PROCESS EVALUATION FOR WHEAT GERM

OIL DEGUMMING

By

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ABSTRACT

The objectives of this study are to optimize a degumming process that will efficiently remove phospholipids from crude wheat germ oil (CWGO) with minimal oil loss and to examine the effect of degumming processes on oil quality and bioactive compounds naturally present in wheat germ oil (WGO). The following methods were used to remove phospholipids: water, acid (citric and phosphoric acids) and enzymatic (Lecitase Ultra and Lysomax) degumming processes. The phosphorus content was 1,860 mg/kg in hexane- extracted and 1,360 mg/kg in mechanically pressed CWGO. After the water degumming, about 56 and 23% phosphorus reduction were achieved in hexane-extracted and mechanically pressed WGO, respectively. Both citric and phosphoric acid degumming were less effective than water degumming and reduced the amount of phosphorus about 39 and 47% in hexane- extracted and 16 and 13% in mechanically extracted WGO, respectively. Enzymatic degumming, which decreased the amount of phosphorus content about 88, 84 and 69% in hexane- extracted WGO and 82, 78 and 53% in mechanically pressed CWGO using Lecitase Ultra, Lysomax, and Gumzyme, respectively. Lecitase Ultra was the most effective enzyme to reduce phospholipid content of both hexane-extracted and mechanically pressed CWGO. Enzymatic degumming significantly increased the FFA content of the oil. All degumming processes resulted in tocopherol loss.

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NOMENCLATURE

ANOVA	Analysis of variance
CWGO	Crude wheat germ oil
ELSD	Evaporative light-scattering detector
EDTA	Ethylene Diamine Tetraacetic Acid
FD	Fluorescence Detector
HPLC	High-performance liquid chromatography
PA	Phosphatidic acid
PDA	Photo diode array detector
PC	Phosphatidylcholine
PE	Phosphatidylethanolamin
PI	Phosphatidylinositol
RSM	Response Surface Method
SC	Supercritical fluid
SLS	Sodium lauryl sulfate
TAG	Triacylglycerides
v/v	Volume/volume
WG	Wheat germ
WGO	Wheat germ oil
w/w	Weight/weight
w/v	Weight/volume

UNITS

%	Percentage
°C	Degree centigrade
g	Gram
h	Hour
kg	kilogram
mbar	Millibar
meq	Milliequivalent
mg	Milligram
mL	Milliliter
min	Minutes
MPa	Mega pascal
µm	Micrometer
mm	Millimeter
nm	Nanometer
ppm	Parts per million
rpm	Rotation per minute

CHAPTER I

INTRODUCTION

1.1 STATEMENT OF PROBLEM

Phospholipids are one group among the many compounds present in crude wheat germ oil (CWGO). The presence of phospholipids in oil leads to undesirable reactions that adversely affect both the color and flavor of the oil during storage, shipping, and cooking. The phospholipid content in crude vegetable oil can be minimized by degumming, which is the first step in the refining process to obtain edible oil. Conventional degumming processes are water and acid degumming. Hydratable phospholipids can be removed by water treatment, while removal of nonhydratable phospholipids requires acid degumming using phosphoric or citric acid. The water and acid degumming for removing phospholipids from CWGO has been reported to be inefficient. Hence, there is a need for development of an efficient CWGO refining process. Recently, enzymatic oil degumming has been attracting a lot of attention. Phospholipases are used for crude oil degumming. Although enzymatic degumming of a few major vegetable oils has been studied, the information on enzymatic degumming of CWGO is lacking.

1.2 HYPOTHESIS

Enzymatic degumming is a more efficient method than the conventional methods used for removing phospholipids from CWGO. It reduces oil losses that occur during refining.

1.3 OBJECTIVES

The main objective of this study is to optimize a degumming process that will efficiently remove phospholipids from CWGO with minimal oil loss. The specific objectives are as follows:

1. Examine the efficiency of water degumming for removing phospholipids from CWGO. Optimize process variables, water to oil ratio, time, and temperature for water degumming to achieve the lowest residual phospholipids with minimum oil loss.
2. Examine the effect of acid degumming on the residual phospholipid content in wheat germ oil (WGO). Determine the optimum conditions (type and concentration of acid, oil to water ratio, time, and temperature of the process) for high oil yield and low residual phospholipids.
3. Examine the effect of enzymatic degumming on the residual phospholipid content in WGO. Optimize the processing variables (enzyme concentrations, time, temperature and oil to water ratio) for two types of enzymes, Lecitase® Ultra, and Lysomax, to achieve minimum residual phospholipids in the degummed oil with minimal oil loss. Test the optimum condition with enzyme Gumzyme.
4. Examine the effect of degumming processes on oil quality and bioactive compounds naturally present in WGO.

CHAPTER II

LITERATURE REVIEW

2.1 WHEAT GRAIN AND GERM

Wheat is one of the most abundant and widely used cereal crops in the world. The wheat grain consists of 81-84% endosperm, 14-16% bran, and 2-3% germ (Atwell 2001). The goal of the wheat milling process is to produce flour with the maximum amount of endosperm and the minimum amount of bran and germ. In wheat flour production, except for whole wheat-flour, the germ and the bran are considered by-products (Atwell 2001).

Wheat germ is a by-product of the wheat milling industry (Dunford and Zhang 2003; Eisenmenger 2005). Germ contains 8–14% oil and can be separated from the grain during the milling process (Dunford and Zhang 2003). The chemical composition of wheat germ varies depending on variety and grade (Al-Kahtani 1989). The typical composition of commercial wheat germ is as follows: 6% moisture, 26% protein, 10% oil, 4% ash, 20% starch, 3% crude fiber, and 15% other substances (Barnes 1983; Zhu and Zhou 2005).The germ contains several essential nutrients, including vitamin B, vitamin E, calcium, magnesium, folic acid, essential fatty acids, and phosphorous (İbanoglu 2002).

2.2 EXTRACTION OF WHEAT GERM OIL

Wheat germ oil (WGO) can be extracted by mechanical pressing, solvent extraction, supercritical carbon dioxide, or aqueous extraction of the germ. Solvent extraction results in high oil yield (> 90% recovery), while the oil yield by pressing is approximately 50% (Singh and Rice 1979). Pressed oil is commonly preferred by consumers as it is free of hazardous chemicals (Wang and Johnson 2001). Mechanically pressed WGO is commercially available. Aqueous extraction, which utilizes water rather than organic solvents to extract oil, was recommended as an alternative to the hexane extraction in the 1950s. Aqueous oil extraction is safe and inexpensive (Rosenthal and others 1996). However, low oil yield and emulsion formation during extraction that makes oil recovery difficult are some of the disadvantages of aqueous oil extraction. Enzyme-aided aqueous extraction improves oil yields. Although aqueous and enzymatic extraction of WGO have been examined, these processes have not been commercialized yet (Xie and others 2011). There are also studies reporting utilization of supercritical fluid technology for the extraction of WGO (Eisenmenger and Dunford 2008; Piras and others 2009). Eisenmenger (2005) has reported that supercritical carbon dioxide extracted oil had a lighter color and less phosphorus compared to oil produced by conventional solvent extraction. Hexane extracted CWGO is commonly dark-colored and sometimes has a strong odor and flavor depending on the degree of oil oxidation (Eisenmenger 2005).

2.3 CHEMICAL COMPOSITION OF WHEAT GERM OIL

Wheat germ oil has a number of nutritional and health benefits such as reducing plasma and liver cholesterol levels (Ge and others 2000) and improving physical

endurance of humans. The latter effect is attributed to its high policosanol content, specifically its high octacosanol content (Cureton and Pohndorf 1972). WGO has been used as a food additive for its antioxidant activity (Krings and others 2000; Wang and Johnson 2001), as a fertility agent in the pharmaceutical industry and in cosmetic formulations (Kahlon 1989). Wheat germ products are also sold as dietary supplements for farm animals, racehorses, and pets (Kahlon 1989). Wheat germ is one of the richest natural sources of α-tocopherol, which possesses vitamin E activity (Ge and others 2002). WGO has the highest tocopherol content (vitamin E) of all vegetable oils, about 2,500 ppm or higher (Piras and others 2009). Among the four tocopherols, α-, β-, γ- and δ, α-tocopherol displays the highest biological activity, and represents around 80-90% of the total content of tocopherols in WGO, while β- tocopherol is the second most abundant tocopherol in WGO (Eisenmenger and Dunford 2008). Piras and others (2009) reported that different oil extraction methods did not result in significant change in α- tocopherol content in WGO. The wheat germ market is mainly based on its high vitamin E content. WGO is marketed in bottles or in capsules and also added to lecithin and liver oil (Kahlon 1989). There is an interest in wheat germ octacosanol as a potential nutraceutical and functional food ingredient (Kahlon 1989).

WGO is a good source of polyunsaturated fatty acids (Wang and Johnson 2001). The main fatty acid in WGO, linoleic acid, is an essential fatty acid and may comprise about 60% of the total fatty acids in WGO. Unsaturated fatty acids such as oleic, linoleic and linolenic acids represent over 80% of the total fatty acids in WGO (Barnes 1982). The fatty acid composition of WGO is as follows: 42–59% linoleic acid, 12–28% oleic acid, 11–19% palmitic acid, 2–11% α-linolenic acid and about 1% stearic acid (Chang

and others 2010). Eisenmenger and Dunford (2008) found that supercritical fluid extracted WGO had higher linoleic acid content than commercial oils. This might be due to the compositional variations in wheat germ from different sources.

The free fatty acid (FFA) content of CWGO is usually very high and varies between 5 and 25%, depending on the conditions of germ separation and storage and the oil extraction process. FFA often contributes to a bitter and soapy flavor in food and accelerates oil oxidation (Wang and Johnson 2001). Hence, FFA is removed during the refining process.

WGO is also rich in phytosterols and policosanols (Atwell 2001). WGO contains significantly higher amounts of phytosterols than other commercial oils (Itoh and others 1973). Sitosterol (60-70%) and campesterol (20-30%) are the two major phytosterols present in WGO (Anderson and others 1926; Itoh and others 1973). Hexacosanol, octacosanol and triacosanol are the major policosanols found in WGO (Lin and others 2004).The policosanol contents and compositions of wheat fractions, straw, bran, and germ have been studied (Irmak and Dunford 2005). In the latter study it was found that the precipitate formed during cold storage of commercial hexane-extracted WGO contained the highest total policosanol contents (628 ppm) among the wheat extracts and milling products (germ, bran, shorts and flour) examined. It was also reported that policosanol content of the solid fraction precipitated at the bottom of the container containing CWGO stored in a refrigerator was higher (17 times higher) than that of the clear CWGO oil above the precipitate (Irmak and others 2006). This result was predictable since policosanol is part of the wax that precipitates at cold temperatures.

Although phospholipids are minor compounds in oil, (1-3% of the oil), they play a significant role in oil quality. In general phospholipid content of CWGO (1300-2500 mg/kg) is higher than other vegetable oils (300-800 mg/kg). The major phospholipids found in vegetable oils include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidic acid (PA) (Eisenmenger 2005). PC represents about 40-60% of total phospholipids in WGO. PE (9-15%) and PI (13-20%), are also present in significant amounts in WGO (Hargin and Morrison 1980).

2.4 CHEMICAL STRUCTURE OF TRIACYLGLYCEROL AND PHOSPHOLIPIDS

Triacylglycerides (TAG) are the main components of vegetable oils comprising > 90% of the oil. The chemical structures of TAG and phospholipid molecules are similar; both are built on a glycerol backbone and contain fatty acids of different chain lengths (Figure 1). On a TAG molecule all three positions on the glycerol backbone (1, 2 and 3) are occupied by fatty acid molecules. In the case of phospholipids a phosphorus group is attached to the 3rd position on the glycerol molecule. In most cases phospholipids have a saturated fatty acid in the 1st position and an unsaturated fatty acid in the 2nd position (Figure 2). TAG is a nonpolar molecule while a phospholipid has both a nonpolar (fatty acid region) and a polar (a functional group containing a phosphatide) regions that make the phospholipid molecule an amphiphile (has both hydrophilic and lipophilic properties) (Dayton and Galhardo 2012).

2.5 CRUDE OIL REFINING

The goal of oil refining is to remove undesirable compounds that have adverse effects on total quality, taste, odor, appearance and storability of the oil (Carr 1978).

Because of the presence of a wide variety of undesirable compounds in crude oil such as FFA, metal ions, color and odor compounds and gums, a number of chemical and physical refining processes are required to produce a good quality product (Jahani and others 2008).

2.5.1 DEGUMMING

Phospholipids must be removed to extend oil shelf life, avoid precipitation during storage and transportation and prevent darkening during high temperature oil processing and applications such as deodorization and frying. Degumming, the first step in the refining process, mainly removes phospholipids along with some of the waxes and other impurities (Carr 1978; Subramanian and others 1999). Degumming may be conducted either as a separate operation or simultaneous with neutralization. For oils rich in phospholipids such as soybean and canola oils, degumming is usually a separate operation. There are several degumming techniques used in industry: water degumming, super degumming, acid degumming, total degumming, enzymatic degumming and ultrafiltration (Xu and Diosady 2004; Yang and others 2006a).

2.5.1.1 WATER DEGUMMING

Water degumming is the method that is commonly used in industry. Hydratable phospholipids are removed from the oil by treatment with water or steam. The hydrated phospholipids become immiscible in the oil and are separated from the oil by centrifugation (Subramanian and others 1999). During water degumming, the temperature of the oil must be controlled, usually in the range of 50-80°C. The high temperature helps to break-up any emulsion that might have formed during degumming and therefore lowers the oil viscosity.

Hydration and coagulation steps typically require 15 to 30 min residence time in the mixing tank. De and Patel (2010) reported that the phosphorus content of rice bran oil was reduced from 310 to 95 mg/kg during degumming with 2% (w/w) water in 30 min. In general phosphorous rather than phospholipid content of the oil is monitored to measure the degumming process efficiency. The analytical method used for phospholipid analysis requires an expensive instrument, HPLC, while phosphorous content in the oil can be analyzed by using instruments commonly found in chemistry laboratories, crucibles, an oven, furnace, and a spectrophotometer. The value measured for phosphorus content is converted to phospholipid by multiplying it by a factor (30) that is calculated based on the phospholipid composition of the oil to converting percent phosphorus to percent phospholipid in the oil (Smouse 1995).

Water degumming can be carried out in batch or continuous mode. In the batch treatment, a longer time is needed because of poor contact between water and oil, and the temperature should be 60-70°C. In continuous operations oil is preheated to a higher temperature, 70-80°C, in a heat exchanger before it is mixed with water. A higher temperature in continuous operations lowers oil viscosity, which facilitates better contact and consequently a shorter residence time is needed (Xu and Diosady 2004). About 1-2% water is added to the oil during water degumming for both batch and continuous processes. The quality of water used is of crucial importance; high levels of dissolved minerals in the water could interfere with the degumming process.

Zufarov and others (2008) described the water degumming process for crude rapeseed and sunflower oils that was carried out by heating the oils to 80°C, mixing with water (about 5% by volume) and stirring the mixture for 15 min. Then the mixture was

centrifuged for 20 min. After water degumming the amount of phosphorus decreased from 864 to 70 mg/kg in rapeseed oil extracted with solvent and from 156 to 61 mg/kg in pressed oil. In solvent extracted and pressed sunflower oils, the amount of phosphorus also significantly decreased from 294 to 57 mg/kg and from 96 to 50 mg/kg, respectively (Zufarov and others 2008). Water degumming may be sufficient for vegetable oils with low nonhydratable phospholipid content and 99% of phospholipids can be removed by heating oil to 50–80 °C with water to the oils with high hydratable phospholipids. However, water degumming does not remove nonhydratable salts of phosphatidic acids; therefore, it is not efficient for oils with high nonhydratable phospholipid content.

2.5.1.2 ACID DEGUMMING

Oils with high nonhydratable phospholipid content require acid degumming, which can be applied directly to crude oil or as a second treatment after water degumming. Nontoxic acids such as phosphoric or citric acid are commonly used (Dijkstra and Van Opstal 1989). Citric acid is more expensive than phosphoric acid, but it is more efficient at removing nonhydratable phospholipids. Citric acid forms a complex with Ca and Mg and also works as a chelating agent to keep the metals in a water-soluble complex (Zufarov and others 2008).

Acid degumming was more effective in reducing phosphorous content in rapeseed and sunflower oil than water degumming (Zufarov and others 2008). The acid degumming was carried out at 80 °C by initially adding 2% (based on volume of oil) aqueous citric acid solution (30% acid in water) to oil and stirring the mixture for 20 min. Then 1% water (based on oil/acid volume) was added and the mixture was stirred for 1 h prior to centrifugation for 20 min. The amount of phosphorus in solvent extracted and

pressed sunflower oils significantly decreased from 294 to 12 mg/kg and from 96 to 7 mg/kg, respectively, after acid degumming. Higher residual phosphorous amount in the solvent extracted oil may be due to the significantly higher phosphorous in the solvent extracted oil than that in pressed oil. For rapeseed oil acid degumming reduced phosphorous content of hexane extracted and pressed oils from 864 and 156 mg/kg to 22 and 17 mg/kg, respectively. Therefore, acid degumming is more effective than water degumming and the efficiency of degumming process is affected by the amount of phosphorous present in the starting material (crude oil).

Acid degumming of CWGO is generally carried out by adding 0.15% (based on oil volume) of phosphoric acid (85% concentration) to the heated oil (60°C) followed by 4% (based on oil weight) distilled water addition to the mixture (Wang and Johnson 2001). The oil is mixed at 250 rpm for 1 h, and then mixing speed is gradually reduced over a one hour period. This process which reduced the phosphorus content of the oil from 1,428 to 1,082 mg/kg was not very effective because over 1,000 mg/kg phosphorous in oil is still too high. The difficulty in degumming WGO is due to very slow and incomplete hydration of the phospholipids. It is probable that a large amount of nonhydratable phospholipids is formed in oil during wheat milling and/or WG storage and handling.

2.5.1.3 ENZYMATIC DEGUMMING

As the name implies, enzymatic degumming utilizes enzymes, which are proteins with the ability to catalyze chemical reactions. In general enzymes are nontoxic and are preferred to traditional food processing approaches using harmful chemicals.

2.5.1.3.1 PHOSPHOLIPASES

Enzymes that react specifically with phospholipids are known as phospholipases. The biochemistry of phospholipases are reviewed in more detail elsewhere (Servi 1999). The term phospholipase refers to five different enzyme types (phospholipase A1, A2, B, C, and D, abbreviated as PLA1, PLA2, PLB, PLC, and PLD, respectively) based on the bond they react on a phospholipid molecule (Dijkstra 2010). Figure 3 shows the bonds on a phospholipid molecule that different types of phospholipases act on (Dijkstra 2011; Dayton and Galhardo 2012). PLA1 removes the fatty acid attached to the1st position on a phospholipid molecule and produces a 1-lyso-phospholipid with one fatty acid remaining in the 2nd position (Figure 4). PLA2 removes the fatty acid attached to the phospholipid on the 2nd position and produces a 2-lyso-phospholipid with one fatty acid remaining on the 1st position (Figure 5). PLB, also known as a lyso-phospholipase, reacts with lyso-phospholipids removing the remaining fatty acid attached to the glycerol backbone and produce a glycerol- phospholipid (Figure 6). Both PLC and PLD attack the phosphoric di-ester bond (Figure 3). PLC cleaves the phosphate group from the phospholipid creating a diacylglycerol (Figure 7) while PLD cleaves the functional group creating a phosphatidic acid (Figure 8) (Dayton and Galhardo 2012). According to the literature five commercial phospholipases have been used for oil degumming: Lecitase® 10L (pancreatic PLA2), Lecitase® Novo (PLA1 from *Fusarium oxysporum*), Lecitase® Ultra (PLA1 from *Thermomyces lanuginosus*/*F. oxysporum*), Purifine® (PLC from *Bacillus anthracis*/*Pichia pastoris*), and LysoMax® (PLA2/LAT from *Streptomyces violaceoruber*). But not all these enzymes are currently available, only Lecitase® Ultra,

LysoMax® and Purifine® are currently available for commercial degumming (Yang and others 2008; Dijkstra 2010).

Earlier commercial phospholipases were produced from porcine or bovine pancreases. Later microbial phospholipases became commercially available. PLA1 and PLA2 are suitable for the enzymatic degumming of edible oils. Also, PLC is used as a yield-enhancing processing aid in edible oil degumming because it produces diacylglycerides that are soluble in oil and stay in oil after degumming, increasing product yield. When crude oil is degummed by using PLC and water (2-4%) at 60°C, PLC efficiently hydrolyzes the major phospholipids and generates 1,2-diacylglycerol oil and water soluble phosphate ester (Hodgson 1996). Phospholipase D (PLD) is an enzyme that catalyzes the conversion of phosphatidylcholine to choline and phosphatidic acid (PA) (Dayton and Galhardo 2012). PLD is present in seeds and is responsible for the destruction of PC and formation of PA during storage and processing.

Lecitase® Ultra is a microbial lipase developed by Novozymes, and it is a protein-engineered carboxylic ester hydrolase from *Thermomyces lanuginosus/ Fusarium oxysporum* (Yang and others 2006b). This enzyme, which is a food-grade PLA1, has activity towards both phospholipids and TAG (Dijkstra 2011; Casado and others 2012). Lecitase Ultra was used in this study for degumming of CWGO.

In 2009 Danisco® introduced a new enzymatic degumming method using the enzyme LysoMax, which is a lipid acyl transferase (PLA2) from *Bacillus licheniformis* (Dijkstra 2010). According to the company, LysoMax® improves the release of oil from the gum. This enzyme acts on all phospholipids and catalyzes the hydrolysis of the acyl moiety at the 2-position and produces a lysophospholipid which is more hydrophilic than

its non-hydrolysed precursor. Free fatty acid produced during hydrolysis can be esterified with a free sterol (Dijkstra 2011). LysoMax was also used in this study for degumming of CWGO.

Recently, DSM Food Specialties (Delft, Netherlands) introduced GumZyme™, which is a PLA2 produced by microbial fermentation of a selected strain of *Aspergillus niger.* This enzyme is Kosher and Halal certified and was claimed to result in high oil yields by promoting a more efficient oil and gum phase separation (Schulze B. and others 2011). This study also examined the efficiency of GumZyme for degumming CWGO.

2.5.1.3.2 ENZYMATIC DEGUMMING PROCESS

The first enzymatic degumming process was reported in the 1990s by the German company Roehm and Lurgi (Aalrust and others 1992). The latter process used an aqueous solution containing enzymes PLA1, PLA2 or PLB. The enzyme was mixed with water and the pH was adjusted to a desired value by using citric or phosphoric acid. Then the enzyme mixture was mixed with oil preheated to 50-75°C. A high speed shear mixer was used to form a water-in-oil emulsion with the phospholipids distributed in the water-oil interphase. The enzyme was allowed to react with the phospholipids for 5 to 6 h before centrifugation to obtain the degummed oil. The optimum pH for enzymes used in the degumming process varied between 4.0 and 5.0 (Dayton 2010).

Most enzyme reactions are reversible, therefore reaction conditions need to be controlled to minimize product dissociation (Servi 1999; Dayton and Galhardo 2012). Enzymatic degumming uses less water, acid, and base and generates less wastewater during crude oil refining than traditional degumming methods (Aalrust and others 1992). Depending on the site of hydrolysis during the enzyme treatment lysophospholipids,

FFA, diacylglycerols, choline phosphate, and other phosphatides are produced. Enzymatic degumming provides economic benefits by reducing the amount of oil loss (Dahlke and others 1995).

Enzymatic degumming of rapeseed oils with different phosphorus contents was examined with and without prior water degumming (Clausen 2001). Two types of enzymes, Lecitase 10L from porcine pancreas and Lecitase Novo from *Fusarium oxysporum,* were used in the process. Lecitase 10L decreased the phosphorus content in the water degummed rapeseed oil from 175-250 mg/kg to less than 10 mg/kg in 3.5 h and the optimum pH was 5.5. Lecitase Novo was also used for the water degummed rapeseed oil with phosphorus content of 175-250 mg/kg at 40-45°C and pH 5. The phosphorus content of the oil could be reduced to less than 10 mg/kg within 2 h by using 200 LEU/kg oil enzyme (LEU, one unit is equivalent to the amount of enzyme producing 1 μmole of free fatty acid per minute). Yang and others (2006a) described an enzymatic degumming process for rapeseed oil with phosphorus content of 212 mg/kg. The phosphorus content of the oil decreased to 34 mg/kg when citric acid was added to the oil and mixed at 80°C for 20 min. After the reaction mixture had cooled to 50°C and then reacted with 0.9-1.3 mL of 4% NaOH and 3 mL water, the phosphorous content of the oil could be reduced to less than 10 and 20 mg/kg with 30 mg/kg of enzyme and without enzyme, respectively, within 6 h. In another study crude rapeseed and soybean oils were degummed by using Lecitase Ultra (Yang and others 2006b). A stable emulsion was created by homogenizing the oil and citric acid mixture at 10,000 rpm at 80°C for 20 min. The phosphorus content in the rapeseed and soybean oils decreased from 123 and 150 mg/kg, respectively, to 35 mg/kg, prior to enzyme addition. Then the oil was degummed by adding the enzyme (30

mg/kg). The oil was degummed to less than 10 mg/kg phosphorous within 2 h, and then further reduction in phosphorus content, 8 mg/kg (rapeseed oil) and 6 mg/kg (soybean oil), was achieved within 5 h. Jahani and others (2008) optimized an enzymatic degumming process for rice bran oil. In the latter study, the phosphorus content of crude rice bran oil was 196 mg/kg, and Lecitase Ultra was used. When citric acid was added to the oil and mixed at 70°C for 30 min, the phosphorus content decreased to 39 mg/kg. After enzymatic degumming, the phosphorus content decreased to 5- 10 mg/kg in 5 h. In another study Lecitase Ultra was used to degum soybean oil (122 mg/kg phosphorous content) (Yang and others 2008). After the oil was treated with citric acid, the phosphorus content was reduced to 61 mg/kg due to coagulation and the precipitation of part of the phospholipids. The phosphorus content decreased to 10 mg/kg after enzymatic degumming (enzyme dosage 40 mg/kg) at pH 5. The gum and degummed oil could easily be separated by centrifugation with only 0.6% loss of oil in the gum by using the enzyme, while 15% oil was lost in the gum during acid degumming (citric acid). Bleaching process further reduced the phosphorus content to about 3 mg/kg. After the deodorization process, the phosphorus content was further reduced to 1-2 mg/kg. The enzymatic degumming of crude soybean oil was studied by Prabhaharan and Rakshit (2009). Crude oil (500 mL) was degummed with 3% citrate buffer solution (based on oil amount) and 1 mL phospholipase A1 (Lecitase Novo) by mixing the mixture at 1,000 rpm and 40°C to remove hydratable and nonhydratable phospholipids in the oil. The phosphorus content in the oil was reduced from 160-180 mg/kg to less than 10 mg/kg after 7 h of reaction. It was found that 3% total water level (1.5% water and 1.5% buffer) enhanced the degumming process when compared to 4 and 5% total water. The studies discussed above

clearly demonstrate that enzymatic degumming process parameters, oil/water ratio, temperature, reaction time and enzyme type need to be optimized for each application to achieve low phosphorous content and minimal oil loss.

2.5.1.4 OTHER METHODS OF DEGUMMING

Dry degumming combines degumming and bleaching steps in oil refining and is used for oils with low levels of phospholipids such as palm oil, lauric oil and coconut oil. The acid (phosphoric acid) is added to the hot oil (80–100 °C), at a concentration of 0.05–1.2% (based on volume of oil) and some water may also be added to enhance the bleaching efficiency. Then bleaching earth is added (about 1–3% of the oil weight). The amount of bleaching earth to be used in the dry-degumming process increases with increasing phospholipid content in the crude oil. The costs associated with high amount of bleaching earth use, disposal of spent earth and high oil loss during dry degumming limit the use of this method to oils with low phospholipid content (Čmolík and Pokorný 2000).

The total degumming process is designed to further treat the oil that has already been water degummed. The process has two variations to cater to different needs. In the first approach a dilute acid is dispersed into the oil, and high shear mixing is used. After sufficient contact time, a base (sodium hydroxide, sodium carbonate or sodium silicate) is added and mixed into the acid-in-oil dispersion to adjust the pH of the aqueous phase to between 5 and 7, where an emulsion is obtained without much soap formation. During the process the acid initially decomposes metal/phosphatidic acid (PA) complexes into insoluble metal salts and PA. PA is then hydrated by partial neutralization with the base added, and removed from the oil by centrifugation. The second approach uses a

combination of two centrifuges to remove the hydrated phospholipids with high efficiency and minimal losses. The first centrifuge removes the bulk of the gum phase. Since the gums are sticky and very viscous, their removal is largely incomplete, particularly during high-throughput operations. The second centrifuge is therefore put in line downstream from the first one to remove the remaining gums in the oil. This process is suitable for high phospholipid content oils such as sunflower, corn, soybean, peanut, and rice bran oils.

In super-degumming process, a concentrated (50%) solution of citric acid is added to crude oil (2% based on volume of oil), either water degummed or not, and heated to 70°C. After about 3 h reaction period, the mixture is cooled to below 40°C and held at this temperature for 30 min to 1 h. Then water (2.5% based on volume of oil) is added to the acid-oil mixture and the liquid phospholipid crystals formed during about one hour hydration period are removed by centrifugation (Čmolík and Pokorný 2000). The difference between super-degumming and acid degumming processes is the cooling step (below 40°C) to facilitate crystal formation, which leads to lower residual phosphorus levels in the degummed oil than the standard acid degumming. Extensive industrial experience has shown that super-degumming is applicable to almost all vegetable oils to achieve final phosphorus contents well below 30 mg/kg. This is a low cost method because of the low energy, lower chemical reagent requirement for the subsequent refining, and less loss of oil than other degumming processes (Xu and Diosady 2004).

Soft degumming process, or Ethylene Diamine Tetraacetic Acid (EDTA) degumming, is a process that facilitates phospholipid removal by a chelating metal ion (iron, calcium and magnesium) present in crude oil to form metal/EDTA complexes. The

nonhydratable phospholipids, mainly present as Ca or Mg salts of phosphatidic acid (PA/M^{2+}) and phosphatidylethanolamine (PE/M^{2+}), are more oil soluble. EDTA is an effective chelating agent because it forms very stable complexes with all polyvalent metal ions (M^{2+}), including Ca^{2+}, Mg^{2+}, and Fe^{2+}. In contact with the nonhydratable phospholipids, EDTA breaks down phospholipid/metal complexes (PA/M^{2+} and PE/M^{2+}) and forms water soluble metal complexes. Hence, nonhydratable phospholipids are converted to their hydratable form and removed from the oil by centrifugation.

The efficiency of soft degumming process depends on the degree of dispersion and contact between the chelating agent and nonhydratable phospholipids. In the soft degumming a detergent, sodium lauryl sulfate (SLS), is used to facilitate the contact between the nonhydratable phospholipids in the oil phase and the water phase containing the chelating agent (Zin 2006). In a soft degumming process an aqueous solution of 5% EDTA is mixed with water degummed soybean oil and heated to 75^{o}C in the presence of an emulsifier (SLS), the mixture is then homogenized for 1 min at 9500 rpm. The emulsion obtained is stirred for approximately 2 min and then centrifuged for 20 min at 5000 rpm. Soft degumming reduces the phosphorus content of soybean oil to 2 mg/kg (Choukri and others 2001). The disadvantage of soft degumming process is the cost of EDTA. Accordingly, the process is far too expensive for treating oils with a high nonhydratable phospholipids content (Dijkstra 1998).

During hexane extraction of oil from oilseeds a mixture containing 25-30% of crude oil and 70- 75% hexane is obtained. This mixture is referred to as miscella. Phospholipids can be separated from TAG in miscella using an appropriate membrane. The molecular size of TAG and phospholipids are approximately 900 and 800 Da,

respectively. Theoretically they could be separated by membrane filtration even if the particle size difference is small (Xu and Diosady 2004). Membrane separation is primarily a size exclusion based and pressure driven process. It separates different components of a mixture according to their molecular weights or particle sizes and shapes and their interactions with the membrane surface and other components of the mixture. Performance of a membrane separation is affected by the membrane construction material, process temperature, and pressure. The selection of the membrane for a given oil degumming process depends on membrane hydrophobicity, hexane/solvent resistance, and pore size of the construction material. The membrane-based crude oil degumming produces two fractions, permeate and retentate. The retentate contains mainly TAG and low levels of phosphorous and metals. A majority of the coloring materials and some of the FFAs and other impurities are removed in permeate along with phospholipids. The membrane degumming process is usually installed in extraction plants. Currently membrane degumming is too expensive for commercial operations, but it could be viable for specialty oils (Lin and others 1997).

A supercritical (SC) fluid is a substance at a temperature and pressure above its critical point. A SC fluid can diffuse through solids like a gas, and dissolve materials like a liquid. SC fluid processes are suitable as a substitute for organic solvents in a range of industrial and laboratory processes. Carbon dioxide and water are the most commonly used SC fluids. Solubility of not only phospholipids, but also pigments, trace metals, and FFA are low in SC carbon dioxide. Therefore, TAG can be extracted with SC carbon dioxide leaving undesirable FFA, metals and pigment behind. A SC process carried out at 55 MPa and 70 °C for 4 h was able to reduce phosphorus content of crude soybean oil

from 620 to less 5 mg/kg, removed pigments and reduced the FFA from 0.39 to 0.1% (List and others 1993). The SC degumming applications can be characterized as being fast, reliable, clean, and applicable for numerous food and industrially related applications. These applications have been fairly limited because of the sophisticated and expensive high-pressure equipment that is required. However, it may be used for specialty oils and pharmaceuticals.

2.5.2 NEUTRALIZATION

Oil neutralization is also known as deacidification or refining. Oil neutralization can be achieved by chemical or physical methods. Chemical or alkali neutralization, which follows degumming, neutralizes FFA in the oil by using dilute caustic soda. The caustic soda is mixed with the degummed oil at 85-90^{o}C for 5-10 min in a high shear mixer in order to hydrate the residual phospholipids and remove the FFA (Carr 1978). Caustic reacts with FFA to form soap-stock, hydrolyzes phospholipids and removes un-saponifiable matter from the crude oil. Soap can easily be removed from oil by centrifugation. The neutralized oil is then washed and dried. Chemical neutralization can be carried out by using different neutralizing agents like NaOH, Na_2CO_3, $NaHCO_3$ (De and Patel 2010). Effective neutralization results in enhanced bleaching, deodorization and produces a high quality product with high yield. In general, chemical refining involves several unit operations such as neutralization, washing and drying (Zin 2006). In physical refining, undesirable compounds are removed from crude oils by physical processes like steam stripping and membrane processing (Stage 1985; Ghosh 2007).

2.5.3 BLEACHING

The term bleaching refers to the process that removes colored substances and further purifies oil. Three types of bleaching methods, heat bleaching, chemical oxidation and adsorption, are used in edible oil refining (Brien 1998). When many oils are heated sufficiently, a phenomenon known as heat bleaching takes place. Heat decomposes some pigments, such as carotenoids, and converts them to colorless materials. However heat bleaching leaves the pigment molecules in the oil. If heat bleached oil comes into contact with air, colored degradation products react with tocopherols naturally present in the oil and form chroman-5, 6-quinones, which are very difficult to remove and reduce oil quality. Chemical oxidation also produces colorless or less colored compounds from pigments. During chemical oxidation acylglycerides are adversely affected and natural antioxidants are destroyed. Hence, oxidative bleaching is never used for edible oils, but is restricted to oils produced for non-food applications, such as soap-making. The common method of bleaching is the adsorption of color producing substances on an adsorbent material. Acid activated bleaching earth, natural bleaching earth, activated carbon and synthetic silicates are commonly used for edible oil bleaching. Degummed and neutralized oil is treated with 0.01-0.05% (based on volume of oil) bleaching material to assist in the removal of any trace metals present in the oil such as iron and copper, pigments, phospholipids and oxidation products. The oil and silica mixture is dried and then silica is removed by filtration (Dayton and Galhardo 2012).

2.5.4 DEODORIZATION

Deodorization removes odoriferous materials, FFA and other undesired minor components to produce a bland oil with good shelf life (Brien 1998). The deodorizer

operates under very high vacuum (2-10 mm Hg) and high temperature, (200-230°C). High pressure steam, commonly direct steam, is passed through the oil and volatile materials are volatilized and carried out by steam. The oil is then cooled and clarified through a filter press to produce clear oil. In this process, the peroxide value of oil is brought down as much as possible (0.01- 0.03 meq/kg). Typically, bleached oil from a caustic refining operation will enter the deodorizer with 0.05-0.15% FFA, while bleached oil from an enzymatic degumming process may contain higher FFA (0.4-1.6%) (Dayton and Galhardo 2012). While deodorization removes FFA from the oil, the FFA content cannot be reduced below about 0.05% because hydrolysis of the oil by the stripping steam is continually producing more FFA.

2.5.5 DEWAXING

Dewaxing, also referred to as winterization, is designed to remove waxes that cause cloudiness in oil. Vegetable oils, including sunflower, corn and linseed oils, contain some wax from the seed shell that makes the oils cloudy at low temperatures. Waxes are esters of fatty alcohols and fatty acids and have high melting points and low solubility in oils. The amount of wax in crude oils varies from a few hundred to over 2,000 mg/kg (Brien 1998). To produce oils with acceptable cold stability (i.e. salad oils), the wax content has to be reduced to about 10 mg/kg. During the dewaxing process oil is tempered at refrigerated conditions, 4-10 °C for 4-6 h, to allow waxes to crystalize and precipitate out of the oil. The precipitate may contain saturated long chain acylglycerides and waxes (Haraldsson 1983).

CHAPTER III

MATERIALS AND METHODS

3.1 SOURCES OF WHEAT GERM AND OIL

Both hexane-extracted and mechanically pressed CWGO were used in the study. Hexane extracted CWGO was a donation from Vitamin Inc. (Chicago, IL. USA). Wheat germ (WG) was purchased from ADM Milling Company (Enid, OK. USA) and mechanically pressed in our laboratory by using a heavy-duty press (Model L250, French Oil Mill Machinery Company. Piqua, OH. USA). The pressed oil was a mixture of oils obtained from an optimization study that examined different conditions of extraction. The process variables were cage temperature, wheat germ pretreatment shaft speed, back pressure and shaft arrangement](Al-Obaidi 2012).

3.2 ENZYMES

Lecitase Ultra, (PLA1) was a donation from Novozymes North America, Inc. (Franklinton, NC). The enzyme has a declared activity of 10 KLU/g (Kilo Lipase Unit/gram) and 1LU (Lipase Unit) is defined the amount of enzyme which releases 1 μmol of titratable butyric acid from tributyrin substrate in 1 min at 40°C with pH 7. The term KLU denotes 1000 LU. This enzyme is an acidic lipase that exhibits optimal activit at pH 5.0 and is marketed in liquid form. Lacitase Ultra is food grade and Kosher/ Halal approved.

LysoMax (PLA2) was donated by Danisco U.S. Inc. (Rochester, NY, USA). The reported enzymatic activity is 900-1,100 units/g [one unit is the amount of the LysoMax that catalyzes the conversion of 1 μmole min^{-1} of substrate (lecithin) into 2-lysolecithin and fatty acids]. It is marketed in liquid form and optimum pH is 6.3-6.7. GumZyme was donated by DSM Food Specialties B.V. (Delft, Netherlands). It is marketed in liquid form and has standardized activity ≥ 4,000 LCU/g [LCU: Lecithin Conversion Unit (one unit is the amount of the GumZyme that catalyzes the conversion of 1 μmole min^{-1} of substrate (lecithin) into lysolecithin], and optimum pH range is 4.8-5.2.

3.3 METHODS

3.3.1 WATER DEGUMMING

CWGO was degummed by adding water and heating the oil (250 g)-water mixture in a 500 mL glass reactor. The reaction conditions were as follows: temperature, 30, 50, or 70°C; deionized water amounts, 2.5, 5, or 7.5% (based on oil weight); 500 rpm stirring speed; reaction time 30, 40, or 50 min. After the completion of the reaction, the mixture was centrifuged for 20 min at 5,000 rpm and the gum phase was separated (Scheme 1). The degummed oil was dried under vacuum using a Rapid-Vap Evaporation System (Model 7900002, Labconco, Kansas City, MO.USA) (40°C, vacuum pressure 210-240 mbar, for 2 h).

3.3.2 ACID DEGUMMING

CWGO was degummed by heating the oil (250 g) in a 500 ml three neck double jacketed reactor. The reaction temperature was kept at 30, 50 or 70°C. Deionized water, 2.5, 5 or 7.5% (based on oil weight) and phosphoric or citric acid at concentration of 0.05, 0.1, or 0.2% (based on oil volume) were added. The mixture was stirred at 500 rpm

for 60 min or homogenized at 20,000 rpm for 2 min (to form an emulsion quickly). Then the acid degummed oil was recovered by centrifugation at 5,000 rpm for 20 min (Scheme 2). The degummed oil was dried by using a Rapid-Vap Evaporation System (Model 7900002, Labconco, Kansas City, MO.USA) (40°C, under vacuum, 210-240 mbar, for 2 h).

3.3.3 ENZYMATIC DEGUMMING

Three enzymes, Lecitase Ultra, Lysomax and Gumzyme, were examined in this study. Optimization study was carried out for Lecitase Ultra and Lysomax. Then, optimum enzymatic degumming process conditions determined by using Lecitase Ultra (enzyme concentration of 750 mg/kg, reaction time 390 min, water: oil ratio 20%, and temperature 60°C) were used for degumming CWGO using Gumzyme.

CWGO (250 g) was heated in a 500 mL reactor at 40, 50, or 60°C. Then citric acid was added at a concentration of 0.065% (based on oil weight). The acid, water and oil mixture was first homogenized at 20,000 rpm for 2 min then stirred at 500 rpm for 1 h. After adjusting the pH of the mixture to 5 by adding 4N NaOH, deionized water (10, 20, or 25% based on weight of oil) and enzyme (Lecitase Ultra or Lysomax at the concentration of 500, 750, or 1000 mg/kg based on weight of oil) were added. The mixture was stirred for 4.5, 5, or 6.5 h at 500 rpm with 2 min homogenization at 20,000 rpm every hour. At the end of enzyme treatment, the enzyme-oil mixture was held in a water bath at 80°C for 30 min to deactivate the enzyme. Then the mixture was centrifuged for 20 min at 5,000 rpm to separate the degummed oil (Scheme 3). The degummed oil was dried by using a Rapid-Vap Evaporation System (Model 7900002, Labconco, Kansas City, MO.USA) (40°C, 210-240 mbar for 2 h).

3.3.4 OIL YIELD

After degumming oil yield was calculated by using the following formula:

Oil yield (%) = [(weight of oil used for degumming- degummed oil weight)/oil weight used for degumming)] x 100

3.4 ANALYTICAL METHODS

3.4.1 SAMPLE PREPARATION FOR THE OILS USED FOR CHARACTERIZATION

WGO samples used in this study were as follows: 1) CWGO H, commercially hexane extracted CWGO. The details of the processing conditions for this oil were proprietary information, and thus not available. The oil samples were used as received in our laboratory. 2) CWGO SP, CWGO mechanically pressed in our laboratory by using a heavy-duty screw press. 3) WD, hexane extracted and water degummed WGO. 4) WD SP, mechanically pressed and water degummed oil. Water degumming process conditions were 30°C, water: oil ratio 7.5% and reaction time of 40 min. 5) ADC, hexane extracted and citric acid degummed oil. 6) ADC SP, mechanically pressed citric acid degummed oil. Mechanical extraction conditions were the same as described earlier in this chapter. Citric acid degumming process conditions were 30°C, 2 min homogenization, citric acid concentration 0.05% and water: oil ratio 7.5%. 7) ADPH, hexane extracted and phosphoric acid degummed oil. 8) ADPH SP, mechanically pressed and phosphoric acid degummed oil. Phosphoric acid degumming process conditions were 70°C, 2 min homogenization, phosphoric acid concentration 0.05% and water: oil ratio 7.5%. 9) EDLU, hexane extracted oil that is degummed using Lecitase Ultra. 10) EDLU SP, mechanically pressed oil that is degummed using Lecitase Ultra. 11) EDL, hexane

extracted oil that is degummed using Lysomax. 12) EDL SP, mechanically pressed oil that is degummed using Lysomax. 13) EDG, hexane extracted oil that is degummed using Gumzyme. 14) EDG SP, mechanically pressed oil that is degummed using Gumzyme. Enzymatic degumming process conditions were the same for all enzymes; expect temperature, enzyme concentration of 750 mg/kg, reaction time 390 min and water: oil ratio 20%. The reaction temperature was 60^{o}C for Lecitase Ultra and 40°C for Lysomax and Gumzyme.

3.4.2 PHOSPHORUS CONTENT ANALYSIS

Phosphorus content of the samples was determined as follows: about 3.0 -3.2 g of oil was placed in a crucible and then 0.5 g of zinc oxide was added. Initially oil was heated slowly, and then the heat was increased until the mass was charred. The crucible was placed in a muffle furnace (Fisher Science, Model 58 Isotemp® Muffle Furnace 600 Series, Fair Lawn, NJ. USA) at 600^{o}C and held for 2 h until the contents turned into white ash. The crucible was then removed from the furnace and cooled to room temperature. The phosphorus content of the ash was determined according to AOCS method Ca 12-55 (AOCS 1997). Phosphorous was extracted from the ash by adding 5 mL distilled water and 5 mL of concentrated HCl to the ash and then heating the mixture to gentle boiling for 5 min. After the filtration of the ash suspension the filtrate was treated with 8 mL hydrazine sulfate and 2 mL sodium molybdate solutions in a boiling water bath for 10 min. Then the mixture was cooled to 25°C followed by the spectrophotometric (DU 520, Beckman Coulter, Inc., Fullerton, CA, USA) measurement of phosphorus at 650 nm as a blue phosphomolybdic acid complex. The phosphorus content was determined by means of a standard curve using NaH_2PO_4 as a standard.

3.4.3 FREE FATTY ACID CONTENT (FFA)

The FFA content of the oil samples was determined by using a colorimetric method (Lowry and Tinsley 1976). Pyridine was added to an aqueous copper acetate solution (5%, w/v) until the pH of the mixture reached to 6.0-6.2. A standard stock solution of oleic acid (National Formulary/Food Chemicals Codex grade, Fisher Chemical, Fairlawn, NJ, USA) was prepared by dissolving 100 mg of oleic acid in 1 mL of hexane. A standard curve was prepared as follows: 10, 20, 30, and 40 µL aliquots of standard stock solution were transferred to individual centrifuge tubes. Then 5 mL benzene and 1 mL copper acetate solution was added to each tube and the contents were mixed for 2 min. Absorbance of the solutions was read at 715 nm using a UV/VIS spectrophotometer (Beckman DU 520, Fullerton, CA, USA) after 5 min centrifugation. The oil samples were prepared using the same procedure described above. About 0.03-0.05 g of oil sample was used to prepare the solution.

3.4.4 MOISTURE ANALYSIS

The moisture content of the oil samples was determined by using a Karl Fischer Titrator (758 KFD Titrino, Metrohm, Brinkman Instruments Inc. Westbury, NY, USA). The 34811 Hydranal Titrant-2 was used as a titrant and the 34812 Hydranol Solvent was the component solvent. Both solvents were purchased from Sigma (Sigma-Aldrich Corporation, St. Louis, MO, USA).

3.4.5 PEROXIDE VALUE (PV)

The PV of oil samples was determined according to AOCS official method Cd8-53 (AOCS 2003). About 5 g of oil sample was placed in a 250 mL flask. Then 30 mL of glacial acetic acid: chloroform (3:2, v/v) mixture was added to the oil. Both acetic acid

and chloroform were American Chemical Society (ACS) reagent grade and purchased from Fisher Chemical (Fairlawn, NJ, USA). Next, 0.5 mL of saturated potassium iodide solution (ACS grade, Fisher Chemical, Fairlawn, NJ, USA) was added to the oil mixture. After 1 min, 30 mL of distilled water and 2 mL of saturated starch solution was added. The final mixture was titrated with a 0.01 N sodium thiosulfate solution (ACS grade, Fisher Chemical, Fairlawn, NJ, USA) until the color changed from dark blue to colorless. The peroxide value was calculated using the equation:

PV = [(mL of titrant)*(0.01)*1000]/ (Sample weight).

3.4.6 *p*-ANISIDINE VALUE (AV)

p-Anisidine values for oil samples were determined according to AOCS official method Cd 18-90 (AOCS 2003). First, 0.5 g of oil sample was mixed with 25 mL isooctane (ACS reagent grade, Fisher Chemical, Fairlawn, NJ, USA). The absorbance of the mixture was measured at 350 nm using a spectrophotometer (DU 520, Beckman Coulter, Inc., Fullerton, CA, USA). Then, 5 mL of oil -isooctane mixture and 1 mL of *p*-anisidine solution (0.25 g/100 mL glacial acetic acid) (ACROS Organics, Morris Plain, NJ, USA) were added to the test tube. The mixture was allowed to stand for 10 min until it produced a colored complex. Finally, the absorbance of the solution was measured at 350 nm. The AV was calculated using the following formula:

AV = [25 * (1.28 * As- Ab)] /m

Where:

As = absorbance of the oil solution

Ab = absorbance of the initial solution (prior to color development)

m = weight of the sample in g.

3.4.7 TOCOPHEROLS

The tocopherol (α, ß, γ and δ) content of the oil was analyzed by HPLC following the method of Katsanidis and Addis (1999). The oil samples were dissolved in hexane (0.20 g/mL) and filtered through a 0.2 μm filter (Iso–Disc filter, Supelco, Bellefonte, PA, USA). The HPLC system (Alliance 2690 Waters Corp., Milford, MA, USA) consisted of a separation module (Model 2695), a Photodiode Array Detector (PDA) (Model 2996, Waters, Milford, MA, USA) and a Multi Wavelength Fluorescence Detector (FD) (Model 2475, Waters, Milford, MA, USA). Two μL of sample were injected onto a normal phase HPLC column, (Zorbax RX-SIL 5 μm particle size, 4.6 x 250 mm, Agilent Technologies, Santa Clara, CA, USA), and the separation of the oil sample was achieved by using a mobile phase consisting of hexane (HPLC Grade, Fisher Scientist, Fairlawn, NJ, USA) and isopropanol (HPLC grade, Pharmco Co. Brookfield, CT, USA) at a ratio of 99:1(v/v). The isocratic flow rate was 1.3 mL / min. The column temperature was set at 35°C. Total run time was 15 min. The fluorescence detector was set at 290 nm excitation and 400 nm emission wavelengths. The fluorescence detector gain was set for 1. An external calibration curve was prepared for each tocopherol standard (α, ß, γ and δ tocopherol standards, Sigma-Aldrich Corporation, St. Louis, MO, USA) to calculate the amount of tocopherols present in the oil sample. The tocopherols content was computed by using the response from the florescence detector.

3.4.8 PHOSPHOLIPIDS

The oil samples were dissolved in chloroform: methanol (2:1, v/v) at a concentration of 0.5 g/mL and filtered through a 0.2 μm Iso Disc filter (Supelco, Bellefonte, PA, USA). A normal phase silica column, μPorasil 10 μm (3.9 mm i.d x 300

mm) from Waters (Milford, MA, USA) was used for the analytical separation of the compounds. The mobile phase consisted of A: chloroform and B: methanol: water (95:5, v/v). The following binary gradient elution program was used: 0-15 min, 99% A to 1% B; 15-20 min, 75% A to 25% B; 20-25 min , 10% A to 90% B; 25-30 min10% A to 90% B; and 30-35 min, 99% A to 1% B. Total run time was 35 min and the mobile phase flow rate was 1.0 mL/min. The detector system was an evaporative light scattering detector (ELSD) (Model 2000, All Tech Associates Inc., Deerfield, IL, USA). The ELSD set points were as follows: nitrogen flow rate 3.5 mL/min, impactor ON, and drift tube temperature of 80^{o}C. Identification and quantification of chromatographic peaks were based on external standard curves prepared for individual standards. Phospholipid standards L-α phosphatidylcholine (PC), L-α phosphatidic acid (PA) sodium salt, and L-α-phosphatidylethanolamine (PE) were isolated from egg yolk and phosphatidylinositol (PI) sodium salt was from soybean. All the standards were purchased from Avanti Polar lipids, Inc, Alabaster, AL, USA.

3.5 STATISTICAL ANALYSIS

A 3^3 factorial design was used for water degumming experiments. Each variable had three levels, temperature (30, 50 and 70°C), time (30, 40 and 50 min), and water: oil (2.5, 5 and 7.5%). The acid degumming experiments were based on a ½ fraction of a 2^2 x 3^3 factorial design. Five variables examined in this experiment: temperature (30, 50 and 70°C), Acid type (phosphoric acid and citric acid), time (homogenized for 2 min and stirred for 60 min), acid concentration (0.05, 0.1 and 0.2%) and water: oil (2.5, 5 and 7.5%). For enzymatic degumming experiments 1/3 fraction of a 2 x 3^4 factorial design was used. Five variables examined in these experiments were as following: temperature

(40, 50 and 60°C), enzyme type (Lecitase Ultra and LysoMax), time (270, 300 and 390 min), enzyme concentration (500, 750 and 1000 mg/kg) and water: oil (10, 20 and 25%). All the experiments were carried in a randomized order.

All analytical tests for oil characterization were carried out in duplicate. In this study, the means were compared using least significance difference (LSD) method. The analysis of variance (ANOVA) of the experimental data and the analysis of Response Surface Method (RSM) experiments were performed using SAS/STAT software, version 9.3 (SAS Institute Inc., Cary, NC, USA). All statistical tests were performed at the 0.05 level of significance.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 DEGUMMING PROCESS

4.1.1 WATER DEGUMMING

The effects of three variables, temperature, time, and water: oil ratio on water degumming efficiency was observed (Table 1). Process efficiency was monitored by measuring the phosphorus content of the degummed oil and the oil yield. Response Surface Methods (RSM) were used to develop mathematical models to describe the processes. Nine of the 27 experiments (chosen randomly) were carried out in duplicate to estimate experimental error.

The lowest phosphorous level in the oil (817 mg/kg) was obtained under the flowing condition: the lowest process temperature, 30°C; the highest water: oil ratio, 7.5%; and a reaction time of 40 min. The high residual phosphorous in the oil confirms the low efficiency of water degumming for CWGO (Wang and Johnson 2001). The effects of all variables except time ($p = 0.0632$), on the residual phosphorous content in oil were significant.

The following quadratic model was developed to explain the relationship between the phosphorus content and processing parameters:

Y_1 (%) = 1704.55309+9.57105X_1- 44.03690X_2+0.68046X_2^2-29.76785X_3-0.17401X_1X_2 (1)

Where Y_1 is the predicted phosphorus content and X_1, X_2, and X_3 represent temperature, time, and water: oil ratio, respectively.

The coefficient of the determination $R^2 = 0.6228$ and the model was significant ($p < 0.0001$) (Figure 9). The coefficients for the temperature and time interaction ($p = 0.0472$), temperature ($p = 0.0093$), and water: oil ratio ($p < 0.0001$) were significant (Table 2). At a given time, the predicted residual phosphorus content in the oil after water degumming decreased with increasing water: oil ratios and decreasing temperature (Figure 10). This might be due to better contact between gum and water phases facilitating efficient hydration of the phospholipids. In this study, the significant effect of added water amount points to the fact that at high water levels, more gums are expected to hydrate, resulting in lower phosphorus content in the oil. Indira and others (2000) reported a similar trend for water degumming of rice bran oil.

In summary, although the water degumming may be effective for vegetable oils with a low nonhydratable phospholipid content such as rice bran oil (De and Patel 2010), rapeseed and sunflower oils (Zufarov and others 2008), it is not suitable for oils containing high levels of nonhydratable phospholipids, such as CWGO.

The oil yield from water degumming was over 91% for all the conditions examined in this study (Table 1). Although the highest oil yields, 98.2% (w/w) was obtained at 70^{o}C, 30 min and water: oil ratio of 2.5%, these conditions did not reduce the phosphorous content of the crude oil (residual phosphorous 1240 mg/kg). The oil yield

under the conditions that resulted in the highest phosphorous removal (30^{o}C, water: oil ratio of 7.5%, and reaction time of 40 min) was 92.8%.

The following quadratic model was developed to explain the relationship between the oil yields and processing parameters:

$$Y_2\ (\%) = 99.07998 - 0.15612X_1 + 0.00256X_1^2 + 0.07408X_2 - 0.58677X_3 - 0.00155X_1X_2 \quad (2)$$

where Y_2 is the predicted oil yield and X_1, X_2, and X_3 represent temperature, time, and water: oil ratio, respectively.

All the coefficients of the model were significant except time ($p = 0.0650$) (Table 3). Although the model (Equation 2) was significant ($p < 0.0001$) and had a high coefficient of determination ($R^2 = 0.8628$), the lack of fit was also significant ($p = 0.0012$) (Figure 11). Oil yields predicted by the model decreased with increasing temperature at 30 and 50^{o}C (Figure 12). At higher temperatures (>50^{o}C) oil yield increased, probably due to the decreased viscosity of the gum, leading to better oil-gum separation. It can be concluded that water degumming of CWGO at a lower temperature (30^{o}C) and water: oil ratio of 7.5% is preferable for achieving low residual phosphorous and oil loss.

4.1.2 ACID DEGUMMING

According to the experimental design, 54 experiments were carried out (Table 4). The effects of five variables on acid degumming efficiency were examined: temperature, time (mixing type - 60 min stirring vs. 2 min homogenization), water: oil ratio, acid type (phosphoric and citric acids) and acid concentration. A heterogeneous variances model

was adopted to determine the optimum conditions that resulted in the lowest residual phosphorus and oil loss in degummed oil.

The lowest phosphorus content (989 mg/kg) was obtained under the following conditions: 70°C, 2 min homogenization, 0.05% phosphoric acid, and water: oil ratio of 7.5% (Table 4). This value was higher than the lowest phosphorous level obtained by water degumming (Table 1); hence, the addition of phosphoric acid did not improve degumming efficiency. The effects of acid type ($p < 0.0001$), time ($p = 0.001$) and acid type and temperature interaction ($p = 0.0060$) on residual phosphorous level were significant (Table 5). The phosphorous content in the oil decreased with increasing temperature when phosphoric acid was used for degumming (Figure 13). There is no need for a long reaction time during phosphoric acid degumming because 2 min homogenization was more effective than 60 min stirring ($p = 0.001$) in reducing the residual phosphorous in oil. Wang and Johnson (2001) also showed that acid degumming (phosphoric acid) was not effective for removing phospholipids from CWGO. Although in this study citric acid addition did not significantly reduce the phosphorous level in CWGO, it was effective for reducing the phosphorus content of rapeseed and sunflower oil (96-97 phosphorous reduction) (Zufarov and others 2008).

Oil yield from acid degumming was over 90% for all the conditions examined in this study (Table 4). Although the highest oil yield, 99.9% was obtained at 70°C, 60 min, water: oil ratio of 2.5% and 0.1% citric acid, these conditions did not reduce the phosphorous content (1240 mg/kg) of the crude oil. The oil yield under the conditions that resulted in the highest phosphorous removal (989 mg/kg) was 93.5%. Time was the

only variable that had a significant effect ($p = 0.0002$) on oil yield during acid degumming (Table 6).

The current study confirms that acid degumming is not effective for CWGO. Hence, there is a need for development of alternative techniques to effectively reduce phosphorous levels in CWGO.

4.1.3 ENZYMATIC DEGUMMING

The effects of five variables on enzymatic degumming efficiency were examined: enzyme type (Lecitase Ultra and Lysomax), enzyme concentration, temperature, time, and water: oil ratios. Then, the optimal enzymatic degumming conditions determined for Lecitase Ultra were employed for CWGO degumming using Gumzyme. Response Surface Methodology was performed to optimize the processing parameters for high oil yield and low residual phosphorous in the oil. According to the experimental design, 27 experiments were carried out for each enzyme (Lecitase Ultra and Lysomax) (54 experiments total).

The lowest phosphorus contents, 219 mg/kg for Lecitase Ultra and 294 mg/kg for Lysomax (Table 7), were obtained at 390 min reaction time, enzyme concentration of 750 mg/kg and water: oil ratio of 20%. Lecitase Ultra required a higher reaction temperature, 60^{o}C, than did Lysomax, 40 °C. Enzymatic degumming was more effective than either water or acid (phosphoric and citric acids) degumming for the removal of phosphorus from CWGO (Tables 1 and 4). Time and enzyme concentration interaction ($p = 0.0445$), enzyme type ($p < 0.0001$) and concentration ($p = 0.0302$) had significant effects on the residual phosphorous level in oil. Lecitase Ultra was more effective than Lysomax removing phospholipids from CWGO (Table 8).

Analysis of the data using Response Surface Methodology indicated that, for both enzymes, the predicted phosphorous content in the oil would increase with increasing enzyme concentrations at 270 and 300 min reaction times (Figures 14-16) while a slight decrease in phosphorous content with increasing enzyme concentration was predicted at 390 min. These results might be due to the increased esterification rather than hydrolysis activity at high enzyme concentration and shorter reaction time. The model also predicts that both enzymes will result in lower phosphorous content in the oil at lower reaction times (270 and 300 min) and water: oil ratio of 20% rather than 10% (Figures 17-19). A further increase in water: oil ratio from 20 to 25% was not predicted to improve phosphorous removal significantly. At a longer reaction time, 390 min, the predicted phosphorous content of degummed oil would be lower at 10% water: oil ratio than at 20 and 25% (Figures 20-22). This can be attributed to partial denaturation of the enzyme and loss of its hydrolytic activity at high moisture content and long reaction time. Hence, a longer reaction time reduces water requirements for enzymatic degumming. According to the model, the optimum enzymatic degumming conditions were as follows: 1000 mg/kg Lecitase Ultra, 40°C, 390 min reaction time, and 10% water: oil ratio. These conditions would produce oil with 288 mg/kg phosphorous content (Table 9), which is higher than the lowest observed phosphorous content with Lecitase Ultra, 219 mg/kg. However, production of oil with 219 mg/kg phosphorous content would require a higher reaction temperature, 60^{o}C, and doubles the water usage for degumming. Yang and others (2006b) and Jahani and others (2008) also showed that Lecitase Ultra was very effective in reducing the phosphorous content in rapeseed, soybean and rice bran oils (95-96% phosphorous reduction).

The effects of all the processing parameters and their interactions, except enzyme concentration ($p = 0.3007$), water: oil ratio and concentration interaction ($p = 0.0689$) on oil yield were significant (Table 10). Oil yield from enzymatic degumming was over 85% for all the conditions examined in this study (Table 7). The highest oil yield for Lecitase Ultra, 92.8 % (w/w), was obtained at 40^{o}C, reaction time of 390 min, enzyme concentration of 1000 mg/kg and water: oil ratio of 25%. To get a similar oil yield, 92.9%, with Lysomax, a higher temperature, 60^{o}C, and lower reaction time, 300 min, and lower water: oil ratio, 10%, were required at the same enzyme concentration, 1000 mg/kg. However these conditions did not reduce the phosphorous content of the degummed oil. The oil yield under the conditions that resulted in the highest phosphorous removal was about 88.7 and 89.6% for Lecitase Ultra and Lysomax, respectively.

Predicted oil yield for Lecitase Ultra was significantly lower than that for Lysomax at 40°C, because the lower phosphorus content with using Lecitase Ultra compared to the Lysomax (Table 11). Analysis of data shows that the interaction between the enzyme type and water: oil ratio significantly ($p = 0.0065$) influences the oil yield. In general, oil yield decreased with decreasing enzyme concentration and water: oil ratio (Figures 23-28), however at the lowest enzyme concentration and water: oil ratio, a slight increase in oil yield was observed. This might be due to the low gum removal from the oil under these conditions. When Gumzyme was used for CWGO degumming the phosphorus content in hexane-extracted and mechanically pressed oils could be reduced to 584 and 645 mg/kg, and the oil yield under this conditions was about 89.12 and 89.88% respectively. Gumzyme was the least effective enzyme in reducing phosphorous content in CWGO.

In summary, this study demonstrated that enzymatic degumming of CWGO is more effective than water and acid degumming. Higher oil yields from water and acid degumming as compared to yields from enzymatic degumming are due to low gum removal during the latter processes. CWGO degumming with Lecitase Ultra was more effective than Lysomax and Gumzyme.

4.2. CHEMICAL CHARACTERIZATION OF WHEAT GERM OIL

Chemical characterization tests were carried out for the oils obtained by using the optimum degumming process conditions determined in this study.

4.2.1 FREE FATTY ACID CONTENT (FFA)

Hexane-extracted crude oil had significantly higher FFA content (15.2%) than did mechanically pressed oil (3.2%) (Table 12). The difference in FFA content might be due to different extraction methods and the original FFA content of the feedstock used for extraction. High FFA content in CWGO has also been reported by other researchers (Wang and Johnson 2001; Eisenmenger and others 2006). The enzymatic degumming process significantly increased the FFA content of the oils (Table 12). This result was expected because phospholipases hydrolyze mainly phospholipids and produce FFA (Yang and others 2006b). Gumzyme produced less FFA than both Lecitase Ultra and Lysomax during degumming of hexane-extracted CWGO. However, residual phosphorous content in Gumzyme was higher than that for other enzymes. There was no significant difference between the FFA contents of oils mechanically pressed and degummed using Gumzyme and Lysomax. Previous studies have reported that an increase in FFAs was the effect of the hydrolysis of phospholipids rather than triacylglycerides (Winter and others 1998; Jiang and others 2011). Even during acid and

water degumming, a slight increase in FFA content of the oils was observed (Table 12). These results are comparable to Wang and Johnson (2001) observations; they found that the FFAs increased during acid degumming. The latter study attributed the increase in FFAs to the presence of acid in the mixture (acid hydrolysis). Our study does not support the latter argument because FFA content of the water degummed hexane- extracted oil was similar to that of the acid degummed oils. The differences might be due to the degumming conditions used in these studies.

4.2.2 PEROXIDE VALUE (PV)

The peroxide value (PV) is an indicator of the presence of primary oxidation products in the oil. High PV indicates that oil was extracted, stored, and /or processed under improper conditions, and high PV designates low oil quality. The PV of the CWGO H was significantly higher than that of CWGO SP (Table 12). It is important to note that these oils were extracted from different batches of WG; hence the results might have been influenced by the feedstock quality, not only by the extraction method. The PV content of oils increased significantly during degumming because of the heat exposure during processing. The results obtained in this study conflicted with Wang and Johnson (2001), which reported that no significant difference ($p > 0.05$) was detected in PV content of CWGO after phosphoric acid degumming at 60 °C and reaction time of 60 min. Iwuoha and others (1996) reported that the PV content of palm and palm kernel oil decreased after degumming at 65 °C for 30 min with H_3PO_4. The variations among the results reported in the literature might be due to the differences in fatty acid composition (unsaturation level) and initial quality (degree of oxidation) of the oils used for the experiments. The PVs of the oils degummed using Lecitase and Lysomax were

substantially higher than those for the acid and water degummed oils. This is due to exposure of the oils to heat for an extended time during enzymatic degumming (≥ 270 min). It is interesting to note that the PV of the Gumzyme treated oil was not as high as the PV of the oils treated with other enzymes. This might be due to slightly lower FFA in the Gumzyme treated oil than in the other enzyme treated oils. FFAs accelerate oxidation reactions in oil.

4.2.3 *p*-ANISIDINE (AV)

The *p*-anisidine value (AV) is a measure of the amount of secondary oxidation products present in oil. The AVs of CWGO H and CWGO SP were not extremely high indicating that secondary oxidation (conversion of primary oxidation products to secondary oxidation products such as aldehydes and small volatile compounds) was not at an advanced stage (Table 12). There was no significant difference in AV of pressed and hexane extracted oils. Water degumming significantly increased the AV of hexane-extracted oil but not pressed oil. Similar to our findings, Brevedan and others (2000) reported that water degummed pressed sunflower oil had lower AV content than water degummed hexane-extracted oil. Degumming with phosphoric acid produced more secondary oxidation products than did degumming with citric acid from both hexane-extracted and pressed oils. Because of the very high FFA and PV of the oil that was extracted with hexane and degummed using Lecitase Ultra, the AV of this oil was higher than the other samples examined in this study.

4.2.4 MOISTURE CONTENT OF OIL

Moisture content is one of the most commonly measured properties of oil. High moisture content leads to hydrolysis during high temperature applications, promotes

microbial growth, and causes cloudiness and phase separation in the oil (Eisenmenger and Dunford 2008). All the oil samples had relatively low moisture content (< 1%) (Table12). A slight increase in moisture content in the degummed oils might be due to the incomplete removal of the water added to the oils during the degumming process.

4.2.5 TOCOPHEROLS

The CWGO examined in this study had a higher total tocopherol content than the values reported in the literature. This high level might be due to variations in sources of wheat germ, oil extraction methods, and conditions used for storage and handling. CWGO SP had significantly higher total tocopherol content (5.2 mg/g oil) and α-tocopherol (3.9 mg/g oil) than did other oil samples examined in this study (Table 13). This result is similar to the result presented by Wang and Johnson (2001), who stated that cold-pressed WGO had higher α-tocopherol (3.5 mg/g) content than CWGO extracted by hexane (1.8 mg/g) and acid degummed oil (1.7 mg/g). As expected, α-tocopherol was the major tocopherol (75% of the total tocols) followed by β- and δ-tocopherol (17 and 8%, respectively) found in the samples. All the degumming processes examined in this study lowered the tocopherol content in the oil. There was no significant difference in the α-tocopherol between WD and WD SP and between EDG and EDG SP. The ADPH, EDG SP, EDG and EDLU ($p > 0.05$) had lower α-tocopherol content (0.6, 0.7, 0.8 and 0.9 mg/g oil) than all other samples. The reduction of α-tocopherol content in these degummed oil samples was 82, 82, 76 and 73.5%, Wang and Johnson (2001) reported similar results, lower β-tocopherol content,in degummed WGO.

4.2.6 PHOSPHOLIPID COMPOSITION

The HPLC method used in this study did not separate PI from PA; hence the results are expressed as PI + PA. Hexane-extracted crude oil (13 mg/g oil) had significantly higher total phospholipid content than did mechanically pressed oil (12.1 mg/g oil) and as expected, the phospholipids content of both of them was higher than that of degummed oil samples examined in this study (Table 14). It is important to note that hexane- extracted oil was centrifuged before the degumming experiments and analytical tests. Hence, some of the phospholipids precipitated during storage and handling is removed during centrifugation. The original phospholipid content of hexane- extracted was expected to be higher than the values reported in this study. CWGO had higher phospholipid content than other vegetable oils (Wang and Johnson 2001). About 76 and 59% of the total phospholipids was PI + PA for pressed and hexane- extracted oils, respectively. The presence of a high level of PI + PA explains the difficulty in reducing the phospholipid content of CWGO. PI is a nonhydratable phospholipid and has higher solubility in oil than other phospholipids. Water (68.6 vs. 57.7%), citric acid (55.4 vs. 44.6 %) and phosphoric acid degumming processes (71 vs. 67.8%) were more effective in removing phospholipids from pressed oil as compared to hexane-extracted oil. A similar trend was observed for PI + PA removal. More PI + PA were removed from pressed oil as compared to hexane- extracted oil during water and acid degumming processes. Both Lesitase Ultra and Lysomax removed more PI + PA (79.2% removal based on the original PI + PA amount in the starting material) than Gumzyme (68.8%) from hexane-extracted oil. All three enzymes removed similar amounts of PI + PA from pressed oil (80.4-82.6%). These results were also confirmed by a significantly lower phosphorous

content in enzyme treated oil than in water and acid degummed oil samples (Table 12). Enzymatic degumming processes reduced the phosphorus content in hexane-extracted and mechanically pressed oils using Lecitase Ultra (88.2 vs. 81.6%), Lysomax (84.2 vs. 78%) and Gumzyme (68.6 vs. 52.6%).

CHAPTER V

CONCLUSION

This study examined the efficiency of water, acid, and enzymatic degumming processes for removing phospholipids from CWGO. The lowest residual phosphorous content, 817 mg/kg, after water degumming was obtained at 30°C, reaction time of 40 min and highest water: oil ratio 7.5%. The oil yield under these conditions that resulted in the highest phosphorous removal (56.1% removal) was about 92.80%. The water degumming process is not efficient for oils with high nonhydratable phospholipid content such as CWGO.

Phosphoric acid addition was more effective than citric acid addition for removing phospholipids from CWGO. However, acid degumming with phosphoric acid at 70°C, 2 min homogenization, phosphoric acid concentration 0.05%, and water: oil ratio 7.5% still resulted in higher residual phosphorus content in the oil, 989 mg/kg, than that of water degumming. The oil yield for phosphoric acid degumming was 93.54%. The acid degumming process is not efficient for oils with high nonhydratable phospholipid content such as CWGO.

CWGO degumming with Lecitase Ultra was more effective than Lysomax. Both enzymes, Lecitase Ultra (219 mg/kg residual phosphorous in the oil) and Lysomax (294 mg/kg residual phosphorus), were more effective than water and acid degumming.

Optimum conditions for enzymatic degumming were as follows: enzyme concentration of 750 mg/kg, reaction time of 390 min, water: oil ratio 20%, and 60 and 40 °C for Lecitase Ultra and Lysomax, respectively. The oil yields under the latter conditions that resulted in the highest phosphorous removal (84-88% removal) were about 88.7 and 89.6% for Lecitase Ultra and Lysomax, respectively. All enzymatic degumming oil yields are lower than that for water and acid degumming. This is partly due to higher amounts of gum removal during enzymatic degumming as compared to acid and water degumming. Although there was no significant difference in the final residual phosphorous content in hexane- extracted and pressed oils after enzymatic degumming, more phosphorous was removed from hexane- extracted oil (88%) than from pressed oil (84%). On the other hand both water and acid degumming removed more phosphorous from hexane- extracted oil (45-56% phosphorous removal) compared to pressed oil (16-22%) and resulted in lower phosphorous content in hexane- extracted oil.

As expected, enzymatic degumming increased the FFA of oils more than acid and water degumming. Gumzyme produced less FFA from hexane- extracted oil than did Lecitase and Lysomax. Significant tocopherol loss was observed during all degumming processes because of the exposure of the oil to heat for extended time.

This study demonstrated that enzymatic degumming is more effective in removing phospholipids from CWGO than acid and water degumming. Higher oil yields from acid and water degumming as compared to enzymatic degumming is due to the significantly lower gum removal during acid and water degumming. It is expected that oil loss will be lower during downstream processing of enzymatically degummed oil during neutralization, bleaching, and deodorization due to the lower residual phosphorous

content in the oil. Although the lowest phosphorus content achieved in this study is still higher than the commodity oil industry standards, less than 30 mg/kg, relatively higher phosphorous levels in enzymatically degummed WGO could be acceptable for the specialty oil industry. Indeed, most of the specialty oils are marketed as virgin oils, which are obtained by filtering the crude oil without further processing. However, considering the extremely high phosphorous content in CWGO, enzymatic degumming would be beneficial to improve the storage properties and appearance of the final product.

FUTURE WORK

The mathematical models developed to describe various CWGO degumming processes had significant lack of fit. Further research is needed to develop better models to optimize CWGO degumming processes. This study examined the effect of degumming processes on tocopherols. The effect of oil refining on other health beneficial bioactive compounds naturally present in WGO should also be investigated. Considering that CWGO has high FFA, it has to be neutralized. The effect of enzymatic degumming on WGO downstream processing, specifically on neutralization, needs to be studied.

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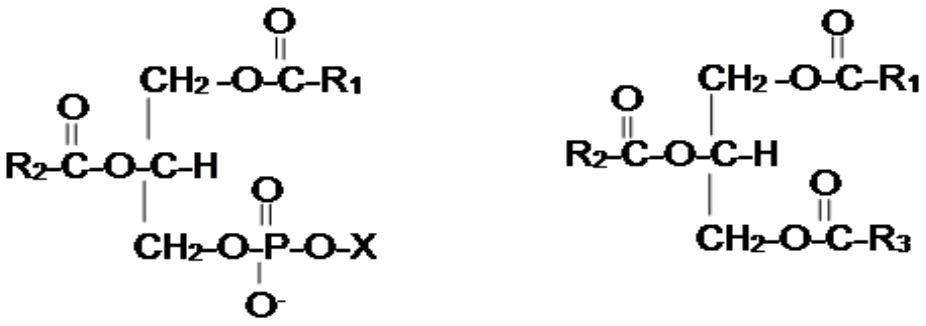


Phospholipid **Triacylglycerol**

Figure 1: Structure of a phospholipid and triacylglycerol molecule.

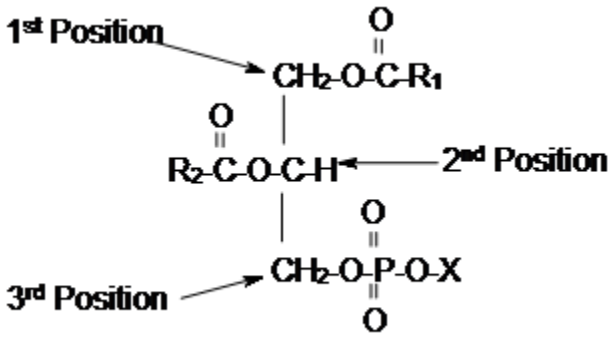


Figure 2: The numbering system for positions on a phospholipid molecule. R=fatty acid chain, X=functional group.

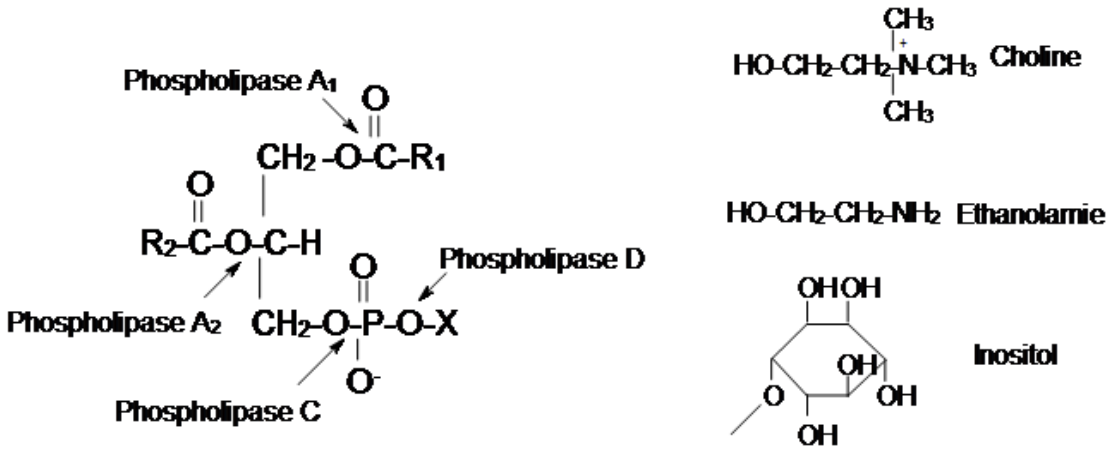


Figure 3: Phospholipase reaction sites (A1, A2, C, and D). R=fatty acid chain, X=functional group X represents: choline, ethanolamine, Inositol or hydrogen. Illustration adapted from (Clausen 2001).

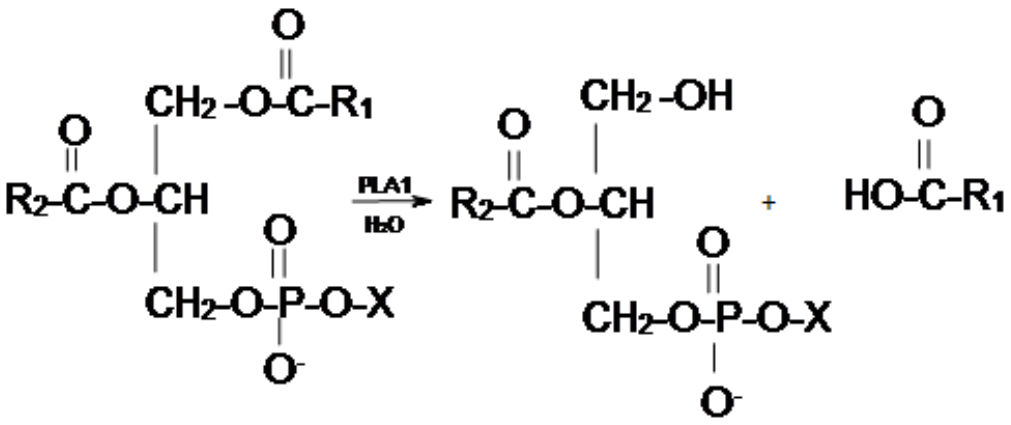


Figure 4: Phospholipase A1 reaction mechanism. R=fatty acid chain, X=functional group.

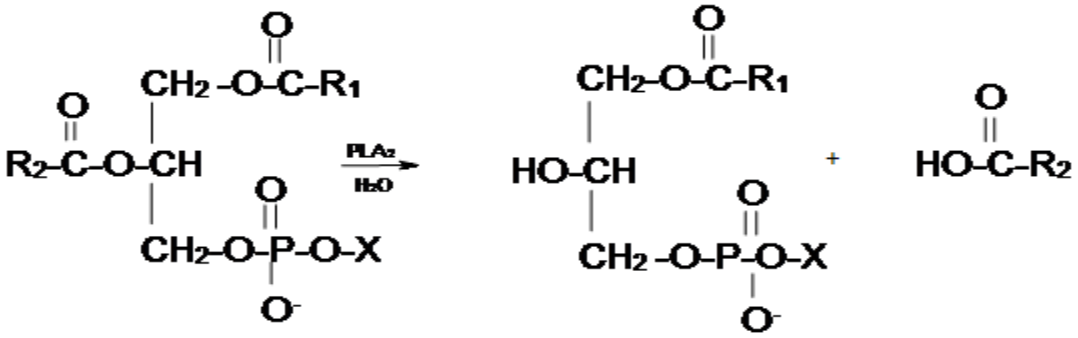


Figure 5: Phospholipase A2 reaction mechanism. R=fatty acid chain, X=functional group.

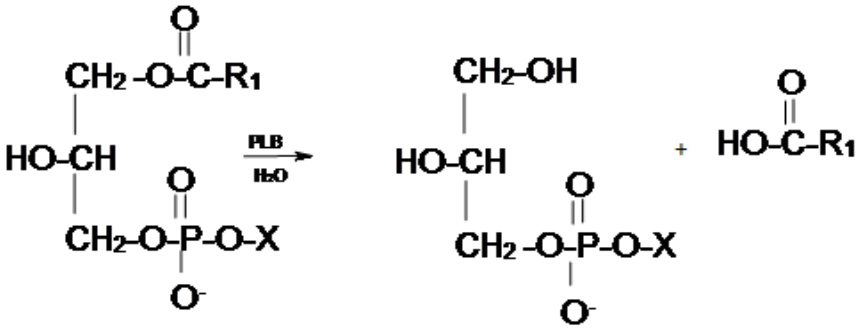


Figure 6: Phospholipase B reaction mechanism. R=fatty acid chain, X=functional group.

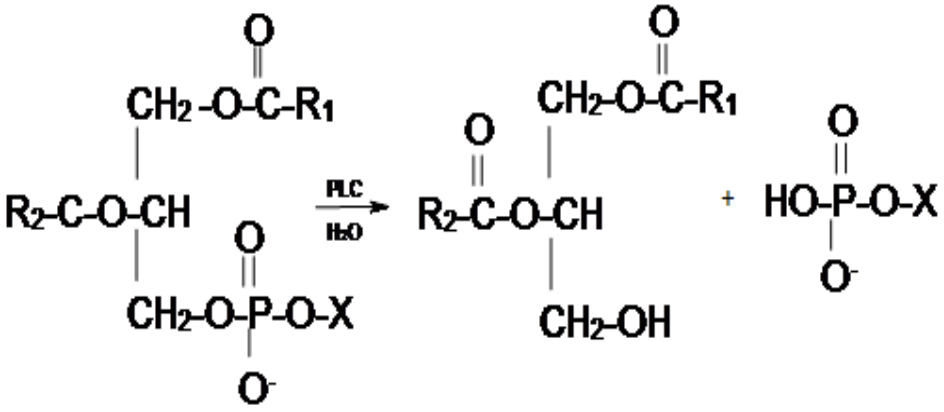


Figure 7: Phospholipase C reaction mechanism. R=fatty acid chain, X=functional group.

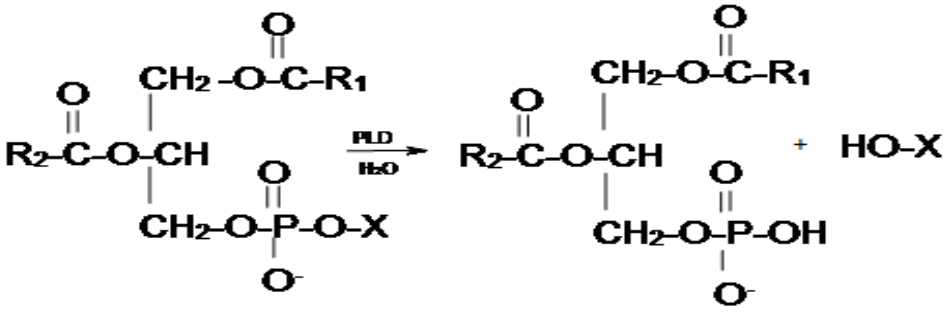


Figure 8: Phospholipase D reaction mechanism. R=fatty acid chain, X=functional group.

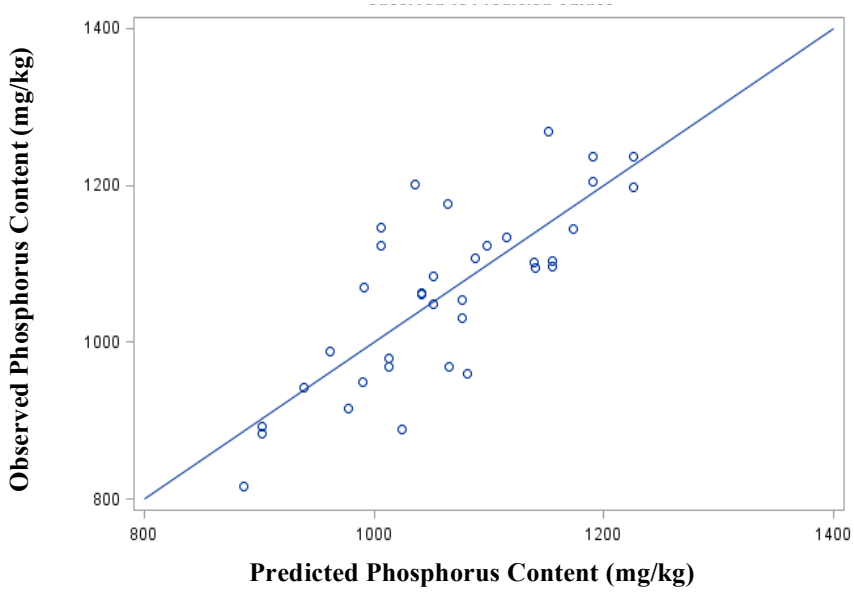


Figure 9: A scatter plot of the observed and predicted residual phosphorus content after water degumming.

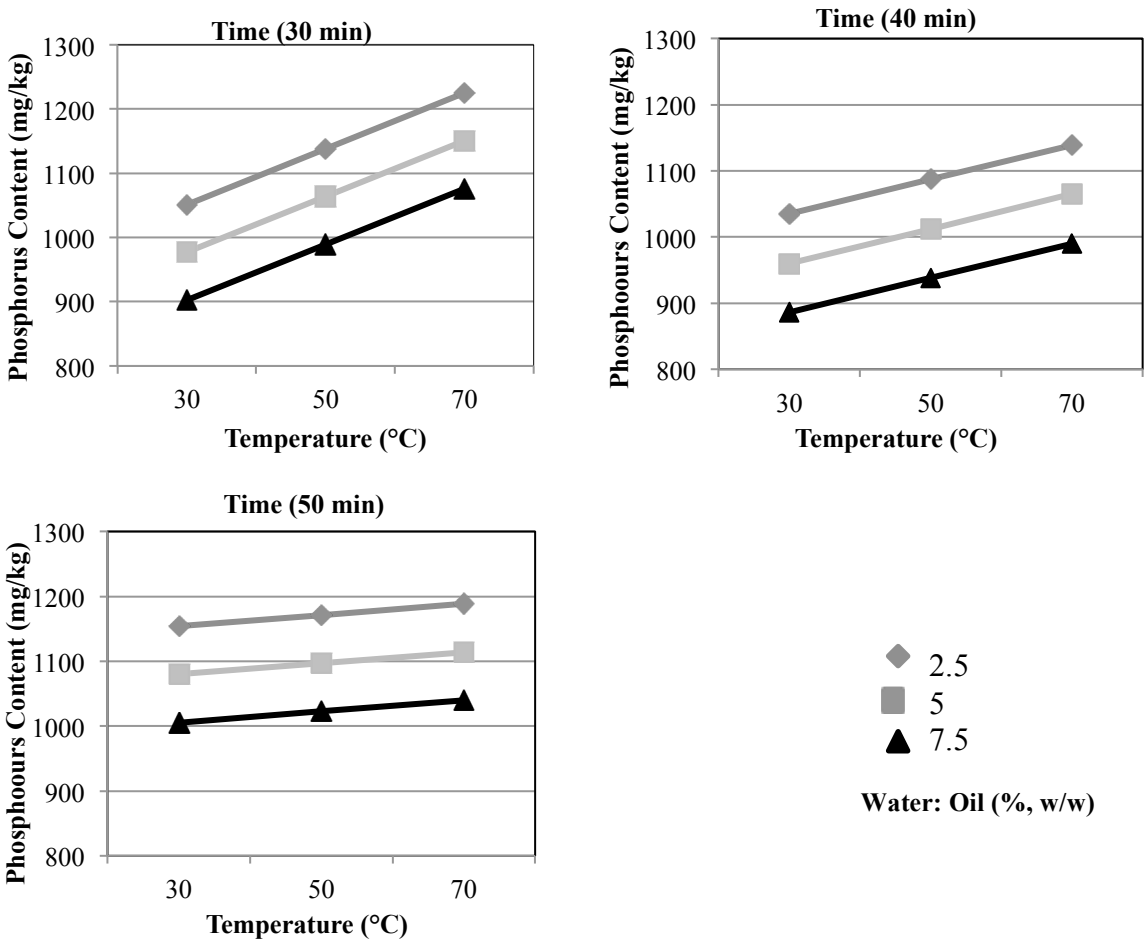


Figure 10: Effect of time, temperature and water: oil ratio on predicted residual phosphorus content after water degumming.

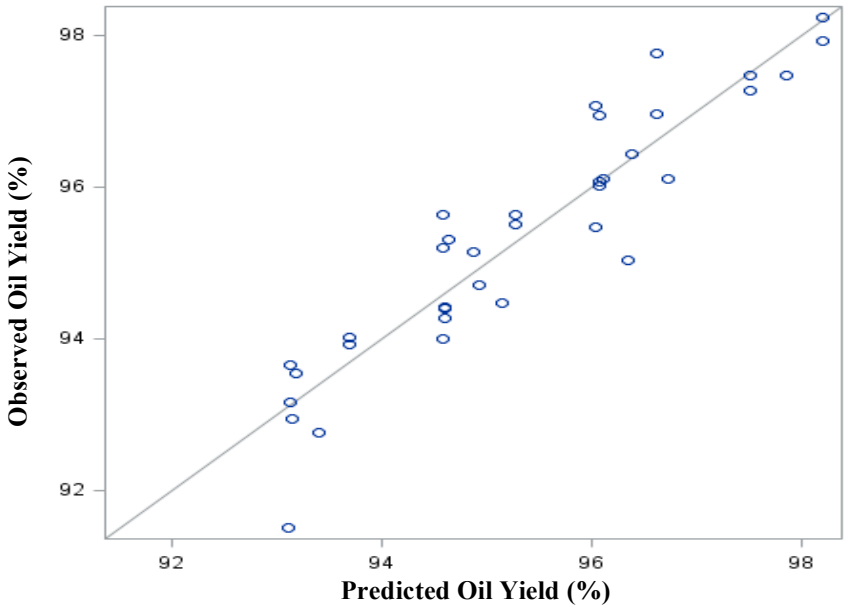


Figure 11: A scatter plot of the predicted oil yield from water degumming.

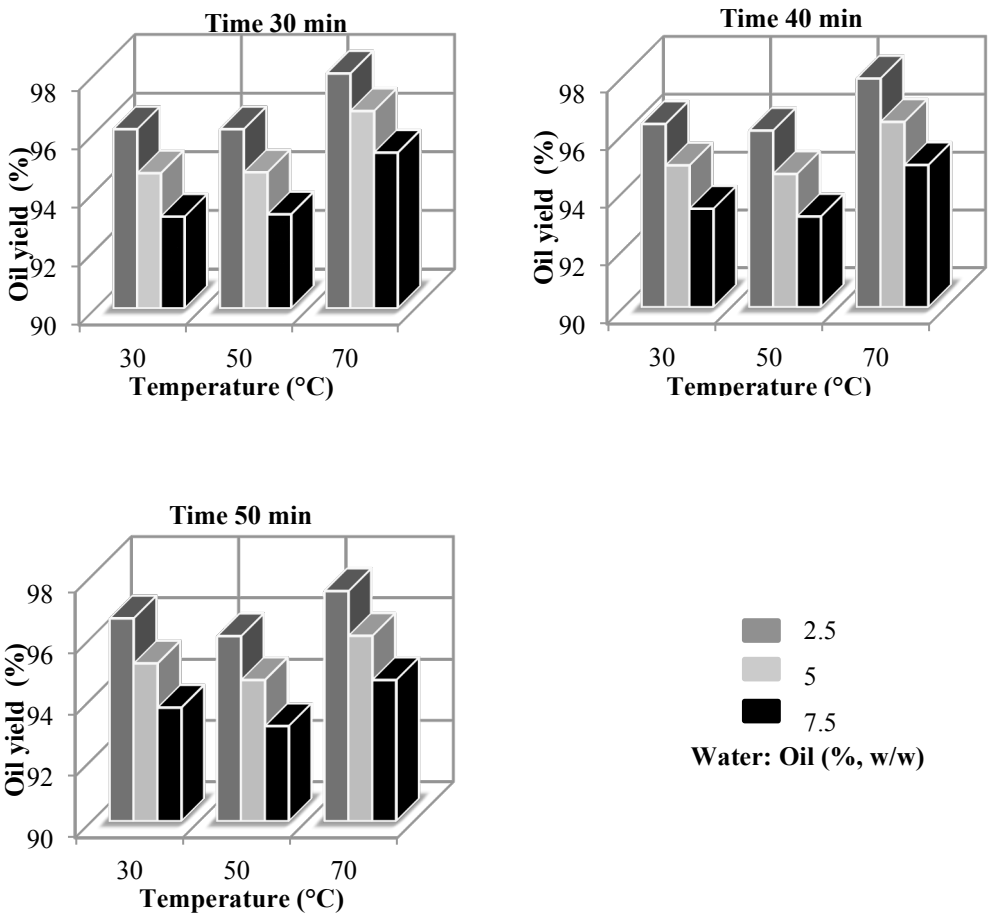


Figure 12: Effect of time, temperature and water: oil ratio on predicted oil yield from water degumming.

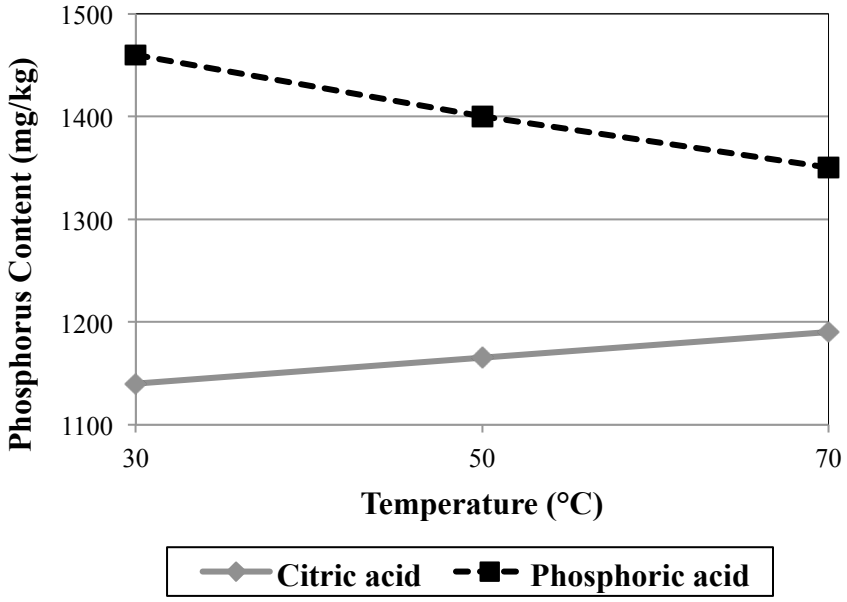


Figure 13: Effect of acid type on residual oil content in oil as a function of temperature.

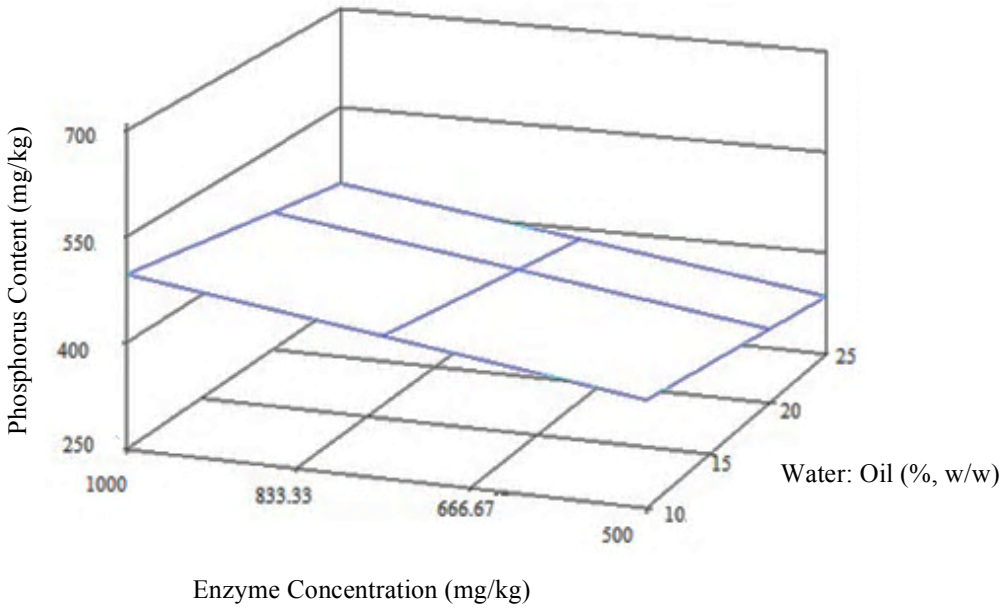


Figure 14: Response surface plot of residual phosphorus content in the oil as a function of Lecitase Ultra concentration and water: oil ratio at 270 min reaction time.

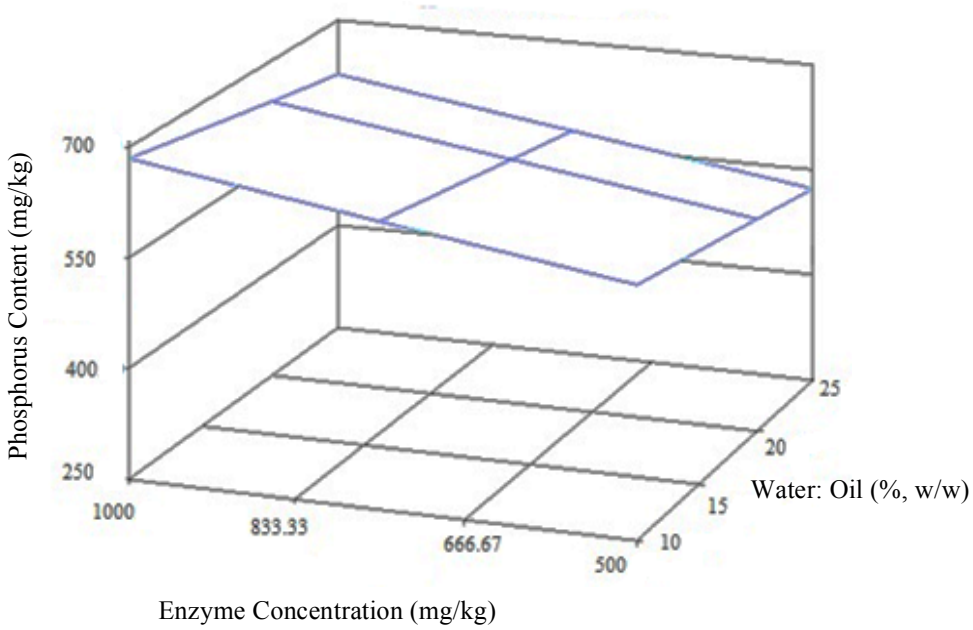


Figure 15: Response surface plot of residual phosphorus content in the oil as a function of Lysomax concentration enzyme and water: oil% at 270 min reaction time.

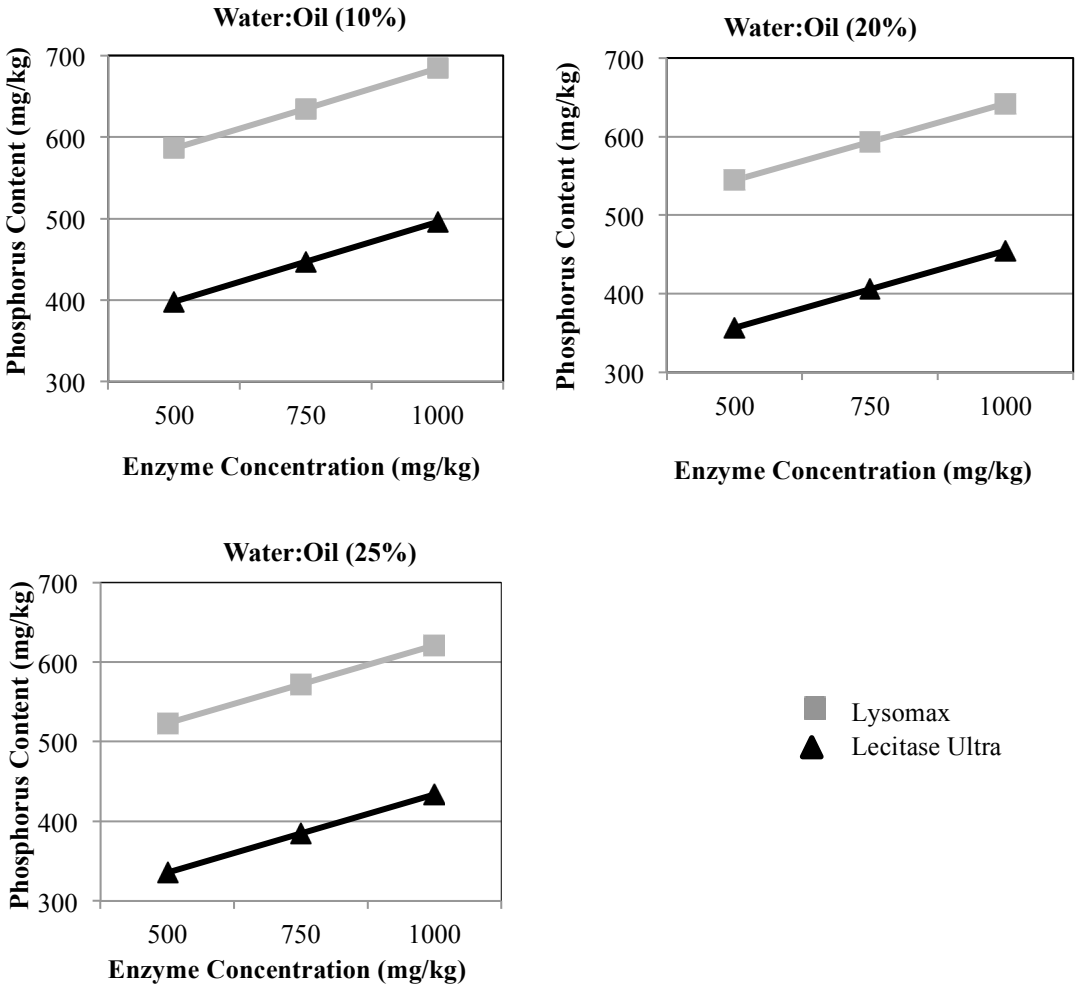


Figure 16: Effect of water: oil ratio, enzyme concentration and two different enzymes on predicted residual phosphorus content after enzymatic degumming at reaction time of 270 min.

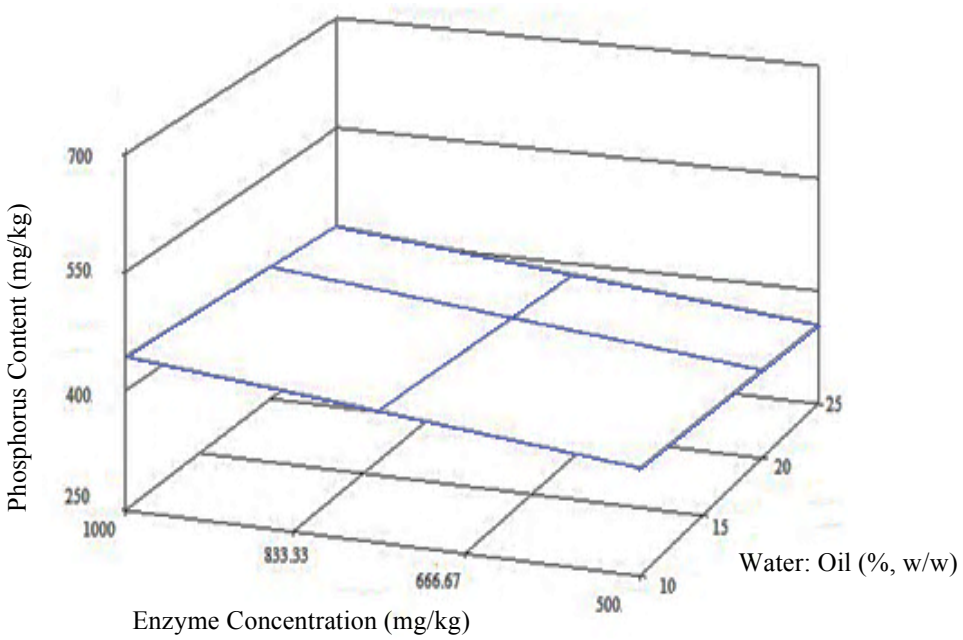


Figure 17: Response surface plot of residual phosphorus content in the oil as a function of Lecitase Ultra concentration and water: oil ratio at 300 min reaction time.

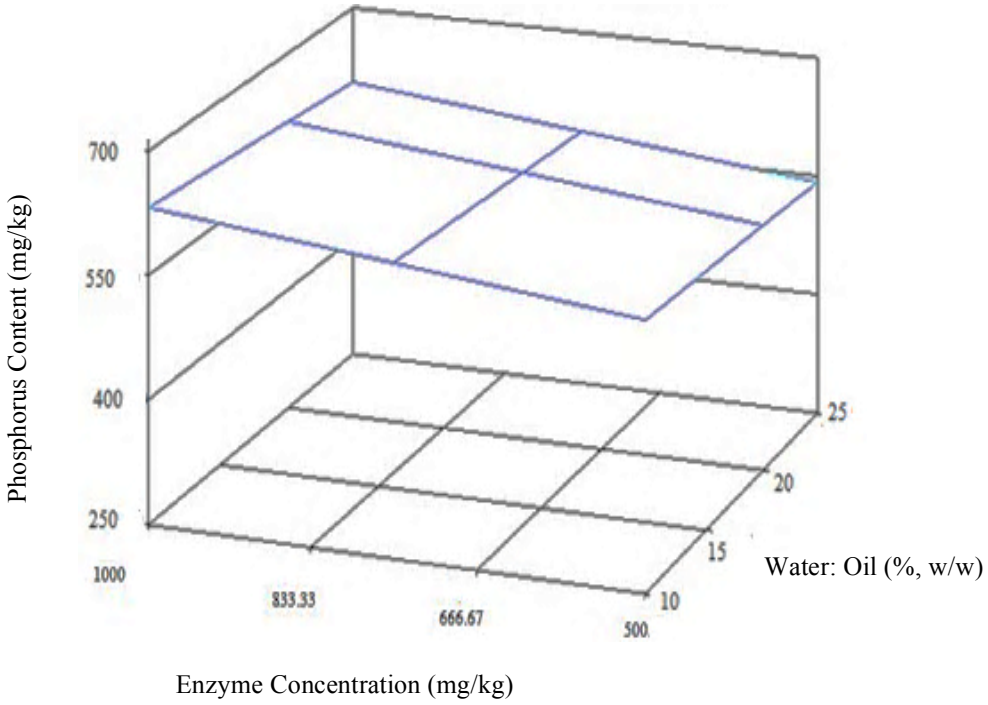


Figure 18: Response surface plot of residual phosphorus content in the oil as a function of Lysomax concentration enzyme and water: oil% at 300 min reaction.

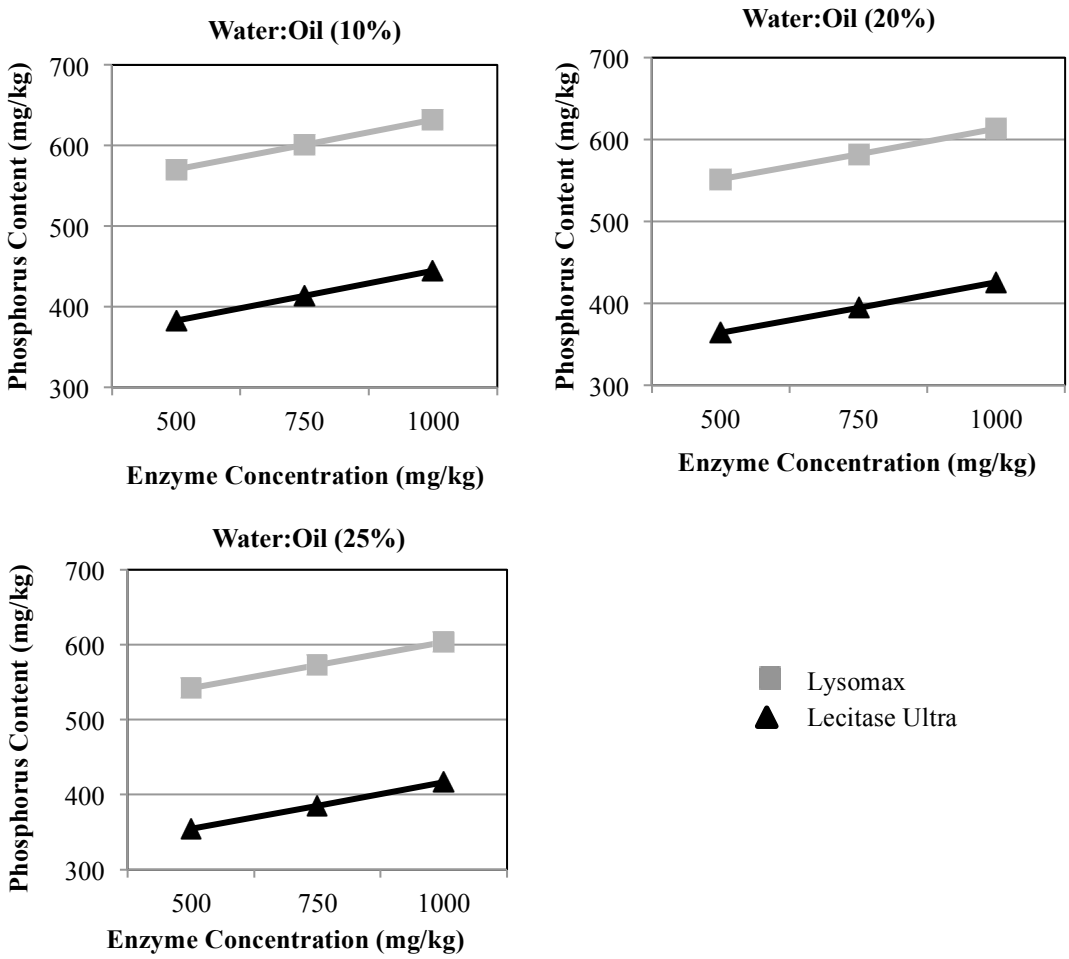


Figure 19: Effect of water: oil ratio, enzyme concentration and two different enzymes on predicted residual phosphorus content after enzymatic degumming at reaction time of 300 min.

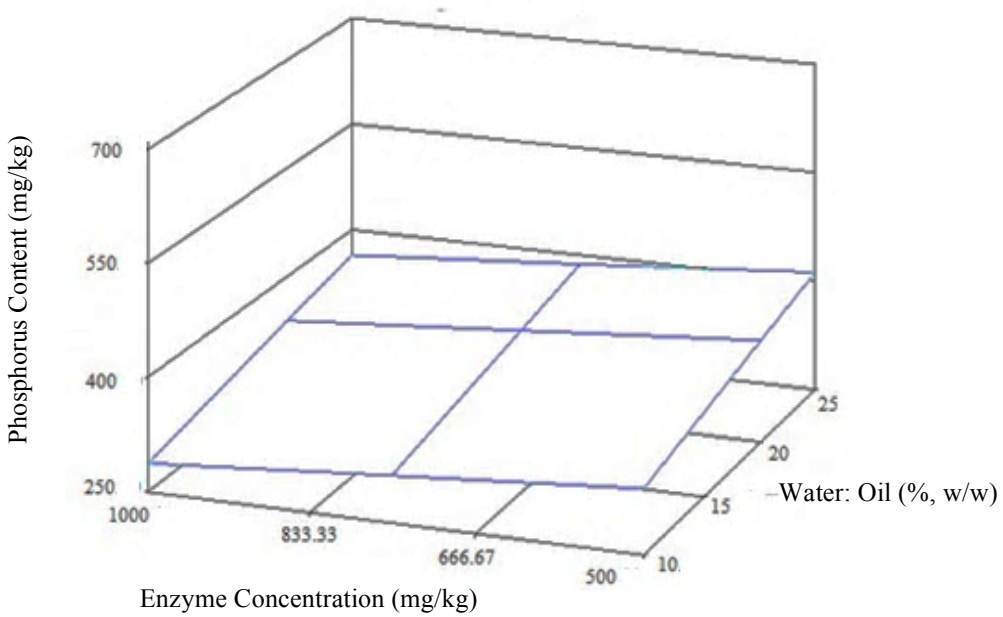


Figure 20: Response surface plot of residual phosphorus content in the oil as a function of Lecitase Ultra concentration and water: oil ratio at 390 min reaction time.

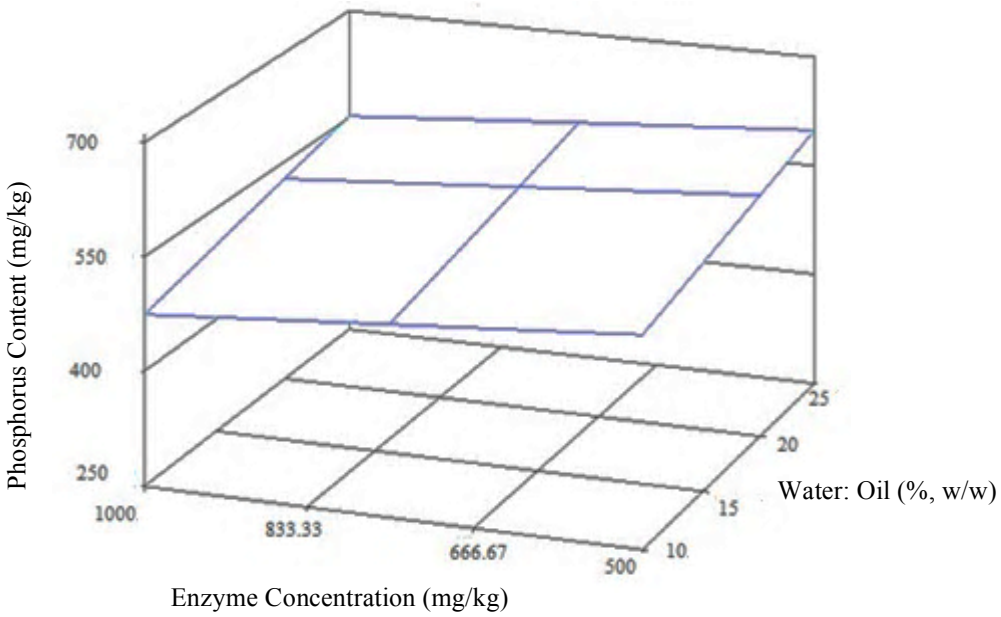


Figure 21: Response surface plot of residual phosphorus content in the oil as a function of Lysomax concentration enzyme and water: oil% at 390 min reaction time.

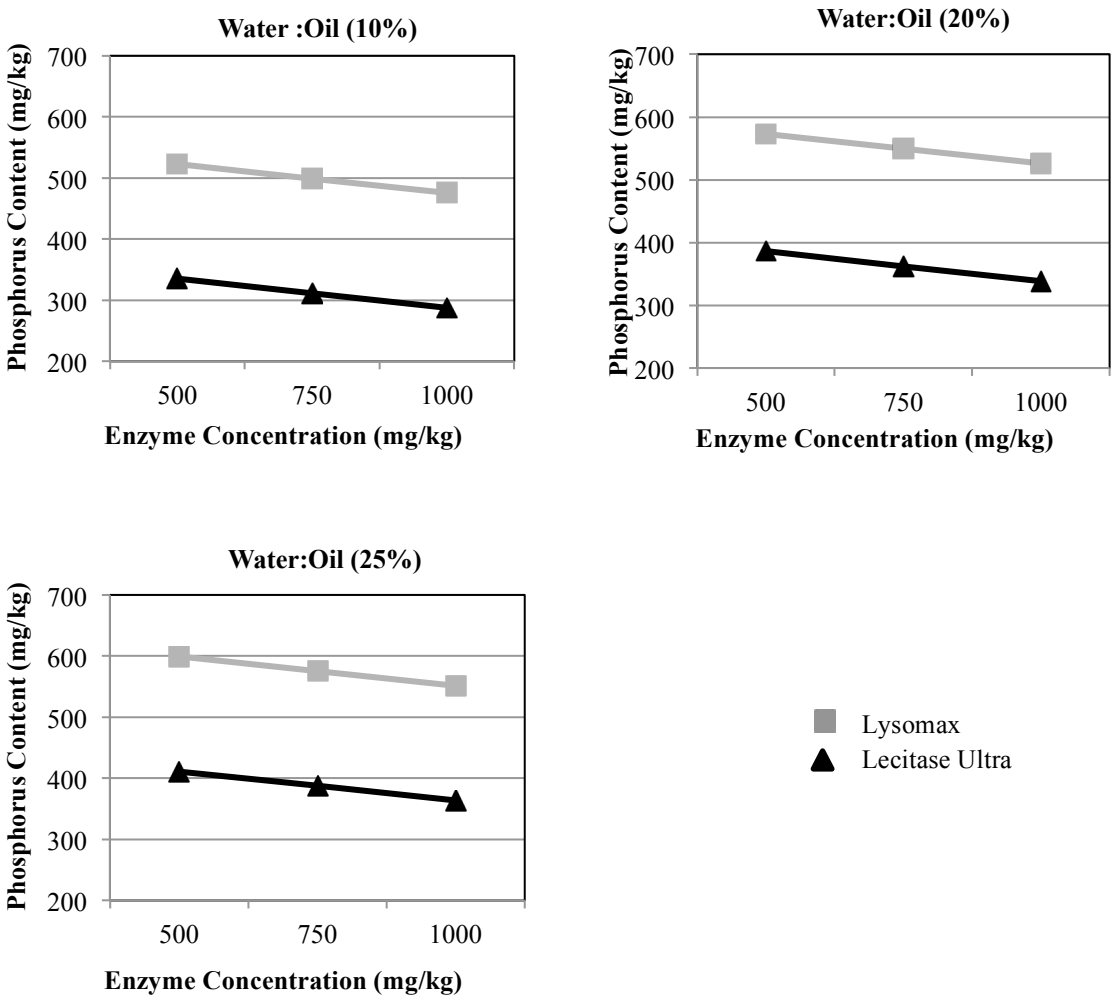


Figure 22: Effect of water: oil ratio, enzyme concentration and two different enzymes on predicted residual phosphorus content after enzymatic degumming at reaction time of 390 min.

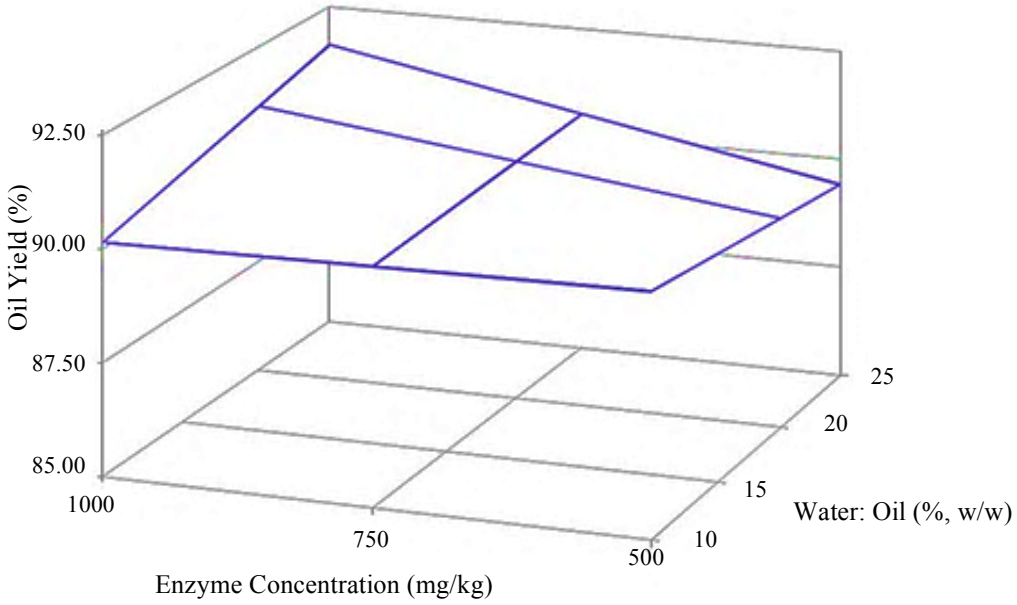


Figure 23: Response surface plot of oil yield as a function of Lecitase Ultra concentration and water: oil ratio at 40°C.

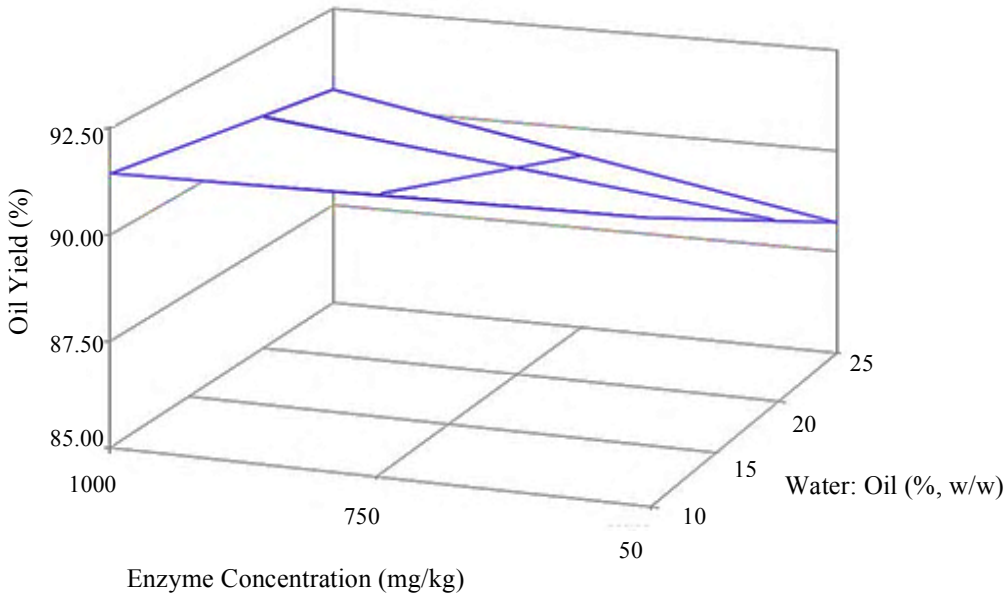


Figure 24: Response surface plot of oil yield as a function of Lysomax concentration and water: oil ratio at 40°C.

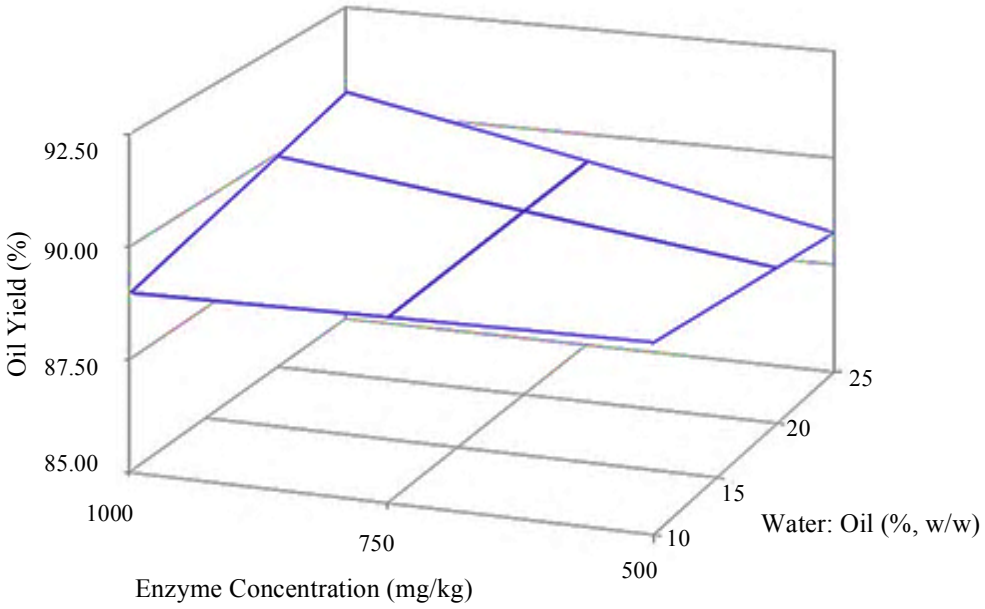


Figure 25: Response surface plot of oil yield as a function of Lecitase Ultra concentration and water: oil ratio at 50°C.

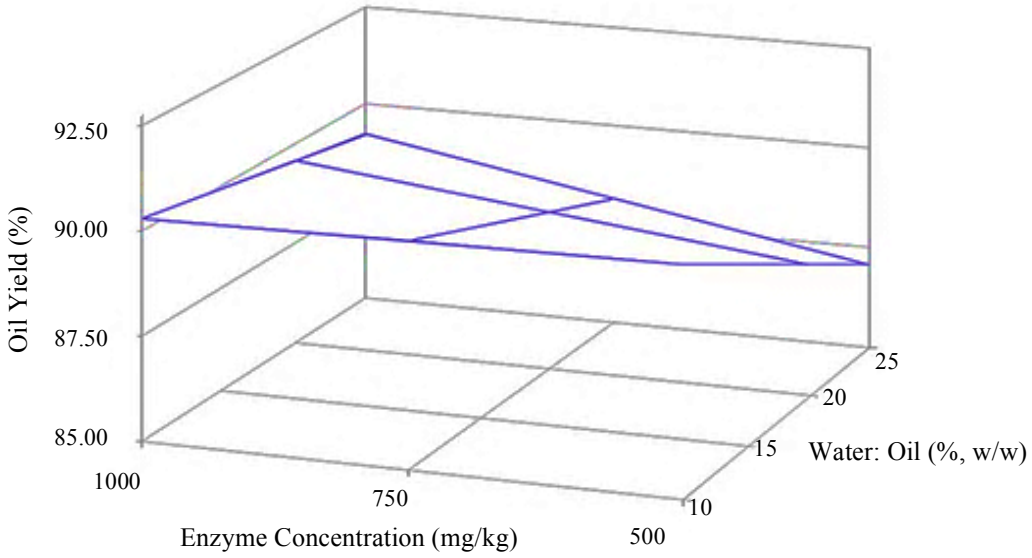


Figure 26: Response surface plot of oil yield as a function of Lysomax concentration and water: oil ratio at 50°C.

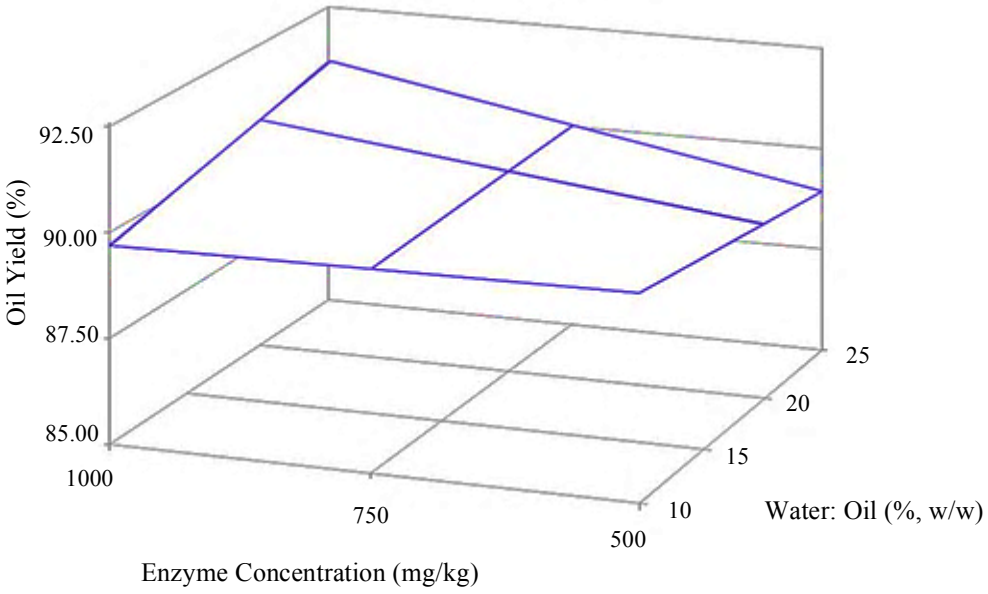


Figure 27: Response surface plot of oil yield as a function of Lecitase Ultra concentration and water: oil ratio at 60°C.

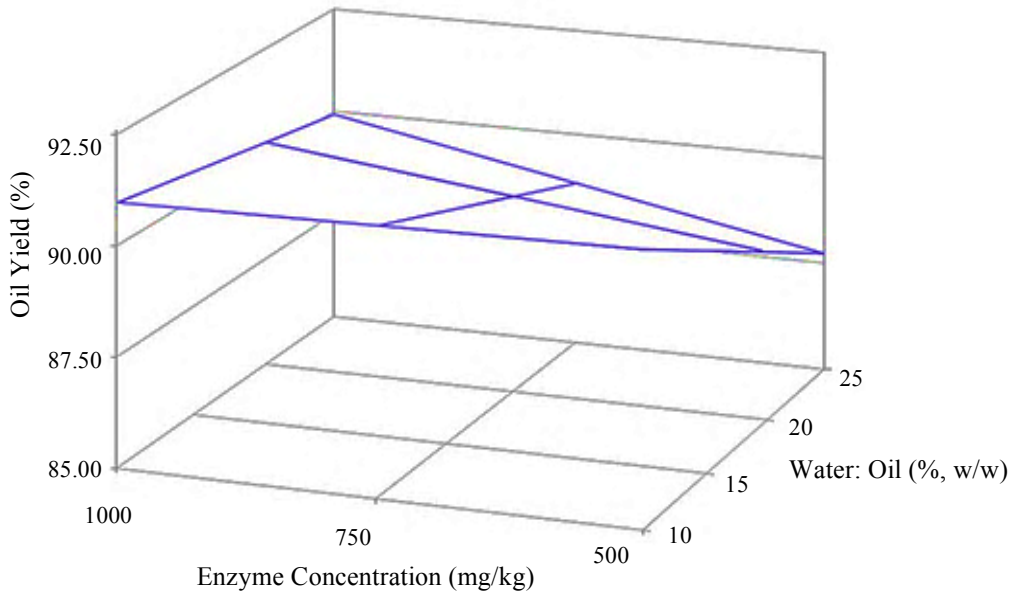
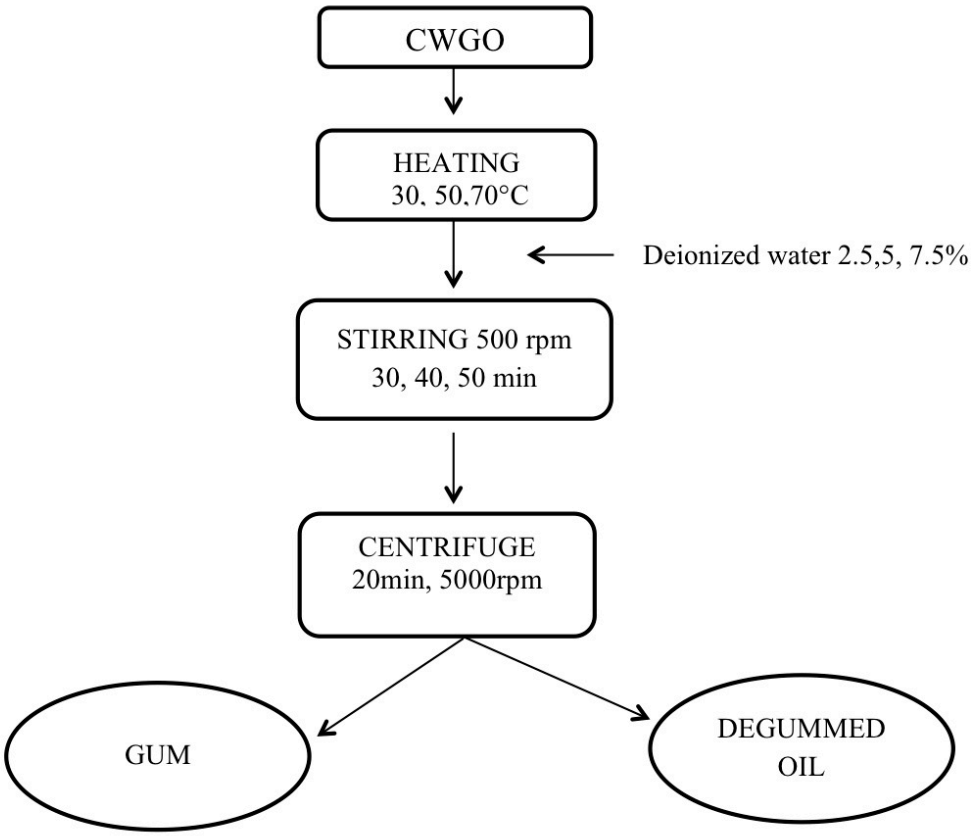
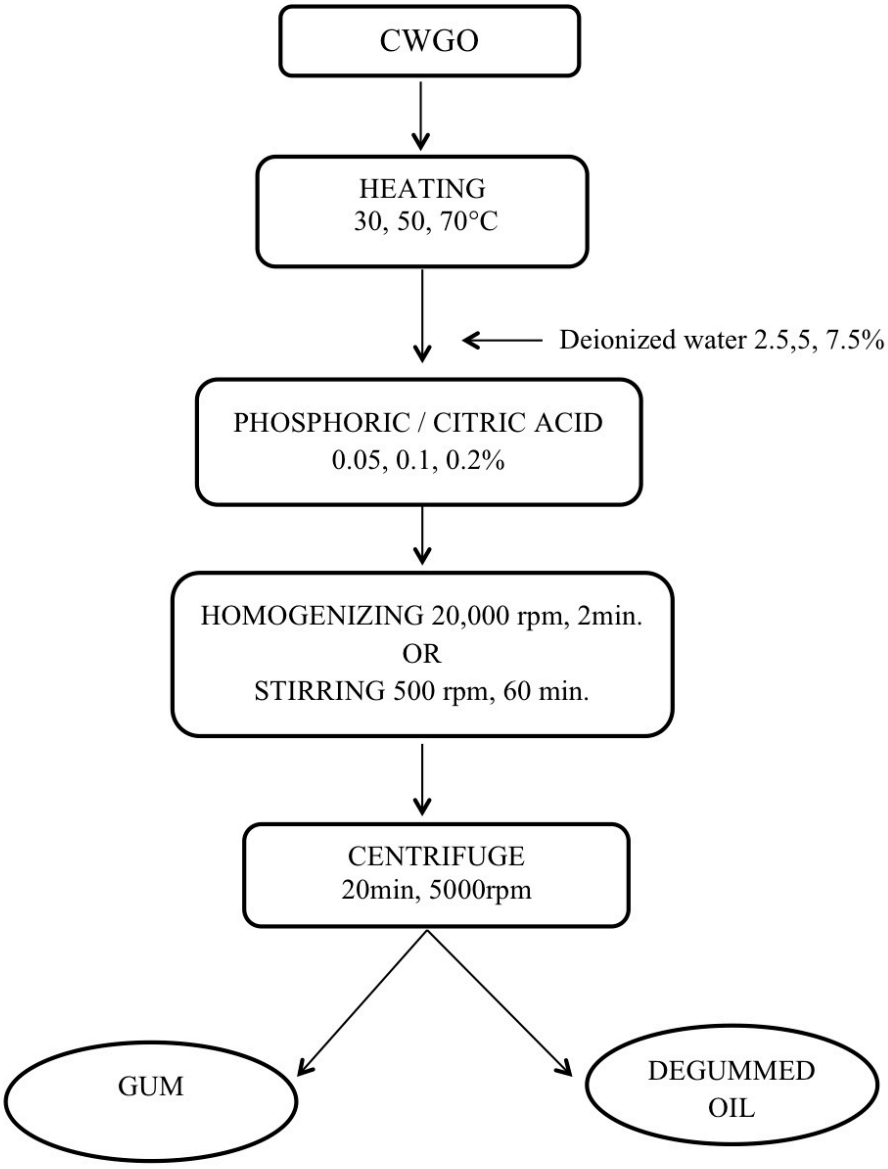


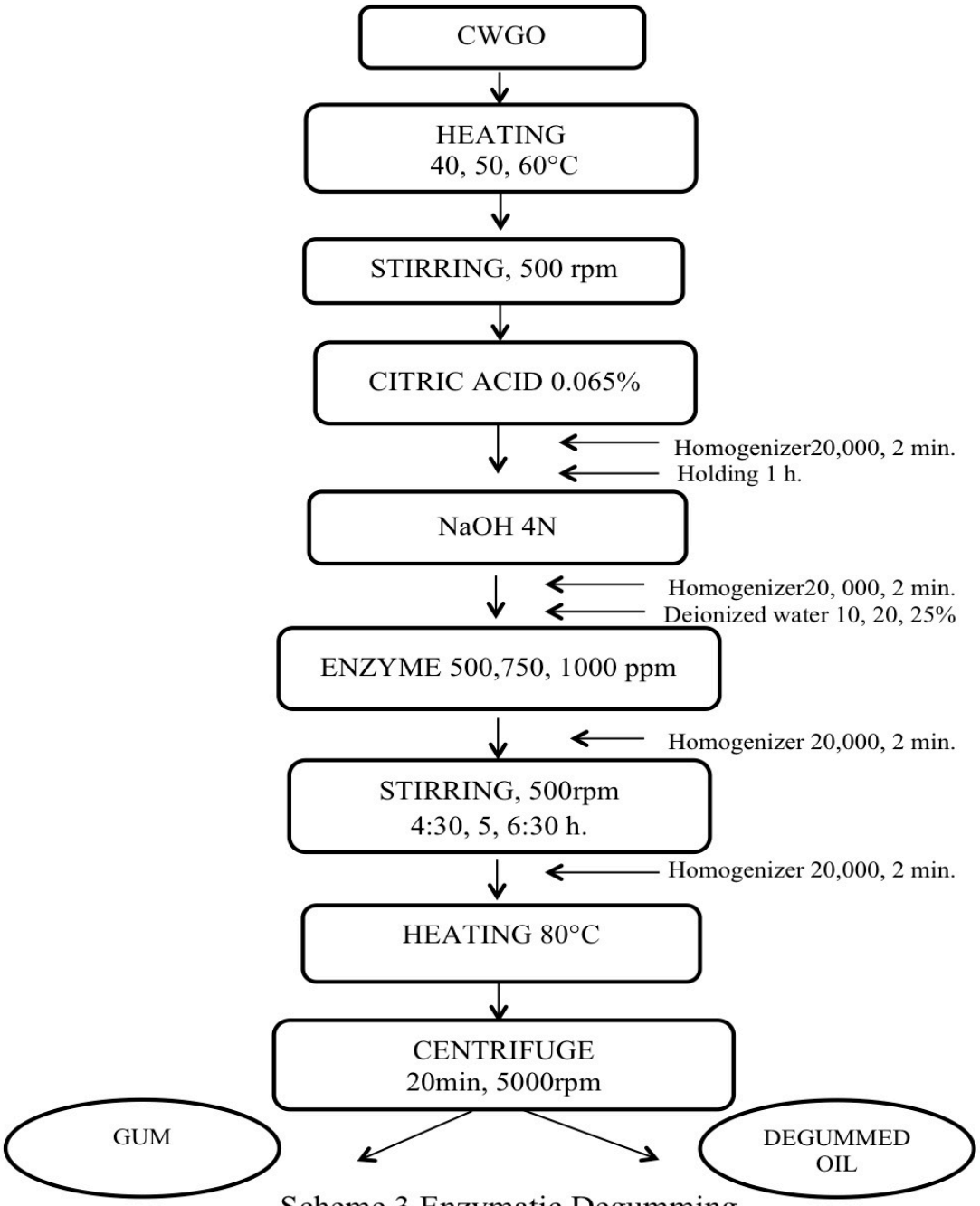
Figure 28: Response surface plot of oil yield as a function of Lysomax concentration and water: oil ratio at 60°C.



Scheme 1 Water Degumming



Scheme 2 Acid Degumming



Scheme 3 Enzymatic Degumming

Table 1: Phosphorus content and oil yields from water degumming process.

Temperature (°C)	Time (min)	Water: oil (%, w/w)	Phosphorus content (mg/kg)		Oil yield (%, w/w)	
			Actual value	Predicted value	Actual value	Predicted value
30	30	2.5	1050	1050	96.1	96.1
30	30	5	915	977	94.4	94.6
30	30	7.5	883	903	93.2	93.1
30	40	2.5	1201	1040	95.0	96.3
30	40	5	988	961	95.1	94.9
30	40	7.5	817	886	92.8	93.4
30	50	2.5	1096	1150	97.8	96.6
30	50	5	960	1080	94.5	95.1
30	50	7.5	1120	1010	93.9	93.7
50	30	2.5	1103	1140	96.1	96.1
50	30	5	1180	1060	95.3	94.6
50	30	7.5	984	990	93.5	93.2
50	40	2.5	1110	1090	96.9	96.1
50	40	5	980	1010	94.3	94.6
50	40	7.5	942	938	92.9	93.1
50	50	2.5	1140	1170	97.1	96.0
50	50	5	1120	1097	94.0	94.6
50	50	7.5	889	1020	91.5	93.1
70	30	2.5	1240	1230	98.2	98.2
70	30	5	1270	1150	96.1	96.7
70	30	7.5	1030	1080	95.5	95.3
70	40	2.5	1090	1140	97.5	97.9
70	40	5	968	1070	96.4	96.4
70	40	7.5	1070	991	94.7	94.9
70	50	2.5	1240	1190	97.5	97.5
70	50	5	1130	1110	95.5	96.0
70	50	7.5	1060	1040	95.2	94.6
30	30	2.5	1080	1050	96.0	96.1
30	30	7.5	893	903	93.6	93.1
30	50	2.5	1104	1150	97.0	96.6
30	50	7.5	1150	1010	94.0	93.7
50	40	5	968	1010	94.4	94.6
70	30	2.5	1198	1230	97.9	98.2
70	30	7.5	1050	1080	95.6	95.3
70	50	2.5	1204	1190	97.3	97.5
70	50	7.5	1060	1040	95.6	94.6

Table 2: Statistical analysis of the estimates of the model parameters determined for residual phosphorus content after water degumming process.

Variable	DF	Parameter Estimate	P-Value
Model	5	-	< 0.0001
Lack of Fit	21	-	< 0.0001
Intercept	1	1704.55309	0.001
Temp.	1	9.57105	0.009
Time	1	-44.03690	0.063
Time * Time	1	0.68046	0.021
Water: Oil	1	-29.76785	<0.0001
Temp * Time	1	-0.17401	0.047

$P < 0.05$ indicates statistical significance.

Table 3: Statistical analysis of the estimates of the model parameter determined for oil yield after water degumming process.

Variable	DF	Parameter Estimate	*P*-Value
Model	5	-	< 0.0001
Lack of Fit	21	-	< 0.0012
Intercept	1	99.08	< 0.0001
Temp.	1	-0.16	0.0282
Temp*Temp	1	0.003	0.0002
Time	1	0.07	0.0650
Water: Oil	1	-0.59	< 0.0001
Temp*Time	1	-0.002	0.0420

$P < 0.05$ indicates statistical significance.

Table 4: Phosphorus content and oil yields from acid degumming process.

Temperature (°C)	Acid Type	Time	Conc. (%)	Water: oil (%,w/w)	Phosphorus (mg/kg)	Oil Yield (%, w/w)
30	Phosphoric acid	Homogenized (2min)	0.1	2.5	1460	96.6
30	Phosphoric acid	Homogenized (2min)	0.1	5	1540	98.7
30	Phosphoric acid	60 min	0.1	7.5	1480	98.5
30	Phosphoric acid	60 min	0.05	2.5	1580	95.2
30	Phosphoric acid	60 min	0.05	7.5	1430	99.7
30	Phosphoric acid	Homogenized (2min)	0.2	7.5	1440	91.2
30	Phosphoric acid	60 min	0.2	2.5	1420	98.4
30	Phosphoric acid	60 min	0.2	5	1490	95.5
30	Phosphoric acid	Homogenized (2min)	0.05	5	1460	94.3
30	Citric acid	60 min	0.2	5	1220	99.0
30	Citric acid	60 min	0.1	7.5	1220	98.9
30	Citric acid	Homogenized (2min)	0.05	5	1120	95.2
30	Citric acid	Homogenized (2min)	0.1	5	1070	96.5
30	Citric acid	Homogenized (2min)	0.1	2.5	1210	94.6
30	Citric acid	60 min	0.05	2.5	1240	95.4
30	Citric acid	Homogenized (2min)	0.2	7.5	1050	92.2
30	Citric acid	Homogenized (2min)	0.2	2.5	1090	94.2
30	Citric acid	Homogenized (2min)	0.05	7.5	1020	92.4
50	Phosphoric acid	60 min	0.2	2.5	1490	99.2
50	Phosphoric acid	60 min	0.05	5	1470	96.2
50	Phosphoric acid	60 min	0.2	7.5	1410	99.2
50	Phosphoric acid	Homogenized (2min)	0.2	5	1210	92.4
50	Phosphoric acid	60 min	0.1	2.5	1410	99.2
50	Phosphoric acid	60 min	0.05	7.5	1450	99.8
50	Phosphoric acid	Homogenized (2min)	0.05	2.5	1495	98.5

50	Phosphoric acid	60 min	0.1	5	1301	96.4
50	Phosphoric acid	Homogenized (2min)	0.1	7.5	1440	95.7
50	Citric acid	60 min	0.1	5	1210	99.3
50	Citric acid	Homogenized (2min)	0.1	2.5	1160	94.3
50	Citric acid	Homogenized (2min)	0.2	5	1060	91.2
50	Citric acid	Homogenized (2min)	0.05	5	1198	93.6
50	Citric acid	60 min	0.05	7.5	1250	95.0
50	Citric acid	Homogenized (2min)	0.2	7.5	1160	91.6
50	Citric acid	60 min	0.2	2.5	1197	97.8
50	Citric acid	Homogenized (2min)	0.1	7.5	1060	92.6
50	Citric acid	Homogenized (2min)	0.05	2.5	1130	94.9
70	Phosphoric acid	60 min	0.05	5	1460	96.4
70	Phosphoric acid	Homogenized (2min)	0.05	7.5	989	93.5
70	Phosphoric acid	Homogenized (2min)	0.1	7.5	1360	99.6
70	Phosphoric acid	Homogenized (2min)	0.1	5	1170	93.6
70	Phosphoric acid	60 min	0.2	5	1410	99.2
70	Phosphoric acid	Homogenized (2min)	0.2	2.5	1470	97.5
70	Phosphoric acid	60 min	0.05	2.5	1460	99.8
70	Phosphoric acid	60 min	0.2	7.5	1320	98.6
70	Phosphoric acid	60 min	0.1	2.5	1430	95.2
70	Citric acid	Homogenized (2min)	0.05	2.5	1190	98.3
70	Citric acid	Homogenized (2min)	0.1	5	1170	98.5
70	Citric acid	60 min	0.2	7.5	1230	96.8
70	Citric acid	60 min	0.1	2.5	1240	99.9
70	Citric acid	60 min	0.05	5	1230	95.2
70	Citric acid	Homogenized (2min)	0.1	7.5	1170	96.6
70	Citric acid	Homogenized (2min)	0.2	2.5	1170	95.2
70	Citric acid	Homogenized (2min)	0.2	5	1160	96.8
70	Citric acid	Homogenized (2min)	0.05	7.5	1090	95.9

Table 5: Statistical analysis of the estimates of the model parameter determined for residual phosphorus content after acid degumming process.

Variable	DF	*F*-value	*P*-Value
Acid	1	101.28	< 0.0001
Time	1	14.90	0.001
Acid*Time	1	0.40	0.5363
Temp	2	1.80	0.1918
Acid*Temp	2	6.69	0.0060
Time*Temp	2	0.22	0.8072
Conc	2	0.18	0.8336
Acid*Conc	2	0.08	0.9229
Time*Conc	2	1.32	0.2887
Temp*Conc	4	1.20	0.3430
Water: oil	2	3.88	0.0376
Acid* Water: oil	2	1.25	0.3071
Time* Water: oil	2	0.96	0.3981
Temp* Water: oil	4	1.24	0.3253
Conc* Water: oil	4	1.73	0.1829

$P < 0.05$ indicates statistical significance.

Table 6: Statistical analysis of the estimates of the model parameter determined for oil yield after acid degumming process.

Effect	DF	*F*-Value	*P* > *F*
Acid	1	1.07	0.3136
Time	1	21.47	0.0002
Acid*Time	1	0.01	0.9107
Temp	2	2.13	0.1447
Acid*Temp	2	1.04	0.3725
Time*Temp	2	0.68	0.5167
Conc	2	1.96	0.1675
Acid*Conc	2	0.50	0.6145
Time*Conc	2	0.91	0.4189
Temp*Conc	2	0.15	0.9631
Ratio	2	0.96	0.4000
Acid*Ratio	2	2.16	0.1416
Time*Ratio	2	0.51	0.6075
Temp*Ratio	4	0.48	0.7481
Conc*Ratio	4	0.79	0.5424

$P < 0.05$ indicates statistical significance.

Table 7: Phosphorus content and oil yields from enzymatic degumming process.

Temperature (°C)	Enzyme	Time (min)	Conc. (mg/kg)	Water: oil (%,w/w)	Phosphorus (mg/kg)	Oil yield (%,w/w)
40	Lecitase Ultra	270	500	10	440	91.8
40	Lecitase Ultra	270	750	25	380	91.6
40	Lecitase Ultra	270	1000	20	496	91.2
40	Lecitase Ultra	300	500	25	378	91.5
40	Lecitase Ultra	300	750	20	503	89.3
40	Lecitase Ultra	300	1000	10	436	90.6
40	Lecitase Ultra	390	500	20	419	92.4
40	Lecitase Ultra	390	750	10	288	88.2
40	Lecitase Ultra	390	1000	25	456	92.8
40	Lysomax	270	500	20	698	87.8
40	Lysomax	270	750	10	747	92.4
40	Lysomax	270	1000	25	553	90.1
40	Lysomax	300	500	10	476	89.2
40	Lysomax	300	750	25	656	87.4
40	Lysomax	300	1000	20	448	90.4
40	Lysomax	390	500	25	700	87.8
40	Lysomax	390	750	20	294	89.6
40	Lysomax	390	1000	10	346	91.9
50	Lecitase Ultra	270	500	25	226	86.0
50	Lecitase Ultra	270	750	20	266	88.4
50	Lecitase Ultra	270	1000	10	392	87.1
50	Lecitase Ultra	300	500	20	378	90.0
50	Lecitase Ultra	300	750	10	353	91.3
50	Lecitase Ultra	300	1000	25	334	91.9
50	Lecitase Ultra	390	500	10	338	87.2
50	Lecitase Ultra	390	750	25	402	87.4
50	Lecitase Ultra	390	1000	20	384	88.4
50	Lysomax	270	500	25	743	87.2
50	Lysomax	270	750	20	643	89.5

50	Lysomax	270	1000	10	757	88.9
50	Lysomax	300	500	20	684	89.2
50	Lysomax	300	750	10	579	89.9
50	Lysomax	300	1000	25	536	92.5
50	Lysomax	390	500	10	509	91.6
50	Lysomax	390	750	25	542	87.8
50	Lysomax	390	1000	20	451	90.7
60	Lecitase Ultra	270	500	20	348	89.6
60	Lecitase Ultra	270	750	10	354	92.7
60	Lecitase Ultra	270	1000	25	451	88.9
60	Lecitase Ultra	300	500	10	417	89.6
60	Lecitase Ultra	300	750	25	392	91.0
60	Lecitase Ultra	300	1000	20	478	91.4
60	Lecitase Ultra	390	500	25	403	88.0
60	Lecitase Ultra	390	750	20	219	88.7
60	Lecitase Ultra	390	1000	10	358	88.4
60	Lysomax	270	500	10	417	90.0
60	Lysomax	270	750	25	506	88.2
60	Lysomax	270	1000	20	670	91.1
60	Lysomax	300	500	25	586	89.0
60	Lysomax	300	750	20	637	88.4
60	Lysomax	300	1000	10	690	92.9
60	Lysomax	390	500	20	678	89.0
60	Lysomax	390	750	10	756	91.8
60	Lysomax	390	1000	25	566	88.6

Table 8: Statistical analysis of the estimates of the model parameter determined for residual phosphorus content after enzymatic degumming process.

Effect	DF	*F*-Value	*P*-Value
Enzyme	1	44.80	<0.0001
Temp	1	0.91	0.3455
Time	1	0.37	0.5467
Conc	1	5.01	0.0302
Water: oil	1	4.06	0.0499
Time* Water: oil	1	4.05	0.0502
Time* Conc	1	4.28	0.0445
Temp* Time	1	0.79	0.3785

$P < 0.05$ indicates statistical significance.

Table 9: The effect of enzymatic degumming process on predicted phosphorus content at 40°C.

Time (min)	Water: oil (%)	Conc. (mg/kg)	Phosphorus content (mg/kg)	
			Lecitase Ultra	Lysomax
270	10	500	399	586
270	10	750	448	635
270	10	1000	497	684
270	20	500	357	544
270	20	750	406	593
270	20	1000	455	642
270	25	500	336	523
270	25	750	385	572
270	25	1000	434	621
300	10	500	383	570
300	10	750	414	601
300	10	1000	445	632
300	20	500	364	552
300	20	750	395	582
300	20	1000	426	613
300	25	500	355	542
300	25	750	385	573
300	25	1000	416	604
390	10	500	335	523
390	10	750	311	499
390	10	1000	288	475
390	20	500	386	574
390	20	750	362	550
390	20	1000	338	526
390	25	500	411	599
390	25	750	388	575
390	25	1000	364	551

Table 10: Statistical analysis of the estimates of the model parameter determined for oil yield after enzymatic degumming process.

Effect	DF	F Value	P > F
Enzyme	1	5.08	0.0290
Temp	1	4.45	0.0403
Temp* Temp	1	4.24	0.0452
Conc	1	1.10	0.3007
Water: oil* Enzyme	2	5.64	0.0065
Conc* Water: oil	1	3.47	0.0689

$P < 0.05$ indicates statistical significance.

Table 11: The effect of enzymatic degumming process on predicted oil yield.

Temperature (°C)	Water: oil (%, w/w)	Conc. (mg/kg)	Oil yield (% ,w/w)	
			Lecitase Ultra	Lysomax
40	10	500	90.3	91.6
40	10	750	90.2	91.5
40	10	1000	90.1	91.5
40	20	500	89.7	89.4
40	20	750	90.4	90.1
40	20	1000	91.1	90.8
40	25	500	89.4	88.2
40	25	750	90.5	89.3
40	25	1000	91.6	90.4
50	10	500	89.1	90.4
50	10	750	89.1	90.4
50	10	1000	89.0	90.3
50	20	500	88.6	88.20
50	20	750	89.3	88.9
50	20	1000	90.0	89.6
50	25	500	88.3	87.1
50	25	750	89.4	88.2
50	25	1000	90.4	89.2
60	10	500	89.8	91.1
60	10	750	89.7	91.0
60	10	1000	89.7	91.0
60	20	500	89.2	88.9
60	20	750	89.9	89.6
60	20	1000	90.6	90.3
60	25	500	88.9	87.7
60	25	750	90.0	88.8
60	25	1000	91.1	89.9

Table 12: Characterization of WGO.

Sample*	FFA (%)	PV (meq/kg)	*p*-Anisidine Value	Moisture content (%)	Phosphorus (mg/kg)
CWGO H	15.2±0.8^{d}	15.6±0.5^{f}	5.3±0.1^{f}	0.4±0.01^{f}	1860±35.6^{a}
CWGO SP	3.2±0.02^{g}	11.9±0.09 ^{j}	5.7±0.4^{f}	0.4±0.007^{ef}	1360±100.5^{b}
WD	16.5±0.7^{c}	17.3±0.08^{e}	8.2±0.09^{e}	0.6±0.05^{d}	817±91.6^{f}
WD SP	3.6±0.1^{fg}	12.1±0.1^{ij}	5.7±0.2^{f}	0.9±0.03^{a}	1050±83.5^{de}
ADC	16.4±0.3^{c}	21.8±0.2^{cd}	15.1±0.5^{d}	0.7±0.06^{c}	1020±10.4^{e}
ADC SP	4.9±0.4^{f}	12.6±0.04^{hi}	6.4±0.02^{f}	0.6±0.02^{d}	1140±29.2^{cd}
ADPH	16.5±0.3^{c}	22.3±0.3^{c}	19.1±0.1^{c}	0.7±0.03^{c}	989±22.1^{e}
ADPH SP	4.3±0.2^{f}	13.2±0.06^{h}	9.1±0.5^{e}	0.3±0.001^{f}	1190±75.01^{c}
EDLU	20.1±0.3^{a}	25.3±0.04^{a}	24.4±0.9^{a}	0.5±0.04^{e}	219±5.6^{h}
EDLU SP	8.8±0.4^{e}	14.0±0.2^{g}	16.1±0.4^{d}	0.5±0.02^{e}	250±13.4^{h}
EDL	19.7±0.1^{a}	24.2±0.2^{b}	19.4±1.5^{c}	0.6±0.02^{d}	294±34.1^{h}
EDL SP	4.5±0.3^{f}	13.8±0.07^{g}	8.0±0.7^{e}	0.7±0.006^{c}	300±6.7^{h}
EDG	17.9±0.3^{b}	21.4±0.5^{d}	20.8±0.9^{b}	0.4±0.01^{f}	584±29.2^{g}
EDG SP	4.3±0.02^{f}	12.8±0.05^{h}	9.1±0.1^{e}	0.8±0.004^{b}	645±5.01^{g}

* The sample abbreviations are as following:
CWGO H: commercially hexane extracted CWGO; CWGO SP: mechanically pressed CWGO; WD: hexane extracted and water degummed WGO; WD SP: mechanically pressed and water degummed oil; ADC: hexane extracted and citric acid degummed oil ; ADC SP: mechanically pressed citric acid degummed oil ; ADPH: hexane extracted and phosphoric acid degummed oil ; ADPH SP: mechanically pressed and phosphoric acid degummed oil; EDLU: hexane extracted oil that is degummed using Lecitase Ultra; EDLU SP: mechanically pressed oil that is degummed using Lecitase Ultra; EDL: hexane extracted oil that is degummed using Lysomax; EDL SP: mechanically pressed oil that is degummed using Lysomax; EDG: hexane extracted oil that is degummed using Gumzyme; EDG SP: mechanically pressed oil that is degummed using Gumzyme.

^{a,b,c,d,e,f,g,h,i,j} Sample means ± SD with the same letter are not significantly different ($p > 0.05$).

Table 13: Tocopherol composition (mg/g oil) of WGO samples processed through various methods.

Sample*	α-Tocopherol	β-Tocopherol	δ-Tocopherol	Total Tocopherol
CWGO H	3.4 ± 0.2^{b}	0.7 ± 0.04^{bcd}	0.5 ± 0.004^{a}	4.6 ± 0.2^{b}
CWGO SP	3.9 ± 0.05^{a}	0.9 ± 0.01^{a}	0.3 ± 0.006^{d}	5.2 ± 0.07^{a}
WD	3.1 ± 0.02^{cd}	0.7 ± 0.03^{cd}	0.09 ± 0.003^{j}	3.9 ± 0.05^{e}
WD SP	3.0 ± 0.06^{d}	0.7 ± 0.0001^{cd}	0.2 ± 0.0002^{e}	4.0 ± 0.06^{de}
ADC	1.2 ± 0.0004^{f}	0.6 ± 0.04^{ef}	0.1 ± 0.02^{gh}	2.0 ± 0.06^{h}
ADC SP	3.2 ± 0.01^{cd}	0.8 ± 0.05^{b}	0.3 ± 0.02^{d}	4.3 ± 0.1^{c}
ADPH	0.6 ± 0.02^{h}	0.3 ± 0.02^{g}	0.1 ± 0.02^{fg}	1.1 ± 0.06^{j}
ADPH SP	3.1 ± 0.08^{cd}	0.8 ± 0.02^{b}	0.3 ± 0.003^{d}	4.2 ± 0.10^{cd}
EDLU	0.9 ± 0.001^{g}	0.1 ± 0.006^{h}	0.1 ± 0.009^{i}	1.1 ± 0.02^{j}
EDLU SP	1.9 ± 0.01^{e}	0.7 ± 0.004^{bc}	0.4 ± 0.004^{b}	3.1 ± 0.02^{f}
EDL	1.9 ± 0.11^{e}	$0.6\pm0.01d^{e}$	0.1 ± 0.02^{gh}	2.7 ± 0.1^{g}
EDL SP	3.2 ± 0.07^{bc}	0.8 ± 0.01^{b}	0.4 ± 0.004^{c}	4.4 ± 0.08^{bc}
EDG	0.8 ± 0.02^{gh}	0.6 ± 0.01^{de}	0.1 ± 0.002^{f}	1.6 ± 0.3^{i}
EDG SP	0.7 ± 0.02^{h}	0.5 ± 0.07^{f}	0.1 ± 0.01^{hi}	1.3 ± 0.1^{j}

*Refer to Table 12 for sample abbreviations

^{a,b,c,d,e,f,g,h,I,j} Sample means ± SD within a column that have the same letter are not significantly different ($p > 0.005$).

Table 14: Phospholipid composition (mg/g oil) of WGO samples processed through various methods.

Sample*	PE[1]	PI+PA[2]	PC[3]	Total Phospholipid
CWGO H	2.1±0.06[a]	7.7±0.2[b]	3.1±0.1[a]	13.0±0.4[a]
CWGO SP	1.9±0.06[b]	9.2±0.5[a]	0.8±0.03[d]	12.1±0.6[b]
WD	1.8±0.007[c]	2.4±0.007[de]	1.2±0.006[c]	5.5±0.02[d]
WD SP	1.1±0.06[e]	2.3±0.03[e]	0.3±0.002[fg]	3.8±0.09[e]
ADC	1.1±0.08[e]	2.8±0.1[c]	1.8±0.1[b]	5.8±0.4[d]
ADC SP	1.1±0.01[e]	2.2±0.01[e]	0.1±0.01[gh]	3.5±0.03[ef]
ADPH	1.3±0.07[d]	2.7±0.06[cd]	3.1±0.3[a]	7.2±0.4[c]
ADPH SP	1.0±0.02[f]	2.3±0.03[e]	0.6±0.05[e]	3.9±0.1[e]
EDLU	0.3±0.007[h]	1.6±0.001[f]	0.1±0.0001[h]	2.0±0.008[g]
EDLU SP	0.5±0.02[g]	1.6±0.001[f]	n.d.	2.2±0.02[g]
EDL	0.3±0.02[h]	1.6±0.01[f]	n.d.	2.0±0.03[g]
EDL SP	0.5±0.002[g]	1.8±0.02[f]	n.d.	2.3±0.02[g]
EDG	0.3±0.02[h]	2.4±0.02[de]	0.4±0.03[ef]	3.2±0.07[f]
EDG SP	0.5±0.02[g]	1.6±0.002[f]	n.d.	2.1±0.02[g]

*Refer to Table 12 for sample abbreviations.

PE[1]: phosphatidylethanolamine; PI+PA[2]: phosphatidylinositol and phosphatic acid; PC[3]: phosphatidylcholine; n.d. not detected.

[a,b,c,d,e,f,g,h] Sample means ± SD within a column that have the same letter are not significantly different (p> 0.005).

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