined miscellae for direct conversion to FAME involved the determination of the FFA and phospholipid contents (Table 5-14). The FFA contents of the oil recovered from homogeneous oil-water-THF combined miscellae produced during 3-stage treatment of the emulsion at 0.5:1 and 0.75:1 THF:oil weight ratios were 0.09%, much lower than the maximum level acceptable for transesterification. The phospholipid content of the oil was ~0.14%, one-tenth the phospholipid content (1.44%) of the crude yellow mustard oil recovered by using hexane in our laboratory.

Table 5-14: The free fatty acid (FFA) and phospholipid contents of the oil recovered through three-stage treatment of the emulsion using tetrahydrofuran

<table>
<thead>
<tr>
<th>Components</th>
<th>Weight Ratios</th>
<th>Recovered Yellow Mustard Oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>FFA Contents</td>
<td>0.5:1</td>
<td>0.09±0.00</td>
</tr>
<tr>
<td>(wt%)</td>
<td>0.75:1</td>
<td>0.09±0.00</td>
</tr>
<tr>
<td>Phospholipid Content</td>
<td>0.5:1</td>
<td>0.14±0.00</td>
</tr>
<tr>
<td>(wt%)</td>
<td>0.75:1</td>
<td>0.15±0.00</td>
</tr>
</tbody>
</table>

All the measurements were performed in duplicate.
All values represent the composition means (n=2) ± standard deviation.

The alkaline emulsion produced during aqueous processing of dehulled yellow mustard flour at pH 11 consisted of not only oil (55.5%) and water (39.8%) but also protein (3.2%) and phospholipids (3.3%). The remarkable stability of this emulsion was due to the presence of high MW protein emulsifiers along with the mixed phospholipid-oleosin layer (Section 5.2, Table 5-4). Once THF was added to solubilize oil and water, the proteins and phospholipids surrounding the oil droplets in the emulsion were broken down and extracted into the polar phase or coalesced to form the solid emulsion precipitate phase. As it is shown in Figure 5-17d, no protein was extracted into the miscellae, leaving all of the proteins in the polar and emulsion precipitate phases. In addition, < 2% of the phospholipids in the emulsion was extracted to the resulting combined miscellae, leaving rest of the phospholipids in the polar and emulsion precipitate phases. The resulting combined miscellae were excellent biodiesel feedstocks after dehydration.
5.3.6 Three-Stage Treatments of Yellow Mustard Emulsion with Dioxane

Single-stage extractions at different dioxane:oil weight ratios (1:1 to 10:1) were examined in section 5.3.3 where statistically similar (P<0.05) quantities of oil of ~71-79% were extracted into miscellae at dioxane:oil weight ratios <6:1. The oil extraction to miscellae decreased significantly to 63% at 7:1 and to 53% at 8:1 (section 5.3.3, Table 5-11). As shown in Figure 5-18, in oil/water/dioxane system, dioxane showed more hydrophilic behaviour than THF indicated by the negative slopes of the tie lines. Therefore, as the dioxane:oil weight ratio was increased from 1:1 to 8:1, the weight ratio of miscella phase to polar phase decreased, thereby reducing the oil extraction into miscellae and preventing complete destabilization of the emulsion. Further increases in dioxane:oil weight ratio (9:1 and 10:1) shifted the location of the emulsion and dioxane mixtures to the single-phase area outside the mutual solubility curve, resulting in the complete destabilization of the emulsion by recovering all of the oil and water from the emulsion to the single-phase oil-dioxane-water miscella. However, the resulting miscella phases had high water contents that render the miscellae unsuitable for producing biodiesel. Therefore, 3-stage extractions of emulsion at lower dioxane:oil weight ratios of 0.25:1, 0.5:1, 1:1, 1.5:1, and 2:1 were examined in this section. The trends in the cumulative oil extraction yields to miscellae in each stage of destabilization are presented in Figure 5-19.
Figure 5-18: Experimental ternary phase diagram of Water (A) + Oil (B) + Dioxane (C) determined in our previous study (Tabtabaei and Diosady, 2012) at room temperature: ×, solubility curve; •, overall composition for the tie lines; and ■, tie line values. The overall compositions of the tie lines were ascertained based on the examined weight ratios during single-phase extraction process.

The amount of dioxane at 0.25:1 weight ratio was not sufficient to destabilize the emulsion. The total amount of dioxane used during the 3-stage extraction at 0.5:1, 1:1, 1.5:1, and 2:1 recovered only 77-86% of the oil from yellow mustard emulsion (Fig. 5-19); the highest oil extraction yields of 85-86% were achieved at the lowest weight ratios. Figure 5-19 shows no significant differences between oil extraction yields achieved during first-stage extraction at 0.5:1, 1:1, 1.5:1, and 2:1 weight ratios, which is consistent with the results obtained during single-stage treatments of the emulsion (Table 5-11). For the second stage, the same oil extraction yields (P<0.05) of 83% were achieved at 0.5:1 and 1:1 weight ratios followed by 77% for 1.5:1, and 74% for 2:1 dioxane:oil weight ratios. For the third stage, the oil extraction yields of 85-86%
achieved at 0.5:1 and 1:1 dioxane:oil weight ratios were significantly higher than those at 1.5:1 and 2:1 (81 and 77%). The oil recoveries achieved at 0.5:1 and 1:1 weight ratios were similar (P<0.05) and significantly higher than those achieved at 1.5:1 and 2:1. As also shown in Figure 5-19, oil extraction yields to miscellae (P<0.05) achieved during second and third steps of destabilization at each specific dioxane:oil weight ratio were similar, indicating that the third-stage extraction contributed little to overall extraction efficiency.

Figure 5-19: Oil extracted from the emulsion to the miscella produced in each stage of the three-stage extraction process using dioxane:oil weight ratios of 0.5:1(◆), 1:1(●), 1.5:1 (▲), and 2:1(×). Results are expressed as means of two independently prepared samples ± standard deviations of the means.

ABC Oil extraction yields in the same dioxane:oil weight ratios with different upper case letters are significantly different (P<0.05).

abc Oil extraction yields in each stage with different lower case letters are significantly different (P<0.05).
The weight fractions of miscella phases in the mixtures and their compositions after each extraction stage are summarized in Table 5-15. Generally, the miscella produced during multi-(3) stage treatment of the emulsion when using dioxane consisted of higher oil content and lower water and dioxane contents than miscella produced during emulsion treatment with THF (Table 5-13). The combined miscella at each weight ratio had high oil content and water content well below or close to the 0.3% limit required for biodiesel production (Table 5-15). For each weight ratio, after the initial oil extraction, a substantially smaller amount of miscella was retrieved in the subsequent stages, causing the gentle incline seen in Figure 5-19.

Table 5-15: Compositions of the miscella and their weight fractions at each stage of the 3-stage treatment of yellow mustard emulsion using dioxane:oil weight ratios of 0.5:1, 1:1, 1.5:1, and 2:1

<table>
<thead>
<tr>
<th>Components</th>
<th>Weight Ratios</th>
<th>Stage 1</th>
<th>Stage 2</th>
<th>Stage 3</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil Content</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(wt%)</td>
<td>0.5:1</td>
<td>88.4±0.0&lt;sup&gt;A&lt;/sup&gt;</td>
<td>77.5±1.1&lt;sup&gt;B&lt;/sup&gt;</td>
<td>73.2±2.4&lt;sup&gt;C&lt;/sup&gt;</td>
<td>86.5±0.5&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>77.3±0.1&lt;sup&gt;A&lt;/sup&gt;</td>
<td>64.7±1.8&lt;sup&gt;B&lt;/sup&gt;</td>
<td>61.3±0.2&lt;sup&gt;C&lt;/sup&gt;</td>
<td>75.4±1.3&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1.5:1</td>
<td>69.9±0.0&lt;sup&gt;A&lt;/sup&gt;</td>
<td>58.2±1.1&lt;sup&gt;B&lt;/sup&gt;</td>
<td>54.9±0.7&lt;sup&gt;C&lt;/sup&gt;</td>
<td>67.3±1.9&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2:1</td>
<td>64.6±0.0&lt;sup&gt;A&lt;/sup&gt;</td>
<td>54.4±0.7&lt;sup&gt;B&lt;/sup&gt;</td>
<td>50.7±0.9&lt;sup&gt;C&lt;/sup&gt;</td>
<td>62.5±1.0&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>Water Content</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(wt%)</td>
<td>0.5:1</td>
<td>0.2±0.0&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.3±0.0&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>0.5±0.1&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.2±0.0&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>0.3±0.0&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.7±0.1&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.6±0.1&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>0.5±0.1&lt;sup&gt;AB&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1.5:1</td>
<td>0.5±0.0&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.7±0.0&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>0.8±0.1&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.5±0.0&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2:1</td>
<td>0.6±0.0&lt;sup&gt;C&lt;/sup&gt;</td>
<td>0.8±0.0&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.9±0.0&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.6±0.0&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dioxane Content</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(wt%)</td>
<td>0.5:1</td>
<td>11.4±0.0&lt;sup&gt;C&lt;/sup&gt;</td>
<td>22.1±1.1&lt;sup&gt;B&lt;/sup&gt;</td>
<td>26.3±2.3&lt;sup&gt;A&lt;/sup&gt;</td>
<td>13.3±0.5&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>22.4±0.1&lt;sup&gt;C&lt;/sup&gt;</td>
<td>34.7±1.7&lt;sup&gt;B&lt;/sup&gt;</td>
<td>38.2±0.1&lt;sup&gt;A&lt;/sup&gt;</td>
<td>24.2±1.1&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1.5:1</td>
<td>29.6±0.0&lt;sup&gt;C&lt;/sup&gt;</td>
<td>41.0±1.1&lt;sup&gt;B&lt;/sup&gt;</td>
<td>44.3±0.8&lt;sup&gt;A&lt;/sup&gt;</td>
<td>32.2±1.9&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2:1</td>
<td>34.8±0.0&lt;sup&gt;C&lt;/sup&gt;</td>
<td>44.7±0.7&lt;sup&gt;B&lt;/sup&gt;</td>
<td>48.4±0.9&lt;sup&gt;A&lt;/sup&gt;</td>
<td>36.8±1.0&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
<tr>
<td>Miscella</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight Fractions&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.5:1</td>
<td>0.35&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.06&lt;sup&gt;C&lt;/sup&gt;</td>
<td>0.02&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.29&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>0.34&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.05&lt;sup&gt;C&lt;/sup&gt;</td>
<td>0.01&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>0.24&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1.5:1</td>
<td>0.27&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.04&lt;sup&gt;C&lt;/sup&gt;</td>
<td>0.01&lt;sup&gt;C&lt;/sup&gt;</td>
<td>0.19&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2:1</td>
<td>0.26&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.04&lt;sup&gt;C&lt;/sup&gt;</td>
<td>0.01&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.16&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All the measurements were performed in triplicate.

All values represent the composition means (n=2) ± standard deviation.

Means sharing the same composition means (n=2) ± standard deviation.

Means sharing the same capital letters in each row are not significantly different (P<0.05).

<sup>a</sup> Miscella weight fraction values represent the weight ratio of the miscella phase to the total mixture after centrifugation during each stage of emulsion destabilization [miscella (g)/(miscella + polar + emulsion precipitate + residual emulsion) (g)].
The phase behaviour of emulsion destabilization through 3-stage treatment was analyzed using the oil/water/dioxane ternary phase diagram. The same behaviour was observed for all of the tested weight ratios. Figure 5-20 shows the phase behaviour of the 3-stage destabilization process at 1:1 dioxane:oil weight ratio. The first-stage extraction of emulsion with dioxane recovered 74% of the oil from emulsion to the miscella phase containing 77.3% oil, 0.3% water, and 22.4% dioxane while leaving rest of the oil in the residual emulsion and emulsion precipitate phases. The composition of the first-miscella and the mixtures, before and after separation, were all located on the same tie line close to the horizontal axis (Fig. 5-20). In the first stage, 34% of the mixture was released in the form of miscella (Table 5-15). The second-stage extraction increased the oil extraction yield to 83% by releasing ~5% of the mixture as the second miscella composed of 64.7% oil, 0.7% water, and 34.7% dioxane. The composition of the second-miscella, pre-separated, and remaining mixtures were all located on the same tie line. The remaining mixture after second extraction stage had ~6.3% oil content, significantly lower than that after first-stage extraction (13.9%), indicating the essential role of the second stage in improving the oil extraction yield. The addition of dioxane to the second remaining mixture did not significantly improve oil extraction yield since only 1% of the mixture was released as the third-miscella, and the composition of the remaining mixture after extraction was located on the composition of the pre-separated mixture (Fig. 5-20), confirming marginal role of the third-stage extraction. Moreover, the compositions of the third-miscella, pre-separated, and remaining mixtures were not located on the same tie line as the third-miscella had lower-than-expected dioxane content. Although the combined miscella with 75.4% oil, 0.5% water, and 24.2% dioxane could be used directly for biodiesel production, its weight fraction was only 0.24 w/w. This indicated that dioxane was more hydrophilic than THF due to the presence of two exposed oxygen atoms available for hydrogen bonding.

Fourth-stage extraction of yellow mustard emulsion for all of the tested dioxane:oil weight ratios resulted in no separation between the miscella and polar phases. Therefore, continuing the extraction to a fourth stage was counterproductive due to the extremely high water content that renders the combined miscella unsuitable for producing biodiesel. In summary, multiple-stage destabilization of the emulsion by using dioxane offered no significant advantage over single-stage destabilization.
Figure 5-20: Three-stage treatment of yellow mustard emulsion at 1:1 dioxane:oil weight ratio on oil/water/dioxane ternary phase diagram: (■), first-stage extraction; (▲), second-stage extraction; (▼), third-stage extraction; and (◆), combined miscella composition. The protein contents of the remaining mixtures after first, second, and third extraction stages were considered too small to be able to represent them on the ternary phase diagram.

The distributions of oil, water, dioxane, and protein between combined miscella, final polar and emulsion precipitate phases produced after the third stage are presented in Figures 5-21a, b, c, and d. Since three stages of extraction were unable to completely destabilize the emulsion, a thick layer of residual emulsion remained in the centrifuge tubes. These were combined with the respective emulsion precipitates for further analysis. Figure 5-21 shows that the majority of oil (77-86%) in the original emulsion was extracted into the combined miscella (Fig. 5-21a), the majority of water (76-98%) to the final polar (Fig. 5-21b), and the majority of protein (77-87%) to the final emulsion precipitate phases (Fig. 5-21d). A major difference was observed between THF (Fig. 5-17c) and dioxane extraction yields (Fig. 5-21c) likely due to the more hydrophilic behaviour of the dioxane that prevents complete oil extraction to the miscellae. While 60-83% of
the total dioxane used for emulsion destabilization was extracted into the polar phase, only 8-9% was extracted into the combined miscella phases.

Figure 5-21  a Oil extraction yields obtained by 3-stage extraction of emulsion at 0.5:1, 1:1, 1.5:1, and 2:1 dioxane:oil weight ratios. b Water extraction yields obtained by 3-stage extraction of emulsion at 0.5:1, 1:1, 1.5:1, and 2:1 dioxane:oil weight ratios. c Dioxane yields obtained by 3-stage extraction of emulsion at 0.5:1, 1:1, 1.5:1, and 2:1 dioxane:oil weight ratios. d Protein extraction yields obtained by 3-stage extraction of emulsion at 0.5:1, 1:1, 1.5:1, and 2:1 dioxane:oil weight ratios. Extraction yields (n=2) for each phase sharing the same lower case letters are not significantly different (P < 0.05).

5.4 Dehydration of Miscellae by Adsorption over 4A Molecular Sieves and Biodiesel Production

We proposed an integrated process (Fig. 5-22) to simultaneously produce food-grade protein products, free of solvent contact/residues, and high-purity biodiesel from dehulled yellow mustard flour. Our process started by two-step aqueous extraction of the flour at pH 11 to extract protein, as the main product, followed by three-stage destabilization of the resulting emulsion
with THF to fully release the oil in the form of oil-THF-water miscella (section 5.3.4). In this section (5.4), I completed the integrated process by using microporous zeolite 4A for dehydration of miscella in both batch and continuous packed column adsorption systems to allow direct production of standard FAME through a single-phase chemical reaction (Fig. 5-22). The results of Section 5.4 have been previously submitted to the Journal of the American Oil Chemists' Society (JAOCS) for publication (Tabtabaei et al., 2014b).

5.4.1 Adsorption Equilibrium Isotherms

Based on the principles of adsorption isotherms described in section 2.8.1, the adsorptive dehydration of oil-water-THF miscella produced during single-stage and three-stage emulsion destabilization were evaluated in the following manner using the Roman numeral classification of Table 4-3.

The adsorption equilibrium studies were performed to evaluate the water adsorption from miscella (I, II, and III) on zeolite 4A as well as comparing the effectiveness of the process based on the oil and THF concentrations of the miscella. The basic and easily linearized Langmuir and Freundlich equilibrium adsorption isotherm models (Table 2-5) were used in interpreting the experimental results. The Langmuir isotherm model is represented by Eq. 18 (Volesky, 2003).

\[
q = q_m \frac{K_L C_e}{1 + K_L C_e}
\]

(18)

where \(q_m\) (g g\(^{-1}\)) and \(K_L\) (wt%\(^{-1}\)) are the Langmuir constants related to the maximum adsorption capacity and energy of adsorption, respectively. These parameters were obtained by fitting the experimental data into the linear form of the Langmuir equation [i.e., \(1/q = 1/q_m K_L C_e + 1/q_m\)].
The empirical Freundlich isotherm model is represented by Eq. 19 (Proctor and Toro-Vazquez, 1996, Volesky, 2003).

\[ q = K_f C_e^{1/n_f} \]  \hspace{1cm} (19)

where \( K_f \) and \( 1/n_f \) are the Freundlich constants, indicating the adsorption capacity and energy of adsorption, respectively. These parameters were determined using the intercept and slope of the logarithmic plot of the Freundlich equation [i.e., \( \log q = \log K_f + 1/n_f \log C_e \)].

The characteristic parameters of the two isotherms and related correlation coefficients were calculated for all miscella (I, II, and III) as summarized in Table 5-16. Although the experimental equilibrium data fitted well to both Langmuir and Freundlich isotherms with correlation coefficient values greater than 0.89, the Langmuir model is the better fitting isotherm in terms of its higher correlation coefficient values obtained in adsorptive dehydration of miscella (I) \( (R^2=0.978) \) and miscella (III) \( (R^2=0.991) \).

Table 5-16: Langmuir and Freundlich parameters for adsorption of water from miscella on zeolite 4A

<table>
<thead>
<tr>
<th>Isotherm Model</th>
<th>Miscella</th>
<th>Estimated isotherm parameters</th>
<th>( q_m ) (g g(^{-1}))</th>
<th>( K_L ) (wt.%(^{-1}))</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Langmuir</strong></td>
<td>I</td>
<td></td>
<td>0.223</td>
<td>44.8</td>
<td>0.978</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td></td>
<td>0.217</td>
<td>50.5</td>
<td>0.953</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td></td>
<td>0.199</td>
<td>73.8</td>
<td>0.991</td>
</tr>
<tr>
<td><strong>Freundlich</strong></td>
<td>I</td>
<td></td>
<td>0.273</td>
<td>0.227</td>
<td>0.912</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td></td>
<td>0.228</td>
<td>0.134</td>
<td>0.984</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td></td>
<td>0.188</td>
<td>0.098</td>
<td>0.891</td>
</tr>
</tbody>
</table>

The correlation of the theoretical Langmuir model with the experimental equilibrium results for the adsorption of water from miscella (I, II, and III) by zeolite 4A is shown in Figs. 5-23a-c. The graphs were plotted based on the water adsorbed per unit mass of zeolite 4A, \( q \), against the concentration of water remaining in the miscella after reaching equilibrium, \( C_e \). The values \( K_L \)
and $q_m$ obtained from the slope and intercept of the linear format of Langmuir model are also listed in Table 5-16.

Figure 5-23 a Adsorption isotherms of miscella (I) on zeolite 4A at room temperature and modeled results using Langmuir equation; ◆, miscella (I) experimental data; −, miscella (I) Langmuir model. b Adsorption isotherms of miscella (II) on zeolite 4A; ▲, miscella (II) experimental data; −−, miscella (II) Langmuir model. c Adsorption isotherms of miscella (III) on zeolite 4A; ■, miscella (III) experimental data; ..., miscella (III) Langmuir model.
The greatest equilibrium adsorption capacity, \( q_m = 0.223 \text{ g g}^{-1} \), was obtained for adsorptive dehydration of miscella (I). Lower values were obtained for miscella (II), 0.217 g g\(^{-1}\), and for miscella (III), 0.199 g g\(^{-1}\). The Langmuir isotherms predicted for the adsorption of water from miscellae were graphically compared in Fig. 5-24a at residual concentrations, \( C_c \), ranging from 0 to 1\%. The factor limiting the uptake of water from miscellae is the presence of high quantities of THF, which results in the formation of hydrogen bonds between water and THF especially at water residual concentrations \( (C_c) \) exceeding 0.05\% (Fig. 5-24a). Consequently, the adsorptive dehydration of dilute miscella phases containing high concentrations of THF and low concentrations of oil was less efficient due to the strong interaction between water and THF molecules. However, at very low water concentrations the adsorptive dehydration of miscella (III) is more favorable, due to the fact that at very low \( C_c \) values, the dilute miscella solutions are less viscous with no significant interactions between water and THF molecules (Fig. 5-24b). The steeper initial slope of miscella (III) adsorption isotherm (Fig. 5-24b) was consistent with its larger \( K_L \) value of 73.8 wt\%\(^{-1}\) (Table 5-16), as high \( K_L \) value indicates high affinity between the water and the adsorbent in the low residual water concentration range (Volesky, 2003).

The adsorption parameter \( 1/n_f \) in the Freundlich isotherm gives an indication of the favourability of the adsorption in which values of \( 1/n_f < 1.0 \) represent favourable adsorption conditions (Proctor and Toro-Vazquez, 1996). The \( 1/n_f \) values obtained for dehydration of miscellae were all less than unity, confirming the favourability of adsorption processes. Larger \( 1/n_f \) and \( K_f \) values obtained for dehydration of miscella (I) compared to miscellae (II) and (III) showed more efficient water removal from concentrated miscella solutions, which is in agreement with the predictions of the Langmuir model.
Figure 5-24 a Comparison of Langmuir equilibrium isotherms predicted for adsorptive dehydration of miscella (I), (II), and (III) at water residual concentrations, $C_e$, of 0 to 1.0 wt%. b Langmuir equilibrium isotherms at low water residual concentrations ($C_e < 0.06$ wt%).

5.4.2 Adsorption of Oil-THF-Water Miscella by Zeolite 4A in a Batch System

The effect of different doses of zeolite 4A on water removal was represented in Fig. 5-25 using miscella (I) and (II) with initial water contents of 1.0 and 1.5 %, respectively. As shown in Fig. 5-25, the water removal efficiencies for the two studied miscella increased significantly from ~ 45 to ~ 97 % at adsorbent doses below 0.1 g/g. This substantial improvement was attributed to the availability of greater surface area and larger number of adsorption sites. The water removal efficiencies were then slightly improved to ~99.5 % as the adsorbent dose was increased to 0.15 g/g. Any further addition of zeolite 4A beyond 0.15 g/g did not result in increased water removal above 99.6-99.7%. Therefore, the value of 0.15 g/g with water removal of ~99.5% was selected as the optimum dosage for both miscella (I) and (II) for subsequent batch treatment studies.

From Figure 5-25, it is also clear that initial water removal efficiencies from miscella (I) were
significantly higher than those obtained for miscella (II). This can be attributed to the more favorable adsorption conditions for miscella (I) due to its lower initial water and THF contents.

Figure 5-25: Effect of adsorbent dose on efficiencies of water removal from miscella (I) (◆) and miscella (II) (☆) at room temperature.

The effect of contact time on the adsorption of water from miscellae (I) and (II) was investigated using zeolite 4A at the optimum dosage of 0.15 g/g (Fig. 5-26). The rate of water removal in the two studied miscellae was similar. The water removal rate was found to be very rapid during the initial 60 min, where water removal efficiencies of 82.6±0.5 and 85.1±0.9 % were obtained for miscellae (I) and (II), respectively. The rate of water removal decreased to zero during the next 70 min until the steady-state condition was achieved at 130 min with no significant change in water removal thereafter. Based on the results in Fig. 5-26, 97.5±0.5 and 97.1±0.3 % of the water was adsorbed in 130 min from miscellae (I) and (II), respectively, only 15.1 and 12 % more than that removed during the first 60 min. This rapid adsorption of water during the early stages of the adsorption process was attributed to the availability of large number of vacant adsorption sites that become occupied with time. The average water removal efficiencies during the last 100 min (140-240 min) of the batch adsorption process increased only to 98.2%. The efficiencies achieved after 130 min were only ~1.3 % lower than the 24h results shown in Fig. 5-25.
After optimization of the adsorbent dose and contact time using the canola oil miscellae, the batch dehydration was repeated with mustard oil miscellae prepared as described in section 4.4.3 and Fig. 4-4. The water removal results with mustard oil miscellae (Table 5-17) were consistent with the results obtained for canola oil miscellae (Fig. 5-26). The water content of miscella (I) after 40 min adsorption was ~0.3 %, the maximum allowable water content in biodiesel feedstock, indicating that 40 min is the minimum contact time required for miscella (I) to be considered as a high-quality starting material for biodiesel production.

Table 5-17: The compositions of the mustard oil miscellae produced by emulsion destabilization after dehydration over zeolite 4A and the equivalent water removal percentages

<table>
<thead>
<tr>
<th>Miscella Number</th>
<th>Adsorption Contact Time (min)</th>
<th>Dehydrated Mustard Oil Miscella Composition</th>
<th>Water Removal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Oil Content (wt%)</td>
<td>Water Content (wt%)</td>
</tr>
<tr>
<td>I</td>
<td>40</td>
<td>69.6</td>
<td>0.3</td>
</tr>
<tr>
<td>I</td>
<td>240</td>
<td>69.8</td>
<td>0.01</td>
</tr>
<tr>
<td>II</td>
<td>240</td>
<td>57.9</td>
<td>0.02</td>
</tr>
</tbody>
</table>

All the measurements were performed in duplicate.
All values represent the composition means (n=2) with standard deviations < 0.05%.
5.4.3 Thermal Regeneration of Zeolite 4A

The regeneration of used zeolite 4A is a critical step that must be considered in designing the adsorptive dehydration of miscella solutions. A series of batch adsorption experiments were performed using miscella (I) at the optimum dosage to estimate the number of cycles that the zeolite 4A could be effectively regenerated at high temperature. The dehydration performances of the multi-cycle regenerated adsorbent were compared to that of fresh adsorbent (Fig. 5-26) and the results are represented in Fig. 5-27 and Table 5-18.

The dehydration performance of miscella (I) decreased significantly after each regeneration cycle as indicated by the reduction in water removal (Fig. 5-27). The maximum level of water allowed in miscellae for standard biodiesel production is 0.3 %, equivalent to 70 % water removal from miscella (I). This was attained in 40 min using fresh adsorbent; however, the time required increased to 130 min in four regeneration cycles. While the thermal regeneration was not effective in fully restoring zeolite 4A to its activated state, the adsorbent can be practically regenerated four times using this simple regeneration technique.

Figure 5-27: Influence of repeated use of zeolite 4A on the water removal efficiencies with respect to time during batch adsorptive dehydration of miscella (I) at optimum dosage of 0.15 g/g and room temperature; ◆, fresh adsorbent; ▣, first-time regenerated adsorbent; ▲, second-time regenerated adsorbent; ○, third-time regenerated adsorbent; ▬, fourth-time regenerated adsorbent.
Table 5-18: Comparison of the effectiveness of regenerated zeolite 4A on water removal

<table>
<thead>
<tr>
<th>Regeneration cycles (#)</th>
<th>Zeolite 4A weight after regeneration (g)</th>
<th>Adsorbent dose* (g/g)</th>
<th>Max. water removal (wt %)</th>
<th>Time for Max. water removal (min)</th>
<th>Time for 70% water removal (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>0</strong></td>
<td>7.5</td>
<td>0.15</td>
<td>98.2±0.3</td>
<td>140</td>
<td>40</td>
</tr>
<tr>
<td><strong>1</strong></td>
<td>7.8</td>
<td>0.16</td>
<td>96.6±0.8</td>
<td>160</td>
<td>60</td>
</tr>
<tr>
<td><strong>2</strong></td>
<td>8.2</td>
<td>0.16</td>
<td>94.4±1.2</td>
<td>190</td>
<td>80</td>
</tr>
<tr>
<td><strong>3</strong></td>
<td>8.4</td>
<td>0.17</td>
<td>92.6±1.2</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td><strong>4</strong></td>
<td>8.5</td>
<td>0.17</td>
<td>87.3±0.6</td>
<td>230</td>
<td>130</td>
</tr>
</tbody>
</table>

*The adsorbent dose was calculated based on the weight of the regenerated zeolite 4A and 50 g miscella (I) solution used in all adsorption processes.

One of the causes for the poor performance of thermal regeneration is related to the structure of the commercial zeolite 4A. It consists of microporous zeolite crystals with pore diameters of 4 Å bound together in the form of beads using a binder that introduces large pores into the overall structure (Jain and Gupta, 1994). The oil-THF-water miscella initially enters the large pores that act as passage way for water molecules to diffuse into the micropores (Do, 1998). The amount of the thermal energy required for regeneration must be high enough not only to strip water from the adsorbents, but also to remove the oil and THF molecules trapped inside the large pores. Apparently, the complete desorption of oil molecules from zeolite 4A were not accomplished at the tested temperature of 275 °C under vacuum. As can be seen in Table 5-18, the weight of the adsorbent increased from 7.5 g to 8.5 g after four cycles of regeneration. The oil molecules remained inside the regenerated adsorbents could block access to micropores, thus reducing the rate of water removal during dehydration of miscellae (Fig. 5-27). Other methods including the use of binderless molecular sieves or alternatively the use of organic solvents such as acetone during regeneration should help improve the life of the molecular sieves. Regeneration of molecular sieves at high temperatures (>315 °C) is not recommended by the manufacturers.

5.4.4 Continuous Adsorptive Dehydration of Miscellae by Zeolite 4A in a Fixed-Bed System

Based on the principles described in section 2.8.2, the continuous adsorptive dehydration of oil-water-THF miscelleae produced during three-stage emulsion destabilization was evaluated in the following manner:
Continuous dehydration was performed by passing miscella (I) or (II) through a column (2.5 cm in diameter and 30 cm in height) packed with zeolite 4A at flow rates of 1.6 or 2.0 mL/min, respectively. The results are depicted in Fig. 5-28 in the form of breakthrough curves (C/C_i vs. time), indicating the loading behavior of water. The properties of fixed-bed adsorption systems were calculated by utilizing the breakthrough curves based on equations 12 to 15 and the equations described in Table 2-6. The performance of the packed bed was evaluated in terms of the breakthrough time (t_b), exhaustion time (t_e), water removal percentages at the point of breakthrough (S_b) and exhaustion (S_e), and the maximum bed capacity (q_e) defined by the total amount of water adsorbed per weight of zeolite 4A at the exhaustion time. The results are summarized in Table 5-19.

The continuous dehydration of both miscellae proved to be highly efficient as relatively long breakthrough times were achieved, representing the ability of the columns to continuously dehydrate the miscellae for hours with nearly 100% efficiency (Table 5-19 and Fig. 5-28). The breakthrough time of miscella (II) was 14.2 h by which time 98.6 % of the water was removed resulting in the production of high-quality biodiesel feedstock. The total water removal at exhaustion time was 46.9 % with the maximum bed capacity of 0.222 g g^{-1}. The continuous dehydration of miscella (I) was more efficient, with a longer breakthrough time of 44.6 h and higher total water removal of 54.2 %. The maximum bed capacity for adsorption of water from miscella (I) was found to be 0.244 g g^{-1}, significantly higher than that obtained for miscella (II). More efficient water removal from miscella (I) can be attributed to its lower initial water and THF contents as well as lower flow rate. Although both flow rates of 1.6 and 2.0 mL/min were low enough to establish equilibrium in the columns, at the lower flow rate the water molecules had more residence time to diffuse into the adsorbent, thus increasing the maximum bed capacity (q_e) and total water removal percentage (S_e).
Figure 5-28: The breakthrough curves for the adsorptive dehydration of miscella at room temperature; ■, miscella (I) (flow rate: 1.6 mL/min); ◆, miscella (II) (flow rate: 2.0 mL/min).

Table 5-19: Different breakthrough parameters for the water removal from miscella (I) and (II) in a fixed-bed adsorption column packed with zeolite 4A.

<table>
<thead>
<tr>
<th>Miscella</th>
<th>( C_i ) (wt %)</th>
<th>( W ) (g)</th>
<th>( Q ) (mL min(^{-1}))</th>
<th>( t_b ) (h)</th>
<th>( t_e ) (h)</th>
<th>( S_b ) (wt %)</th>
<th>( S_e ) (wt %)</th>
<th>( q_e ) (g g(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>111.1</td>
<td>1.6</td>
<td>44.6</td>
<td>103.8</td>
<td>98.1</td>
<td>54.2</td>
<td>0.244</td>
</tr>
<tr>
<td>II</td>
<td>1.5</td>
<td>111.1</td>
<td>2.0</td>
<td>14.2</td>
<td>45.5</td>
<td>98.6</td>
<td>46.9</td>
<td>0.222</td>
</tr>
</tbody>
</table>

1The breakthrough time \( t_b \) and exhaustion time \( t_e \) were defined as the time of adsorption when the effluent water concentration, \( C_e \), was about 10 and 97 % of the initial water concentration, \( C_i \), respectively.

2Total water removal percentage \( S_e \) and water removal percentage at breakthrough \( S_b \) were calculated using Eqs. 14 and 15, respectively.

3The maximum adsorption capacity of the column \( q_e \); g g\(^{-1}\)) at \( t_e \) was calculated using Eq. 13.

While more studies should be performed to optimize the key operating parameters of flow rate, bed height and miscella composition, the observed satisfactory breakthrough times demonstrated the suitability of the process for producing high-quality starting materials for the single-phase base-catalyzed transmethylation process.
5.4.5 Biodiesel from Dehydrated Oil-THF Miscella by a Single-Phase Chemical Reaction

Based on the principles described in section 2.7, the dehydrated miscellae were used as starting materials for biodiesel (FAME) production in a pseudo-single-phase base-catalyzed transmethylation. Reaction conditions were methanol:oil molar ratio of 14:1, the THF/methanol volume ratio of 1.0, and a sodium hydroxide concentration of 1.2 wt% as determined earlier (Mahajan et al. 2006). The dehydrated mustard oil miscella (I) with 0.3% water, 30.1% THF, and 69.6% oil (Table 5-17) was selected as the starting material to evaluate the quality of the resulting biodiesel. The weight percentages of FAME, MG, DG, and TG; the total glycerol (G_T) contents; and the acid number of the biodiesel products are shown in Table 5-20. The results indicated the FAME content of 99.3% after 10 min when the reaction had essentially reached steady state. Approximately the same conversion of ~99.2% was achieved after 10 min using either pure canola oil or mustard as the starting material. This indicates the high-quality of the dried oil-THF miscella as biodiesel feedstock. The biodiesel produced after 10 min of the reaction had G_T concentration of 0.154 wt% and acid number of 0.461 that met the ASTM maximum levels of 0.240 wt% and 0.5 mg KOH/g, respectively. The European standard also prescribes individual limitations on MG (0.8 wt%), DG (0.2 wt%), and TG (0.2 wt%) concentrations. As seen in Table 5-20, the MG, DG, and TG contents of the biodiesel product met the European specifications as well. Therefore, the oil-THF-water miscella solutions produced through three-stage emulsion destabilization at both 0.5:1 and 0.75:1 THF:oil weight ratios are excellent biodiesel feedstocks after dehydration over zeolite 4A.

5.5 Synthesis and Evaluation of the Process Flow Diagram

Figure 5-29 depicts each of the individual steps of our integrated process starting with aqueous processing of dehulled yellow mustard flour at pH 11 to produce high-quality protein products followed by three-stage destabilization of the resulting emulsion with THF to directly produce biodiesel after miscella dehydration.
Table 5-20: Effect of time on total glycerol ($G_T$, wt%), methyl esters (wt%), and glyceride contents (wt%)$^a$

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>FAME (%)</th>
<th>$^bG_T$ (%)</th>
<th>MG (%)</th>
<th>DG (%)</th>
<th>TG (%)</th>
<th>Acid Number (mg KOH g$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>87.68</td>
<td>1.910</td>
<td>3.024</td>
<td>4.447</td>
<td>4.847</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>97.68</td>
<td>0.407</td>
<td>0.749</td>
<td>1.213</td>
<td>0.354</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>99.07</td>
<td>0.210</td>
<td>0.722</td>
<td>0.137</td>
<td>0.064</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>99.30</td>
<td>0.154</td>
<td>0.496</td>
<td>0.141</td>
<td>0.065</td>
<td>0.461</td>
</tr>
<tr>
<td>12</td>
<td>99.38</td>
<td>0.140</td>
<td>0.482</td>
<td>0.074</td>
<td>0.065</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>99.37</td>
<td>0.142</td>
<td>0.488</td>
<td>0.065</td>
<td>0.074</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>99.33</td>
<td>0.154</td>
<td>0.544</td>
<td>0.062</td>
<td>0.065</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>99.30</td>
<td>0.160</td>
<td>0.567</td>
<td>0.065</td>
<td>0.065</td>
<td>-</td>
</tr>
<tr>
<td>60</td>
<td>99.33</td>
<td>0.153</td>
<td>0.543</td>
<td>0.058</td>
<td>0.065</td>
<td>-</td>
</tr>
</tbody>
</table>

All values are averages of two runs and the values for each run lay within ±2% of the mean value for a particular data point.

$^a$14:1 methanol/oil molar ratio, room temperature, 1:1 THF/methanol volume ratio, 1.2 wt% sodium hydroxide (based on the oil).

$^b$ Total glycerol content ($G_T$) was calculated using Eq. 16.

Our integrated process starts by mixing dehulled yellow mustard flour with water using 4:1 water-to-flour weight ratio. The slurry will be extracted at pH 11 for 30 min at room temperature followed by centrifugation. Three-phase decanter centrifuges (e.g. Centrisys Corporation, Beloit, WS, USA) could be used in industry for separating the emulsion and skim fractions from the solid residual fraction. The skim fraction will be further centrifuged to separate the remaining emulsion; however, this step could be eliminated in an industrial scale process as the three-phase decanter centrifuges can provide better separation between the emulsion and skim fractions. The solid residual fraction will be subjected to the second-stage extraction by mixing with the same amount of water used for the first extraction stage. The resulting slurry will be then centrifuged and separated into three different phases including emulsion, skim and solid residual fractions. The emulsion and skim were combined with the respective first-stage fractions. The final skim fraction will be processed by membrane separation technologies to produce high-quality food-grade protein isolates. The final yellow mustard emulsion will be subjected to the three-stage destabilization process using 0.5:1 THF:oil weight ratio. As shown in Fig. 5-29 and based on the procedure described in Section 4.4.3, in each stage of the destabilization process, oil-water-THF miscella solution was produced that will be combined and fed to the adsorptive dehydration column packed with zeolite 4A. The dehydrated oil-THF miscella will be reacted with methanol for 10 min in the presence of 1.2 wt% sodium hydroxide as a catalyst to produce standard FAME.
Figure 5-29: The process flow diagram (PFD) of our integrated technology for the production of biodiesel (FAME) and high-quality protein products from dehulled yellow mustard flour.
To this end, as summarized in Table 5-21, our two-stage aqueous extraction process at pH 11 extracted 64.6% of the oil from yellow mustard flour into the emulsion fraction (Table 5-21, Table 5-2, and Fig. 5-1). The resulting emulsion was destabilized using three-stage THF treatment at 0.5:1 THF:oil weight ratio at which 97.0% of the oil was recovered into the oil-water-THF miscella followed by 99.3% conversion to FAME after dehydration. Therefore, in total, around 62.2% of the oil in the flour was recovered as standard biodiesel.

Extensive studies were also performed in our laboratory by Soltero (2013) who produced soluble protein isolate (SPI) and precipitated protein isolate (PPI) as high-quality food-grade protein products from skim fraction obtained during two-stage aqueous processing of the flour. As shown in Table 5-21, around 83.1% of the crude protein in the flour was extracted into the skim fraction at which 83.0% was recovered as PPI and SPI. So, in general, around 69% of the flour protein was recovered as high-quality protein products.

The fiber-rich solid residual fraction remained after aqueous processing of the flour contained 11.1% of the flour protein and 20.6% of the flour oil, and could be processed as a high-energy animal feed.

Table 5-21: The ultimate recovery of oil and protein from dehulled yellow mustard flour into biodiesel and high-quality food-grade protein products during our integrated process

<table>
<thead>
<tr>
<th>Oil Yields</th>
<th>Protein Yields</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oil Yields</strong></td>
<td><strong>Protein Yields (wt%)</strong></td>
</tr>
<tr>
<td><strong>Process Steps</strong></td>
<td><strong>Oil Distribution</strong></td>
</tr>
<tr>
<td>Two-stage AEP: pH 11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Flour into emulsion</td>
</tr>
<tr>
<td>Three-Stage Emulsion Destabilization&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Emulsion into miscella</td>
</tr>
<tr>
<td>Pseudo-Single-Phase Transmethylation&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Miscella into biodiesel (FAME)</td>
</tr>
<tr>
<td>Integrated Process</td>
<td>Flour into biodiesel (FAME)</td>
</tr>
</tbody>
</table>

<sup>a</sup> AEP:pH 11 is the optimal two-stage aqueous extraction process of dehulled yellow mustard flour at pH 11 (AEP(iii); Table 5-2; Fig. 5-1)

<sup>b</sup> The results are based on the three-stage destabilization of the emulsion using 0.5:1 THF:oil weight ratio (Fig. 5-15)

<sup>c</sup> Pseudo-single-phase transmethylation at 10 min using 14:1 methanol/oil molar ratio, room temperature, 1:1 THF/methanol volume ratio, and 1.2 wt% sodium hydroxide (Table 5-20)

<sup>d</sup> The membrane separation process via ultrafiltration and diafiltration developed in food engineering laboratory (Soltero, 2013) to produce soluble protein isolate (SPI) and precipitate protein isolate (PPI)
Several factors influence the economic analysis of our integrated process. These include cost of the starting materials, market value and acceptance, utilization of secondary products and, of course, the capital, design and operating costs. While the cost of implementation can strongly affect the ultimate process selection, the minimum standard for viability is that the sales value of process products must be able to regain the cost of the starting materials. Table 5-22 shows the breakdown of market prices for mustard seed products. In our process, the protein isolates (PPI and SPI) are the primary products, while biodiesel and fiber-rich residual fraction are secondary products. Table 5-22 reports product margin only, that is, the values of the input seeds versus the final products. The preliminary economic analysis showed an overall positive margin for our integrated process.

Table 5-22: Preliminary economic analysis of our integrated process

<table>
<thead>
<tr>
<th>List of Inputs</th>
<th>Weight (Kg)</th>
<th>Price ($/Kg)</th>
<th>Value ($)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mustard Seed</td>
<td>1000</td>
<td>0.34</td>
<td>340</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>List of Products</th>
<th>Weight (Kg)</th>
<th>Price ($/Kg)</th>
<th>Value ($)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPI</td>
<td>244</td>
<td>4.75</td>
<td>1159</td>
</tr>
<tr>
<td>SPI</td>
<td>43</td>
<td>4.75</td>
<td>204</td>
</tr>
<tr>
<td>Biodiesel</td>
<td>200</td>
<td>(~60 gallon)</td>
<td>(3.86 $/gallon)</td>
</tr>
<tr>
<td>Fiber-rich fraction</td>
<td>247</td>
<td>0.40</td>
<td>99</td>
</tr>
</tbody>
</table>

a Balke, 2006
b Soltero, 2013
c Biodiesel (B100) density was considered as 0.88g/cm³
d US Department of Energy. Alternative Fuels Data Centre
CHAPTER 6

6 Conclusions

This project focused on development of an aqueous extraction process for yellow mustard consisting of aqueous dissolution of the protein for isolate production, and utilization of the oil, which was recovered in the form of a stable oil-in-water emulsion. The main objective was to investigate the use of a single solvent, such as pure THF or dioxane to solubilize the emulsion to recover a homogeneous oil-solvent-water miscella to be used directly in the catalytic transesterification based on Dr. Boocock’s biodiesel production method after water removal if needed.

All of the objectives determined for this project in order to design an integrated process for production of high-quality protein products and biodiesel from dehulled yellow mustard flour were achieved through the course of this investigation. Five different journal papers were prepared. Three papers were published in the Journal of the American Oil Chemists’ Society (JAOCS) based on the single-stage and three-stage destabilization of the emulsion using DMF, THF, and 1,4-dioxane (Tabtabaei and Diosady, 2012, Tabtabaei et al., 2013, Tabtabaei et al., 2014a). One paper was published in the Food Research International based on the optimization of aqueous extraction processes (Tabtabaei and Diosady, 2013), and one paper was prepared based on the adsorptive dehydration of miscellae for production of high-purity biodiesel for submission to the JAOCS (Tabtabaei et al., 2014b). It is also suggested to seek a patent protection on the whole integrated process investigated in this dissertation.

Finally, our integrated process consisting of aqueous contact of mustard flour to recover protein as the main product followed by multi-stage destabilization of the resulting emulsion with THF will produce high-quality protein products that will not have been exposed to any organic solvents throughout the process, while biodiesel will be efficiently recovered using less flammable solvent than conventional hexane extraction. The following conclusions were drawn in attempts to design our integrated process.

1. The two-stage aqueous extraction process at pH 11 (AEP: pH 11) presents an opportunity for simultaneous recovery of oil and protein from yellow mustard flour. In this process, around
64.6% of the oil in the original flour was extracted into the emulsion phase, 14.8% extracted into the skim, and 20.6% remained in the solid residue. Over 83% of the total protein present in the flour was extracted into the skim and 3.2% to the emulsion, while 11.1% remained in the solid residue. Although this extraction process resulted in the formation of stable multilayer emulsion, it was selected as an optimal process due to the desirable distribution of protein between emulsion and skim fractions.

2. Enzymatic demulsification of the emulsion with endoprotease Protex 6L recovered over 94% of the oil in the emulsion. However, enzymatic treatments would be too expensive to be effectively used in industrial scale.

3. Of the three solvents (THF, DMF, and 1,4-dioxane) tested for the chemical destabilization of the alkaline emulsion, THF was the most successful. Single-stage treatment of emulsion at 4:1 THF:oil weigh ratio recovered over 97% of the oil and 30% of the water in the homogenous miscella phase. The application of the oil/water/THF phase diagram enabled us to design a three-stage destabilization process at 0.5:1 THF:oil weight ratio that recovered 97% of the oil and 4% of the water in the oil-rich miscella suitable for direct conversion to biodiesel without additional THF removal after reducing the water content to 0.3%.

The destabilization of the emulsion with dioxane was less desirable. The preparation of oil/water/dioxane ternary phase diagram confirmed the more hydrophilic behavior of dioxane in the oil-water system compared to THF. Furthermore, three- and four-stage destabilization processes of yellow mustard emulsion at lower dioxane:oil weight ratios showed no significant advantages over single-stage destabilization.

DMF was not suitable for destabilizing the emulsion. It recovered only some 38±3% of the oil in the emulsion even at high weight ratios since DMF is widely miscible with water, preventing separation of the oil from the emulsion. To clarify this, the ternary phase diagram of DMF/oil/water was prepared and confirmed the limited solubility of the oil in DMF in the presence of water.

4. The FFAs and phospholipids contents of the oil recovered from homogeneous oil-water-THF combined miscellae were much lower than the needed level for transesterification. Therefore,
these miscella phases proved to be excellent feedstocks for the preparation of high-purity methyl esters through single-phase base-catalyzed transmethylation after reducing the water content to the level required for standard biodiesel (0.3 %).

5. The oil-water-THF miscellae obtained by three-stage destabilization of the emulsion at 0.5:1 and 0.75:1 THF:oil weight ratios were successfully dehydrated on zeolite 4A using both batch and continuous fixed bed systems.

5a. The Freundlich and Langmuir adsorption models were first used for the mathematical description of the adsorption of water by zeolite 4A. The adsorption equilibrium data fitted very well to the Langmuir adsorption model. The Langmuir constants were determined and used to compare water removal from the miscellae based on their compositions.

5b. During the batch studies, the miscellae were successfully dehydrated as water removal percentages of over 97 % were achieved at 130 min and room temperature using the optimum adsorbent dosage of 0.15 g/g. The thermal regeneration of the zeolite 4A at 275 °C for 6 h could be practically repeated for four cycles to successfully dehydrate the miscellae to the quality standards for biodiesel feedstock.

5c. Breakthrough curves were determined during the continuous fixed-bed adsorption studies and long breakthrough times of 14.2 and 44.6 h were obtained using 111.1 g of zeolite 4A for the miscellae produced during three-stage emulsion destabilization at 0.75:1 and 0.5:1 THF:oil weight ratios, respectively.

5d. The dehydrated miscella was reacted with methanol in a single-phase base-catalyzed transmethylation process where 99.3 % of FAME was recovered after 10 min, satisfying the ASTM standard in terms of total glycerol content ($G_T$) and acid number.

Based on the results of this study, yellow mustard could be considered as a renewable biofuel feedstock in which the oil could be directly recovered as standard FAME by a combined THF extraction/adsorption process without any additional refining steps. Our integrated process could be economically very attractive as it produces food-grade protein products as well as high-grade biodiesel from an underutilized Canadian crop.
CHAPTER 7

7 Recommendations

In order to complete our integrated process based on the results reported in the previous sections, some further work should be completed, as discussed in detail in the following subsections.

7.1 Aqueous Extraction of Oil and Protein from Dehulled Yellow Mustard Flour

The commercialization of our integrated process requires the production of both food-grade protein isolates and high-purity biodiesel. While over 96% of the oil in the emulsion was successfully recovered as standard FAME, the production of high-quality protein isolates represents the greatest challenge due to the substantial amounts of oil remained in the protein-rich skim fraction after centrifugation. The oil remaining in the skim fraction during two-stage AEP of mustard flour at pH 11 was 14.8% of the total oil. This is a considerable loss of the oil product as well as a difficult impurity to remove from a skim protein product. Consequently, the quantity of oil remaining in the skim fraction after centrifugation remains a major challenge for aqueous processing of mustard flour; therefore, further investigation is necessary to recover the oil prior to ultra-filtration and dia-filtration processes.

In our group, extensive studies were performed by Soltero (2013) who produced lipoprotein products, soluble protein isolate (SPI) and precipitated protein isolate (PPI), from the skim fraction containing 14.8% of the total oil. These lipoprotein products should be tested in future to evaluate their binding and emulsifying properties.

I previously determined the quantity of the phospholipids in the emulsion fraction; however, it is recommended to evaluate the quantity and composition of the phospholipids in the starting material and all of the fractions (emulsion, skim and solid residual fractions) produced from the AEP of mustard flour. This approach helps us to examine the effect of phospholipids on the stability of the emulsion and skim fractions, thus developing effective destabilization processes to recover oil.
It is also recommended to find an application for the insoluble fibre-rich solid residual fraction to improve the economic viability of our AEP technology. While the solid residual fraction can be processed as a high-energy animal feed, it might be also considered as a renewable feedstock for the production of bio-ethanol.

### 7.2 Treatment of Yellow Mustard Emulsion with Organic Solvents

The emulsion treatments with THF and dioxane were performed at room temperature during the course of this investigation. However, the destabilization of the emulsion at higher temperatures close to the boiling point of THF could improve the recovery of the oil. Therefore, it is necessary to evaluate the role of temperature in oil recovery during emulsion destabilization, and to analyze the quality of the resulting miscellae in terms of FFA and phospholipids.

Three-stage treatment of the emulsion with THF at room temperature was effective in recovering over 97% of the oil from the emulsion into the low-phosphorous and low-free fatty acid (FFA) oil-solvent-water miscella. As described in section 5.3.4, the three-stage destabilization of the emulsion resulted in the formation of three different fractions: an oil-rich miscella phase, a water-rich polar phase, and an emulsion precipitate phase. The water-rich polar phase contains considerable amounts of THF that should be separated from water for recycling. Many studies have been performed previously on THF-water mixtures to separate THF for recycling purposes. THF is miscible with water in all proportion and it forms an azeotrope at 83 mol% THF in water. Thus, conventional distillation cannot be used for separation of aqueous THF. Therefore, various alternative separation technologies including extractive and azeotropic distillation, reactive distillation, pressure swing distillation, liquid–liquid extraction, adsorption, salt addition and membrane based pervaporation are recommended for THF separation from polar phases remaining after emulsion destabilization.

The destabilization of the emulsion resulted in the formation of low-phosphorous and low-free fatty acid (FFA) oil-water-THF miscella suitable for direct conversion to biodiesel without additional refining. Therefore, it is recommended to evaluate the distribution of the phospholipids and FFAs in the miscella, polar, and emulsion precipitate phases to precisely understand the behaviour of the destabilization process.
As described in sections 5.4.5, the addition of methanol to the dried oil-THF miscella resulted in the formation of standard FAME. After biodiesel production through pseudo-single-phase transmethylation, in a large full-scale industrial plant, the reaction mixture is transferred to a rotovapor and the majority of the methanol and THF are separated from biodiesel. In our integrated process, the recovered solution of THF-methanol cannot be reused directly for emulsion destabilization before separation of the methanol from THF. Unfortunately, the separation of methanol-THF mixture is difficult by conventional distillation. Therefore, it is essential to evaluate the prevaporation technology for separation of THF from methanol or to investigate the use of THF-methanol mixture for emulsion destabilization.

### 7.3 Water Removal

The preliminary studies of continuous adsorptive dehydration of oil-water-THF miscellae were conducted in this dissertation. It is recommended to perform more studies using packed bed column to optimize the influencing parameters of flow rate, bed height and miscella composition.

It is also necessary to develop efficient regeneration technologies for 4A molecular sieve since thermal regeneration was not an effective method for multi-cycle regeneration of adsorbents. The use of binderless molecular sieve or organic solvents such as acetone during regeneration might improve the life of the adsorbents.

### 7.4 Assessing Financial Viability of the Process

The preliminary economic analysis of our integrated process for the production of protein isolates and biodiesel was impressive. However, it is essential to perform a simulated economic analysis on the process for commercialization purposes. The evaluation of process economics might be performed by using related capital cost estimation softwares such as CAPCOST to evaluate the rate of return on the investment by including the capital, design, manufacturing and operating costs.
8 References


JUNG, T. Y. M. (2011) Simultaneous Protein and Biodiesel Production from Yellow Mustard Seed with Isopropyl Alcohol Extraction. Toronto, University of Toronto.


PIERCE TECHNICAL RESOURCE Acetone precipitation of proteins.  


APPENDIX A

Analytical and Experimental Methods
A1. Determination of Oil Content using Mojonnier Method

All measurements were performed in triplicate. For all analysis, corks were soaked in water for 1 h to improve seal.

• For solid samples: AOAC Method 922.06. Fat in Flour (acid hydrolysis)

1. Place 2g of sample (weigh to nearest 0.1mg) in 50mL beaker.
2. Add 2mL ethanol and stir to moisten particles to prevent lumping on addition of acid.
3. Add 10mL HCl (25+11), mix well, set beaker in water bath held at 70-80°C and stir in frequent intervals during 30-40min.
4. Add 10mL ethanol and cool.
5. Weigh four 150mL beakers to nearest 0.1mg.
6. Transfer mixture to Mojonnier flask. Rinse beaker into extraction flask with 25mL diethyl ether added in 3 portions, stopper flask (cork stopper) and shake vigorously for 1min.
7. Add 25mL petroleum ether and shake for 1min.
8. Let stand until upper liquid is practically clear. Draw off as much possible of ether-fat solution through filter consisting of cotton ball packed just firmly enough in funnel stem to let ether pass freely into weighted 150mL containing boiling chips.
9. Re-extract liquid remaining in flask twice, each time with only 15mL of each ether. Shake well on addition of each ether and draw off ether solution into same beaker. Wash tip of funnel and end of funnel with few-mL of mixture of ethers in equal volumes.
10. Evaporate ether on steam bath, then dry fat in oven at 100°C for 90min.
11. Remove beaker from oven, let stand and weight.
12. Run blank using only reagents for each set of experiments.

13. Calculation:

\[
\text{Oil (\%)} = \frac{[(\text{weight beaker + fat}) - (\text{weight beaker})] - \text{weight blank}}{\text{weight sample}} \times 100
\]
• For emulsions: AOAC Method 995.19. Fat in cream

1. Place test sample in water bath at 38 ± 1°C. Mix thoroughly and weight aliquot immediately. Do not let samples remain in water bath more than 15min after reaching 38°C.
2. Weight empty flask.
3. Pipet into flask enough cream to yield 0.3-0.6g of extracted fat (0.8g 60% emulsion, 1g 40% emulsion, 2g 20% emulsion) and weight to the nearest 0.1mg.
4. Dilute test portion with 10mL distilled water at room temperature.
5. Weigh four 150mL beakers to the nearest 0.1mg.
6. To test portions in flask add 1.5mL NH₄OH and mix thoroughly.
7. Add 3 drops of phenolphthalein indicator to sharpen visual appearance of interface.
8. Add 10mL ethanol, stopper with water-soaked cork and shake vigorously 15s.
9. For first extraction add 25mL diethyl ether, stopper with cork and shake for 1min. Hold body of flask horizontally with lower bulb and stopper up.
10. Loosen cork gently to release built-up pressure. Add 25mL petroleum ether, shake for 1min.
11. Let stand until ether phase and the pink aqueous phase are separated and transfer ether-fat solution to weighted beaker.
12. For second extraction add 5mL ethanol, stopper with same cork used for first extraction and shake 15s.
13. Add 15mL diethyl ether, replace cork and shake 1min.
14. Add 15mL petroleum ether, stopper with same cork and shake 1min.
15. Let phases separate. If the interface is below neck of flask, add water to bring level half way up neck. Add water slowly to cause minimum disturbance of separation. Decant ether solution into same beaker used for first extraction.
16. For third extraction omit addition of ethanol and repeat procedure for second extraction.
17. Evaporate solvents in the fumehood on hot plate. Dry extracted fat at 100°C for 90min.
18. Remove beaker from oven and place them in a dessicator. Cool down and weigh.
19. Run 1 blank with reagents and substitute emulsion with 10mL distilled water. Reagent blank should be < 0.0020g residue.
20. Calculation:

\[
\text{Oil (\%)} = \frac{[(\text{weight beaker} + \text{fat}) - (\text{weight beaker})] - \text{weight blank}}{\text{(weight flask + sample) - (weight flask)}} \times 100
\]
A.2. Oil Content in Miscella

All measurements performed in triplicate for miscella oil content determination.

1. Weigh 50mL beaker.
2. Make sure miscella is well mixed and take ~ 20-25mL of sample. Weigh to nearest 0.1mg.
3. Evaporate solvents in fume hood on hot plate. Dry fat in oven at 100°C for 1h.
4. Cool samples and record weight.
5. Calculation:

\[
\text{Oil (\%)} = \frac{[(\text{weight beaker} + \text{fat}) - (\text{weight beaker})]}{\text{(weight beaker + sample) - ( weight beaker)}} \times 100
\]
A.3. Determination of Protein Content using Kjeldahl Method (AOCS Method Ba4d-90)

• Kjeldahl Digestion:
1. Preheat the Digestion Unit to setting 4.
2. Place sample in the Buchi tubes. All determinations should be done in triplicate and one tube for the blank. The weight of the sample should be enough to guarantee a reading of 10mL in the titration. If the sample is 100% protein, around 0.1g of sample should be used.
   - For solids samples: For mustard flour (30% protein) weight around 0.3g. For emulsion around 2g. Too much sample for emulsion can make the digestion very long and with too much foaming. Blank: weighing paper.
   - For liquid samples: For extract (4% protein) weigh 2-2.5g. Blank: distilled water.
3. Add 4 Kjeldahl tablets to each tube.
4. Add 25mL concentrated sulphuric acid to each tube.
5. Place the glass manifold on the tubes and make sure there is glass fibre on the side of the blank. Use the clamp wires to tighten the tubes and the manifold.
6. Connect the suction tube to the glass manifold and turn on the tap water.
7. Place the tubes on the digestion unit and run on setting 4 for 20min. Then move to setting 6 for 10min and setting 10 for 35min. The walls of the tubes must be clean and the solution should be colourless for the last 10 min of digestion. This is standard time; however, sometimes samples can take longer. If there is foam formation please removes the samples from the digestion unit and cool down, then place the tubes back on the digestion unit.
8. Remove the tubes form the digester and place in a rack with the water running for 20min until the solution is cool. The tubes can be then removed and the rack placed in the fumehood. When the tubes cool, a white precipitate will appear. Distillation can be performed later on the day or on a different day. If solution is too viscous because digestion took too long, distillation should be done on the same day.

• Kjeldahl Distillation:
1. Remove the glass manifold and be careful not to spill any condensed acid. Rinse with distilled water.
2. Swirl the sample and make sure the white precipitate mixes with the clear liquid. Add 50mL distilled water and mix very well. The solution should be completely clear at this point.
3. Add 25mL sodium thiosulfate (8%), swirl the samples and cover. The solution will turn dark gray. Swirl the samples every 5 min for the next 15 min to increase the extent of the reaction in the tubes.

4. Check the distillation unit and make sure the distilled water compartment is ¾ full and the 32% NaOH solution 1/3 full.

5. Turn on the tap water and turn on the distillation unit and wait until it beeps, indicating it is ready for use.

6. Place 60mL boric acid (4%) into Erlenmeyer flasks and add 3 drops of indicator.

7. After hearing the beep, open the glass compartment and remove tube. Replace water in the tube with fresh distilled water and fill it halfway. Replace water in the Erlenmeyer with distilled water as well.

8. Set distillation time to 2 min by pressing the time button and then press start. The machine will beep when it is finished. Wait until there is no more noise and remove the tube using the tongs.

9. Replace water tube with blank tube and the Erlenmeyer flask with one with boric acid solution. Ensure the tip of the tube is fully submerged in the solution. Place paper towels underneath if necessary.

10. Add 90mL NaOH solution by pressing the reagent button until the 180mL mark. The button should be pressed twice to ensure it is supplied manually, otherwise the machine will do it automatically. Enough NaOH should be added until there are no more bubbles in the solution indicating it has been neutralized.

11. Set time to 5min and press start to run distillation.

12. The machine will beep when it is finished. Wait until there are no more noises and use the tongs to remove the tube and put it back on the rack.

13. Remove the Erlenmeyer flask and rinse the tip with distilled water. The pink solution should be light pink or clear.

14. Repeat steps 9 to 13 by placing a new sample tube and a new Erlenmeyer flask with boric acid solution. The solution in the Erlenmeyer will turn green indicating the presence of nitrogen in the sample.

15. Once all the samples have been distilled, place a tube with fresh distilled water (half way) and an Erlenmeyer with fresh distilled water. Run distillation for 5min.

16. Repeat the cleaning procedure as many times as necessary.
17. When the machine is clean, replace tube and Erlenmeyer with fresh distilled water, turn off the unit and turn off the tap water.

18. Sing the logbook.

• Titration:

1. Pump 0.1N sulphuric acid slowly until the burette is set to 0mL.
2. Titrate the sample until the pink endpoint is reached. The sample will transit from green to pink. The endpoint should be the same as the blank.
3. Record the volume of the sulphuric acid used to reach the endpoint.

• Calculation:

\[
N \, (\%) = \frac{[\text{H}_2\text{SO}_4 \, \text{ml used for sample} - \text{H}_2\text{SO}_4 \, \text{ml used for blank}]}{\text{Weight of sample}} \times 0.1N \times 1.4
\]

Protein \,(\%) = N \,(\%) \times 6.25
A.4. Determination of Moisture Content (AACC Method 44-15A)

Always perform in duplicate or triplicate.

1. Weigh an aluminum tray and record weight.
2. Weight sample in aluminum tray and record weight.
3. Put sample in oven at 105 °C for at least 20h, preferably 24h.
4. Remove samples from oven, cool in a dessicator and record weight.

5. *Calculation:*

\[
\text{Moisture (\%)} = \left[ \frac{(\text{weight sample + tray})_{\text{wet}} - (\text{weight sample + tray})_{\text{dry}}}{(\text{weight sample + tray})_{\text{wet}} - \text{weight tray}} \right] \times 100
\]