PULSED ULTRASOUND-ASSISTED SOLVENT EXTRACTION OF OIL FROM SOYBEANS AND MICROALGAE

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DEDICATION

To my parents.

ABSTRACT

The two biological oils used in this study come from two distinctly different sources; soybeans and microalgae. Yet, both are economically important due to the similarities in their nutritional and industrial value. Besides being the top sources for health-promoting polyunsaturated fatty acids (PUFA), both oils are becoming increasingly important in the production of biodiesel (through transesterification). The microalgal species chosen for the current study was *Nannochloropsis oculata*, a small marine microalga known for its unusually high lipid content. Conventionally, soybean and microalgal oils are extracted using the organic solvents n-hexane and chloroform-methanol (CM), respectively. However, both solvents have several drawback associated with them; n-hexane is highly volatile and flammable, while CM poses high toxicity and safety concerns (due to the presence of chloroform). The alternative solvent mixture of hexane-isopropanol (HIP), as a result of its better safety and efficiency qualities, has been used as the primary solvent in this study.

Ultrasound-assisted solvent extraction or UASE has recently become popular as a novel alternative to traditional standalone solvent extraction, offering numerous advantages such as shorter treatment times, simpler sample preparation and higher efficiency. Hence, pulsed UASE with HIP (3:2 v/v) as solvent was the principal technique investigated in this study. Ultrasonic processing was carried out using an immersed probe (or sonotorode) resonating at a maximum amplitude of 124 µm. Two parameters related to UASE were studied; resonance amplitude and effective treatment time. The oil yields (assessed gravimetrically) and fatty acid profiles (from GC) of the extracted oils were analysed. The study showed that pulsed UASE resulted in high extraction efficiency (upto 19.92% oil yield was obtained for soybeans and upto 69.53% oil was extracted from *N. oculata*). Both amplitude and treatment time were found to have significant effects on oil yield. However, the study also showed that high resonance amplitude and treatment time did not necessarily produce high yields. For soybeans, the oil extracted by pulsed UASE was found to be comparable in fatty acid composition to that extracted by traditional methods such as Soxhlet and room temperature shaking. In contrast, the extracted microalgal oil had a superior PUFA profile compared to these two traditional methods.

RÉSUMÉ

Dans cette étude, deux huiles de sources très différentes sont utilisées; les graines de soja et les microalgues. Chacune de ces huiles possèdent un intérêt économique important en raison de sa valeur nutritive et industrielle. Elles sont connues pour être une des principales sources en acides gras polyinsaturés (AGPI), et deviennent de plus en plus utilisées dans la production de biodiesel (par transestérification). L'espèce de microalgues choisie pour l'étude était *Nannochloropsis oculata*, une petite microalgue marine connue pour sa teneur en lipides anormalement élevés. Traditionnellement, les huiles de soja et de microalgues sont extraites en utilisant respectivement, les solvants n-hexane et le chloroforme-méthanol (CM). Toutefois, ces deux solvants ont plusieurs inconvénients; le n-hexane est très volatil et inflammable, tandis que le CM possède une toxicité élevée et des problèmes de sécurité (en raison de la présence de chloroforme). Un solvant alternatif, l'hexane-isopropanol (HIP), a donc été utilisé comme solvant principal dans cette étude en raison de sa moins grande nocivité et de son efficacité.

L'extraction assistée par ultrason avec solvant ou EAUS est de plus en plus utilisée comme nouvelle alternative à l'extraction par solvant traditionnelle, offrant de nombreux avantages tels qu'un temps de traitement plus court, une préparation de l'échantillon plus simple et une meilleure efficacité. Ainsi, la principale technique utilisée dans cette étude a été la technique EAUS avec HIP (3:2 v/v) comme solvant. Le traitement par ultrasons a été réalisée en utilisant une sonde immergée (ou sonotorode) résonnant à une amplitude maximale de 124 µm. Deux paramètres ont été étudiés; l'amplitude de résonance et la durée de traitement. Les rendements en huile (évaluée par gravimétrie) et la composition en acides gras (par CG) des huiles extraites ont été analysées. L'étude a montré que l'EAUS abouti à des taux d'extraction élevés (jusqu'à 19.92% de rendement en huile pour les graines de soja et jusqu'à 69.53% d'huile extraite à partir de *N. oculata*). L'amplitude et la durée de traitement ont tous deux des effets significatifs sur le rendement en huile. Cependant, l'étude a également montré qu'une amplitude de résonance et un temps de traitement élevé ne donnent pas nécessairement un fort rendement. Pour les graines de soja, la composition en acide gras de l'huile extraite par EAUS est comparable à celle extraite par des méthodes traditionnelles telles que Soxhlet et l'extraction par agitation à température ambiante. En revanche, l'huile extraite des microalgues a une teneur en AGPI plus élevé par rapport à ces deux méthodes traditionnelles.

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LIST OF SYMBOLS AND ABBREVIATIONS

FA	Fatty acid(s)
EFA	Essential fatty acid(s)
MUFA	Monounsaturated fatty acids(s)
PUFA	Polyunsaturated fatty acid(s)
HIP	n-hexane-isopropanol
CM	Chloroform-methanol
UASE	Ultrasound-assisted solvent extraction
PUASE	Pulsed ultrasound-assisted solvent extraction
LA	Linoleic acid
ALA	Alpha-linolenic acid
EPA	Eicosapentaenoic acid
AA	Arachidonic acid
GLA	Gamma-linolenic acid
EDA	Eicosadienoic acid
DGLA	Dihomo-gamma-linolenic acid
DHA	Docosahexaenoic acid
ω-3	Omega-3
ω-6	Omega-6
FAME	Fatty acid methyl ester(s)
GC	Gas chromatography
FID	Flame ionization detector
UWI	Ultrasonic wave intensity
SCO_2	Supercritical carbon dioxide
RT	Room temperature

CHAPTER 1: **INTRODUCTION AND OBJECTIVES**

1.1. GENERAL INTRODUCTION

Throughout the course of natural history, few substances have had a more profound position in human life than *oils*. Even though the 21st century usage of the mononym *oil* is almost exclusively reserved for fossil-based mineral oils or crude oil – the primary source of fuel for the modern age, *biological oils* (i.e., those produced by living organisms) have been an integral part of human life since time immemorial.

Biochemically, oils are usually grouped together with *fats* as a class of compounds under *lipids*, one of the four major groups of large polymeric biological molecules (or *biomolecules*) that make up living organisms; the others being proteins, polysaccharides and nucleic acids. Fats are chemically similar to oils but differ mainly in their physical state; oils are liquid at room temperature, while fats are solids. Oils and fats serve a plethora of functions in all living beings, both organisms that produce them, as well those that consume them (Nelson, Lehninger et al. 2008).

Vegetable oils are the most important class of biological oils and comprise those that are edible. The term could be misleading, as edible oils of both plant and animal origin are considered vegetable oils. *The Lipid Handbook* (Gunstone, Harwood et al. 2007) – one of the most prominent reference works related to edible oils - lists 17 major vegetable oils produced worldwide, based on their biological sources of origin. Oils of plant origin make up the vast majority of these as a result of their diverse and enormous number of applications in food preparation, the most notable of which are: flavor, texture, shortening, and cooking. Few animal fats such as butter and ghee also share similar functions (Gunstone 2005; Gunstone and Harwood 2007). Among vegetable oils, the largest market shares belong to those obtained from oil palm, soybeans, canola (rapeseed), sunflower, peanuts and cottonseed (Foreign Agricultural Service 2011). Oils obtained from tropical plants such as coconut and oil palm are more popular among Eastern cultures. This widespread presence of edible vegetable oils in human diet has given them a significant role in human nutrition and health. One of these important functions is as a source of *essential fatty acids* (EFAs); these are metabolically-important fatty acids that cannot be synthesised by humans and need to be taken through diet (Akoh and Min 2008). In addition to their nutritional significance, vegetable oils also have important industrial functions. The imminent exhaustion of fossil fuels has further pushed biological oils into the limelight as renewable and/or more sustainable sources for the production of next-generation biofuels like biodiesel (Gunstone 2011).

Traditionally the most preferred method of oil extraction involves the use of an organic solvent to selectively transfer (and hence remove) lipids from their biological source material (Birch and Ian 2000; Wang 2004). Hexane is the industrial favourite for soybeans (Hammond, Johnson et al. 2005), while a solvent mixture comprising chloroform and methanol is the preferred solvent for extraction of oil from microalgae (Woertz 2007). However, both these solvents have substantial drawbacks such as high flammability (Russin, Boye et al. 2011) and high toxicity (Radin 1989), respectively. In addition, lipid extraction is not complete as most polar lipids such as phospholipids are left out (Erickson 2008). Among the alternative solvents suggested, a mixture of hexane and isopropanol (HIP) in the ratio 3:2 (v/v) looks most promising due to its relatively safer nature and, comparable or superior extraction efficiency (Hara and Radin 1978; Radin 1981; Radin 1989).

Although standalone solvent extraction is the industrial standard for the extract of several vegetable oils, this method requires long treatment times and several sample preparation steps prior to the actual extraction process. In the case of soybeans, these tedious steps have profound industrial implications; they include cleaning, dehulling, moisture-conditioning and flaking (Wang 2004; Hammond, Johnson et al. 2005). With microalgae, the problem is of a completely different nature; presence of rigid cell walls that make cell disruption difficult (Lewis and Eric 1992; Harun, Singh et al. 2010; Lee, Yoo et al. 2010; Halim, Gladman et al. 2011). Hence, several innovative methods have been suggested, that could be used to 'assist' the solvent extraction process. Among these, two methods have had significant success; microwaves and ultrasound (Pernet and Tremblay 2003; Li, Pordesimo et al. 2004; Wang and Weller 2006; Cravotto, Boffa et al. 2008; Tran, Hong et al. 2009; Lee, Yoo et al. 2010; Halim, Gladman et al. 2011; Prabakaran and Ravindran 2011). Microwave-assisted extraction (MAE) is a thermal process while ultrasound assisted solvent extraction (UASE) is a more mechanical process. Even though MAE has been proven to extract several valuable phytochemicals (Wang and Weller 2006), the thermal nature of the method makes it unsuitable for the extraction of PUFAs due to their high susceptibility to oxidation and degradation. UASE on the other hand, has been successfully used for the extraction of oils from different biomaterials including soybeans and microalgae, to varying degrees of success (Cravotto, Binello et al. 2004; Li, Pordesimo et al. 2004; Luque-García and Luque de Castro 2004; Sharma and Gupta 2004; Ranjan, Patil et al. 2010; Prabakaran and Ravindran 2011).

The economic importance of vegetable and microalgal oils makes their extraction an important process. Although information on the application of UASE in the extraction of these oils is both positive and promising, this novel technique is still far from becoming off-the-shelf (Patist and Bates 2008; Patist and Bates 2011). In addition, literature is limited, especially for microalgae. For instance, there are currently no reports on the UASE of lipids from *N. oculata* even though this marine microalga is a well-known source for the PUFA eicosapentaenoic acid (EPA). Further, HIP is still not very popular among researchers working with either of these oils, in spite of reports about its superior extraction efficiency.

1.2. STUDY HYPOTHESIS AND OBJECTIVES

The hypothesis of this research project is that hexane-isopropanol (HIP) is an efficient solvent for extracting biological oils, and that pulsed ultrasound-assisted solvent extraction (PUASE) with HIP as solvent could be used to extract high-quality biological oils with greater efficiency than conventional methods. The overall objective of the project was to improve the effectiveness of oil extraction in terms of oil yield and fatty acid profile from ground soybeans and microalgae. The specific objectives were:

i. To compare oil yields obtained from ground soybeans and *N. oculata* by room temperature shaking using different solvents such as hexane, petroleum ether, chloroform-methanol (CM) and hexane-isopropanol (HIP).

ii. To investigate the effects of ultrasonic resonance amplitude and treatment time on oil yields during PUASE with HIP (3:2 v/v) using an immersed probe system.

iii. To evaluate the influence of three extraction methods (PUASE with HIP, Soxhlet extraction with n-hexane and room temperature shaking with HIP) on fatty acid composition (especially PUFAs) of extracted oils.

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CONNECTING STATEMENT

Chapter 2 presents a review of the current literature on soybean and microalgal oils, their conventional methods of extraction, and the prospects of using ultrasonic processing to assist the solvent extraction of these oils.

CHAPTER 2: **REVIEW OF LITERATURE**

2.1. OILS & FATTY ACIDS

2.1.1. Oils – food as well as fuel

Oils can be described as hydrophobic (water-insoluble) substances that are liquid at room temperature and comprise mostly triglycerides (Belitz, Grosch et al. 2009). In general, most oils are viscous liquids that are soluble in organic solvents. Crudely classified according to sources of origin, there are mainly two kinds of oils; *organic* and *mineral* oils.

Organic or biological oils are those produced by living organisms (such as plants, animals, microorganisms etc.) through biological or organic process. Bearing in mind the astounding variety of living organisms that are capable of producing or processing them, organic oils are amazingly diverse in source and composition. Often, rather than being called oils, they are grouped along with fats, waxes, sterols and other similarly hydrophobic compounds into a class of organic compounds called ***lipids.*** Fats are very similar to oils with the only difference being their solid nature at ambient temperatures. Considering their biological origin, it is perhaps not surprising that biological oils and fats (in their several different forms) represent almost 40% of our diet. Besides being important sources of nutritionally-significant *unsaturated fatty acids*, these oils and their sources are important agricultural commodities (Gunstone 2011). **Mineral oils** on the other hand, are also of organic origin, but they are the product of geochemical conversions under the influence of various physical factors over several millions of years. More commonly referred to as *crude oil* (or *petroleum* when grouped along with its gaseous counterpart; natural gas) they form the most prominent member of the family of *fossil fuels.* These fossilized oils are primarily found in deep underground reservoirs, although some are obtained from surface sources (such as *oil sands*) (Gluyas and Swarbrick 2004). Starting with the industrial revolution, crude oil and its several refined products have been the primary sources of fuel for the human civilization; making crude oil one of the world's most valuable commodities and giving it the nickname 'black gold'. Naturally, the business of petroleum has had an incredible influence on human history and played an outstanding role in shaping the socio-political climate of the past century (Maugeri 2006).

However, over the last several decades, diminishing crude oil reserves and increasing environmental degradation as a result of the use of fossil fuels have led to an increased interest in alternative, cleaner and efficient sources of energy. It is here that organic oils are beginning to attract fresh attention as sources of new generations of *biofuels*.

2.1.2. Fatty acids – biochemistry and nomenclature

Fatty acids (FAs) are the most significant constituents of almost all vegetable, animal and marine (fish or algal) oils. They are an important class of aliphatic lipids comprised of an alkyl chain (carbon backbone) with a carboxylic acid group (-COOH) at one end and a methyl group (-CH_3) at the other end. The length (i.e., the number of carbon atoms, including that of the carboxylic group) and the level of saturation (i.e., the number of double bonds and their location) in the alkyl chain are the two most important features that affect the physical and functional properties of FAs (Gurr, Harwood et al. 2002; Belitz, Grosch et al. 2004; Casal and Oliveira 2007). Since the polar nature of the –COOH group is balanced out by the non-polar nature of the alkyl chain, FAs are neutral in polarity; hence, they are often classified as ***neutral lipids*** (Akoh and Min 2008).

The most prominent functional role of FAs is as a source of metabolic energy other than or in addition to the usual source – glucose. FAs also play several crucial biological roles including the formation and maintenance of cellular membranes, cell signalling etc. For these reasons, they have a prominent presence and pivotal role in human and animal diet, health and nutrition (Belitz, Grosch et al. 2004).

Considering the complex and often complicated nature of FA nomenclature, this manuscript utilizes the *lipid number* (or shorthand) system, according to which FAs are given the form **C:D(x,y,z)**; C stands for the number of carbon atoms in the alkyl chain; *D* denotes the number of unsaturated (double) bonds within the chain; and *x, y, z* represent the position(s) of the double bond(s), if any (counted as the x^{th}, y^{th} and/or z^{th} carbon atom from the carboxylic acid group). For e.g., the unsaturated FA with the trivial (or common) name ***linoleic acid*** (Fig. 1) is assigned the abbreviated designation **18:2(9,12)**; meaning, this FA has an alkyl chain made up of 18 carbons and contains two double bonds, one each at the 9^{th} and 12^{th} carbons from the carboxylic acid group (Wiese and Snyder 1987; Belitz, Grosch et al. 2004; Casal and Oliveira 2007; Jones, IUPAC Subcommittee on Polymer Terminology et al. 2009).

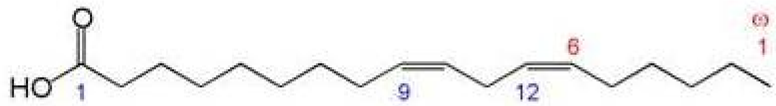

Figure 1: Linoleic acid (LA)

Of the various different FAs found in biological oils, only less than 1% is in their free form. The vast majority are present in the form of **glycerides** i.e., as esters of glycerol. Glycerol (Fig. 2) has three hydroxyl groups, and it reacts with one, two or three FAs to produce mono-, di- or triglycerides respectively. More than 90% of all biological FAs are found in their triglyceride form. Naturally occurring FAs especially in vegetable oils are mostly even-numbered; odd carbon FAs are very rare and are usually only found in foods from animal sources such as milk and cheese. Due to the rarity of odd-numbered FAs, these are often used as *internal standards* in GC analysis of biological oils (Gurr, Harwood et al. 2002; Casal and Oliveira 2007; Gunstone, Harwood et al. 2007).

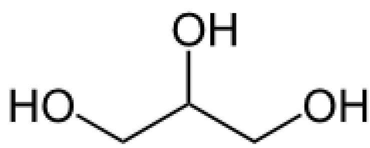

Figure 2: Glycerol structure

2.1.3. Unsaturated fatty acids and human health

These are FAs that contain one or more double bonds in their alkyl chains; the former are called monounsaturated FAs (**MUFAs**) and the latter are called polyunsaturated FAs (**PUFAs**). In order to further differentiate unsaturated FAs from each other biologically, an additional *omega* terminology is used. Here, in addition to lipid numbers, FAs are further classified according to the position of the double bond from the terminal methyl group (called the n^{th} end or the omega terminal), denoted by ***n-x*** or ***ω-x***. Accordingly, three families of unsaturated fatty acids are found in nature: **ω-3, ω-6 and ω-9 FAs**, also known as *linolenic type*, *linoleic type* and *oleic type* respectively. Nutritionally, ω-3 and ω-6 FAs are the most significant and abundant (Holme and Peck 1998; Gurr, Harwood et al. 2002; Belitz, Grosch et al. 2004; Casal and Oliveira 2007).

The presence of double bonds render these FAs with lower melting points than saturated FAs; a property responsible for the characteristic liquid nature (at room temperature) of most vegetable and fish oils (both of which are rich sources of unsaturated FAs) (Belitz, Grosch et al. 2004). In contrast, most fats of animal origin such as butter or lard are solid at room temperature as they are made up predominantly of saturated fatty acids. The *fluid property* of unsaturated FAs, particularly PUFAs is responsible for maintaining the structural integrity of cellular membranes and is of great biological significance (Gurr, Harwood et al. 2002). Besides being vital to membrane fluidity, PUFAs have been proven to possess remarkable health-promoting functions against a wide range of disorders, most notably cardiovascular disease. The therapeutic effects of PUFAs on heart disease has been so well-documented and understood that the American Heart Association (AHA) recently published a set of recommendations for the use of ω -6 FAs (Kris-Etherton, Harris et al. 2003). Other disorders that have been targeted by PUFAs include inflammatory bowel disease (Siguel and Lerman 1996), rheumatoid arthritis (Zurier, Rossetti et al. 1996), cancer (Caygill, Charlett et al. 1996) and even diabetes (Abete, Testa et al. 2009).

The subjects of the current study are themselves renowned sources of these PUFAs; soybean oil is rich in the **ω-6** PUFA ***linoleic acid*** or LA (lipid number – 18:2(9,12)) and microalgal (*Nannochloropsis oculata*) oil is rich in the **ω-3** PUFA ***eicosapentaenoic acid*** or EPA (lipid number – 20:5(5,8,11,14,17)). LA is one of the major ***essential FAs*** (EFAs) for humans, i.e., these must be ingested through diet as the human body is unable to synthesis them. EFAs serve as the precursors to several important anti-inflammatory agents such as eicosanoids, lipoxins (from ω-6 EFAs), resolvins (from ω-3 EFAs) etc. Other cellular functions include cell-signalling and interaction with DNA (Lawrence 2010). Both oils also contain smaller but significant quantities of other nutritionally important unsaturated FAs such as ***alpha-linolenic acid*** or ALA (another essential FA) in soybean oil and ***arachidonic acid*** (AA) in microalgal oil. ALA is an ω-3 PUFA (lipid number – 18:3(9,12,15)) while AA is an ω-6 PUFA (lipid number – 20:4(5,8,11,14)) (Hodgson, Henderson et al. 1991; Zhukova and Aizdaicher 1995; Gunstone, Harwood et al. 2007). The fatty acid composition of these two oils will be discussed more in-depth in the coming sections.

2.1.4. Fatty acid analysis by gas chromatography

Gas chromatography (GC) is the most well-established analytical method for FA analysis of biological oils, so much so that the method was itself developed (over six decades ago) to carry out the separation of FAs in oils (Gutnikov, Scott et al. 2000). However, except for FAs with less than 10 carbons (or oils/samples rich in these), the analysis of FAs in their *free form* by GC is rare. Further, as discussed earlier, in their natural form, FAs are mostly present as triglycerides. Hence, samples are subjected to a derivatization step prior to FA analysis using GC: the most widely used method is ***transesterification*** to produce **fatty acid methyl esters or FAMEs**. According to this process, the FA triglycerides react with methanol to produce a mixture of FAMEs and glycerol. The reaction is catalyzed by either a strong acid (usually hydrochloric or sulphuric acid) or base (such as sodium methoxide or potassium hydroxide) (Casal and Oliveira 2007). The current study utilizes a an acid-catalyzed transesterification protocol with ***methanolic HCl*** as described in earlier reports (Palmquist and Jenkins 2003).

GC is a versatile technique that could be used to separate and analyze any mixture of volatalizable compounds. The separation is accomplished by differences in interaction between the *gaseous mobile phase* (a *carrier gas*) containing the analytes (i.e. the FAMEs to be separated) and the *stationary phase* i.e. the GC *column*. FAME analysis is usually accomplished using ***capillary GC*** where the column is a capillary tube with a fine coating of the stationary phase on its inner surface. Hydrogen and helium are the preferred carrier gases (mobile phases) while the stationary phase is usually a polar polyester compound like a siloxane derivative (as is used in the current study). The most common injector for FA samples is the *split-splitless* type. The different FAMEs (present in the injected sample) are carried through the column in varying velocities and eventually elute out of the column at different times (*retention times*), usually by order of alkyl chain length and then by number of double bonds. At the column exit, these FAMEs are detected according to their non-oxidized carbons by a *flame ionization detector* (FID). As with most modern GCs, the elution peaks are monitored in real-time via an appropriate computer console. FAME peaks are resolved further by employing a optimized and computer controlled *temperature program,* where the column temperature is raised to higher values using isothermal periods and linear (or non-linear) programs with multiple *ramps*. From the resulting chromatograph, the area of each FAME peaks could be found and used to calculate the corresponding FAME's weight percentage of the total FAME content – this generates a ***fatty acid or FAME profile*** for the particular sample (Sewell and Ian 2000; Casal and Oliveira 2007). The GC system used in the current study utilized helium as carrier gas. Hydrogen (different from carrier gas) and air were the FID's detector gases.

2.2. SOYBEAN OIL

2.2.1. Soybean production

Soybeans are one of the world's most important agricultural *commodities*, contributing almost $50 billion to the global economy (Wilson 2008). They are also the world's top oil crops; accounting for more than 57% of all oil crops (including oilseeds) produced worldwide (Fig. 3). In contrast, the next major competitor – palm kernel comes in at second place with a distant 13% (Foreign Agricultural Service 2011).

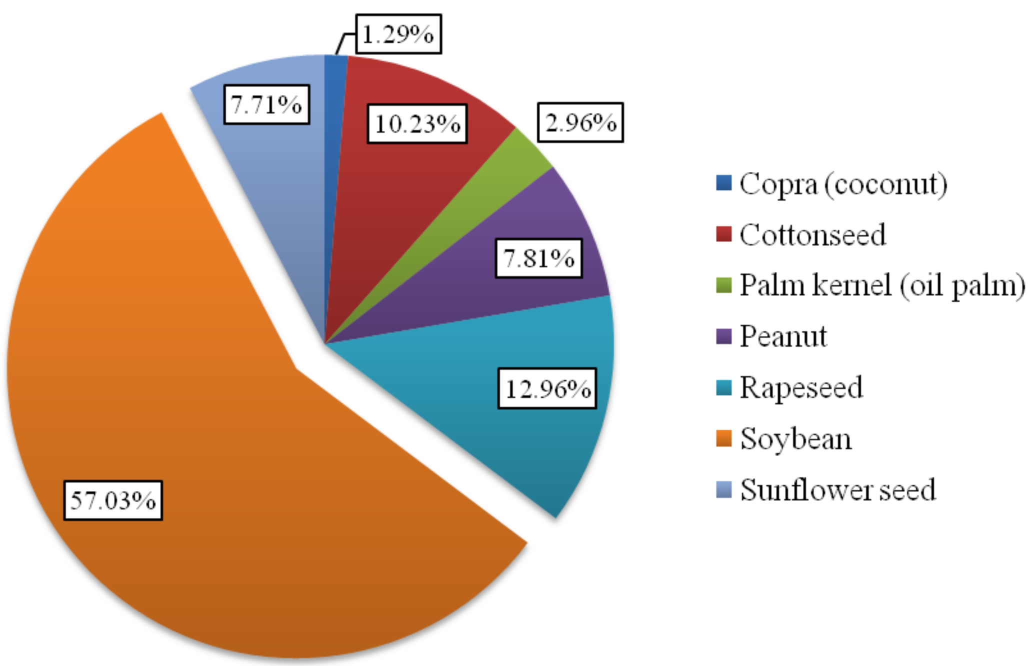

Figure 3: Major oil crops – world supply (August 2011).

It is perhaps surprising to note that much of the value of soybeans *does not* actually come from its oil, but rather from its highly-valued ***protein meal*** (commonly called soy protein) which accounts to more than 69% (quantity) of all soybean products (Fig. 3). Soybeans have the highest protein yield (per area of cultivated land) for any food crop cultivated worldwide. In second place, soybean oil constitutes about 16% of all soybean products. Despite this, soybeans are still classified as oil crops. Due to the presence of a well-balanced mixture of several important amino acids and fatty acids, soybeans serve a diverse array of functions as food/feed for human and animal nutrition. Soybean meal (or soy protein) is the most widely-used ingredient in livestock feeds and is a very important constituent of several different kinds of foods for human consumption (Erickson 1995).

The global demand and production of soybeans and its products have been increasing steadily over the past half century, driven mostly by its protein meal (Fig. 4). Production rates are expected to increase as global population and food demand continue to skyrocket. Further, a recent interest in the use of vegetable oils as feedstock for next generation biofuels is likely to add momentum to this trend. Currently, the United States of America leads the world in soybean production with more than one-third of the global market share, followed by Brazil, Argentina, China and India (Wang 2004; Foreign Agricultural Service 2011).

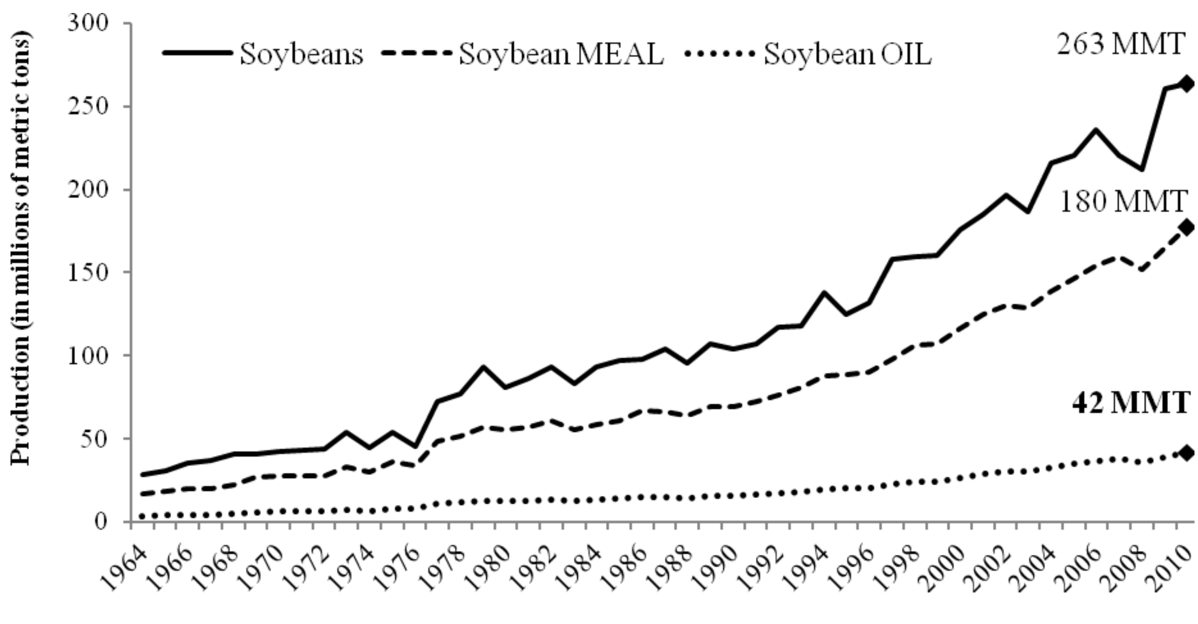

Figure 4: World soybean production (1964 - 2010).

2.2.2. Soybean oil

Apart their high protein content (40.69% w/w dry basis), soybeans contain a substantial amount of carbohydrates (29.4%). Only about 21% of whole dry soybeans is made up of oils (and fats) (Erickson 1995; Hammond, Johnson et al. 2005; Singh 2010). Yet, soybean oil is one of the most important vegetable oils produced worldwide, accounting for almost 30% of the world's supply (Fig. 5). It also ranks a close second among sources of vegetable oil, behind palm oil (Foreign Agricultural Service 2011).

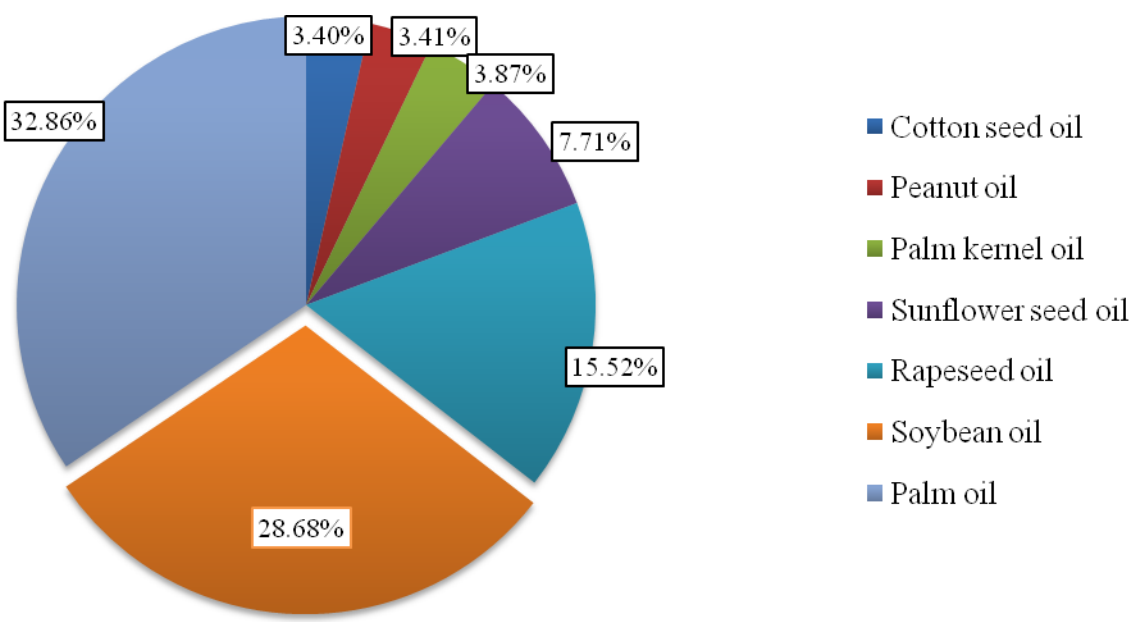

Figure 5: Vegetable oils - world production supply (2010 - 2011).

More than 97% of commercially produced soybean oil is used in food-based applications encompassing a wide range of products such as shortening, cooking and salad oils, salad dressings and margarine. Soybean oil also has several non-food industry applications. Fig. 6 shows the distribution of these applications (Hammond, Johnson et al. 2005).

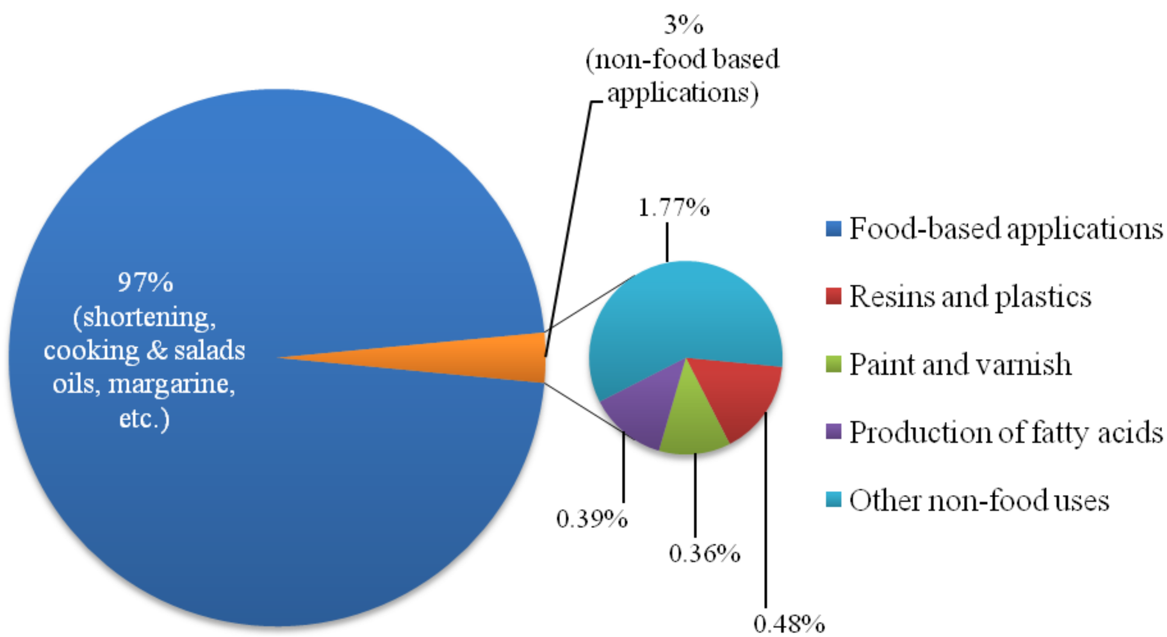

Figure 6: Industrial applications of soybean oil.

Industrially, both mechanical and chemical (solvent) methods are used for extracting (or recovering) oil from soybeans. Among mechanical methods, electrically operated **continuous screw-pressing** is the much more common than other processes such as batch pressing and extrusion-expelling. However, considering the low oil content of soybeans, **solvent extraction** is the most widely used industrial method for the extraction of soybean oil with hexane as the preferred solvent (Hammond, Johnson et al. 2005; Williams 2005). The process is outlined in more detail in section 2.4.3.

2.2.3. Fatty acids in soybean oil

More than 95% of crude soybean oil is composed fatty acid triglycerides. Other components include phospholipids (1.5 to 2.5%), unsaponifiable matter (including sterols, tocopherols and some hydrocarbons – all making up to approximately 1.6%), free fatty acids (0.3 to 0.7%) and trace metals like iron (1 to 3%) and copper (less than 0.05%) (Erickson 1980; Erickson 1995; Hammond, Johnson et al. 2005). *Refining* the crude oil increases the triglyceride content to 99%. Among these, there are five prominent (or major) fatty acids (FAs) – they are listed in Table 1. There is a slight variation in these values as dependent on varietal, geographical and environmental conditions.

Table 1: Fatty acids in refined soybean oil from 27 samples (Hammond, Johnson et al. 2005)

IUPAC name (*trivial name*)	**Lipid number**	**Content* (%)**
<u>Major fatty acids</u>		
cis, cis-9,12-Octadecadienoic acid *(Linoleic acid or LA)*	18:2(9,12)	54.51%
cis-9-Octadecenoic acid *(Oleic acid)*	18:1(9)	22.98%
Hexadecanoic acid *(Palmitic acid)*	16:0	10.57%
9,12,15-Octadecatrienoic acid *(α-Linolenic acid or ALA)*	18:3(9,12,15)	7.23%
Octadecanoic acid *(Stearic acid)*	18:0	4.09%
<u>Minor fatty acids</u>		
Tetradecanoic acid *(Myristic acid)*	14:0	0.04%
Eicosanoic or Icosanoic acid *(Arachidic acid)*	20:0	0.33%
Dodecanoinc acid *(Lauric acid)*	12:0	0.10%

*as % w/w of total FAME content

The fatty acid composition of soybean oil varies with seed maturity, variety and geography among other factors. Of these, seed maturity considerably affects palmitic acid and ALA negatively (i.e., they decrease in content as the seed matures), while it has a positive effect on LA (Hammond, Johnson et al. 2005). As can be seen from table 1, more than 80% of crude soybean oil is made up of unsaturated fatty acids including two PUFAs: linoleic acid (LA) and α-linolenic acid (ALA). Together, LA & ALA make up the PUFA level of soybean oil to over 61% - one of the highest values among vegetable oils. The former is an ω-6 PUFA while the latter is an ω-3 PUFA. In addition, both PUFAs are the two most vital *essential FAs (EFA)* for humans; soybean oil is thus one of the best dietary sources for EFAs (Meydani, Lichtenstein et al. 1991).

Besides contributing to the liquid nature (and hence 'oil') of soybean oil, this high level of unsaturation (especially the high levels of PUFAs) is considered undesirable for certain food applications due to the high susceptibility to oxidation. Additional processing methods like neutralization, bleaching and deodorization are used to improve the purity of soybean oil, as per the requirements of specific applications. Further, hydrogenation is also commonly used to improve the oxidative stability of processed soybean oil (Asbridge 1995).

2.3. MICROALGAL OIL

2.3.1. Microalgae

Microalgae (or microphytes) are a class of algal organisms comprising of microscopic, mostly photosynthetic organisms. Found in both marine (salt water) and fresh water habitats, they measure no more than a few hundred micrometers (µm) in size (Harun, Singh et al. 2010; Wageningen UR 2010). In spite of their miniscule size, microalgae play a pivotal role in the maintenance of life on earth – they alone produce half of all the atmospheric oxygen on the planet. It is also not surprising to note that microalgae possess an incredible biodiversity; estimates vary between 200,000 and a few million different species, and only a tenth of this variety has been scientifically identified to-date. In contrast, the total number of plant species identified number just over 315,000 (Wageningen UR 2010).

Research on microalgae is not new. Much of it had been focused primarily on species known to be of nutritional value such as *Spirulina* and *Nostoc*; the former is a very popular food supplement. Other strains that have also been well studied include *Chlorella* and *Dunaliella* (Wageningen UR 2010). According to Cardozo et al. (2007), more than 15,000 novel compounds have been identified from microalgae; these include lipids, carotenoids, phycocolloids (such as agar and alginate), lectins, amino acids and several other compounds of economic importance. Ofcourse, the most notable amongst these are microalgal lipids which include several of the health-promoting PUFAs mentioned earlier (Cardozo, Guaratini et al. 2007; Chisti 2007). Until recently, fish and fish oil were considered the top sources for PUFAs; however, it is now well-known that, rather than synthesising these on their own (*de novo*), fish accumulate them through the consumption of microalgal plankton. Today, microalgae are the top source for PUFAs in fish and animal feed: the consumption of microalgae-enriched feed leads to the accumulation of these PUFAs in food products derived from animals, such as seafood, meat, milk and eggs (Fredriksson, Elwinger et al. 2006). Fish and animal feed accounts to more than 30% of the world algal production (Spolaore, Joannis-Cassan et al. 2006; Teale 2006).. Further, the recent explosion of biodiesel research has led to the discovery and study of numerous species of microalgae that have the ability to accumulate large quantities of lipids, with some species being composed of upto 80% of their dry biomass weight as lipids (Borowitzka and Borowitzka 1988; Chisti 2007).

2.3.2. *Nannochloropsis oculata*

The microalgal species used in the present study is *Nannochloropsis oculata* (Fig. 7), a unicellular, marine eustigmatophyte measuring between 1 and 2 µm (The Stramenopile Chloroplast Genomics Project 2011). Studies with various species of *Nannochloropsis* have shown than this genus has some of the highest lipid productivities among microalgae. Yet, the fatty acid profile is very much species-specific and varies significantly (Roncarati, Meluzzi et al. 2004).

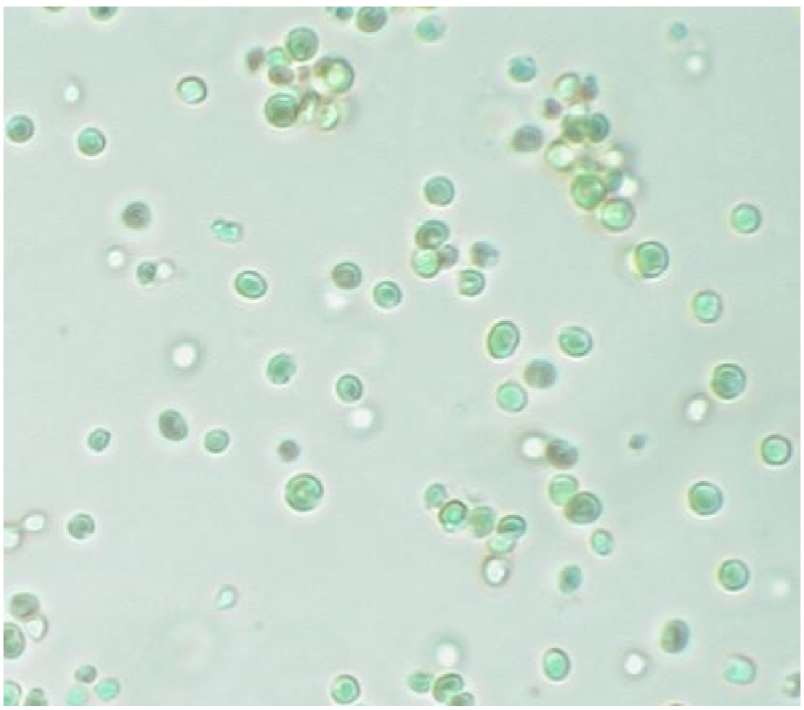

Figure 7: Nannochloropsis sp. (Wageningen UR 2009)

Lipid productivity of microalgal species is affected by several factors such as cultivation stages, light intensity, temperature, dissolved inorganic carbon, nitrogen and phosophorus among others; the latter two seem to be very well-studied (Emdadi and Berland 1989; Sukenik and Carmeli 1990; Hodgson, Henderson et al. 1991; Renaud, Parry et al. 1991; Sukenik 1991; Seto, Kumasaka et al. 1992; Sukenik, Zmora et al. 1993; Chini Zittelli, Lavista et al. 1999; Lourenço, Barbarino et al. 2002; Converti, Casazza et al. 2009; Su, Chien et al. 2010). As a result of this, and due to the lack of an optimized combination of cultivation-harvesting-extraction methods (that has been proven to produce the highest lipid yields), there is a great level of inconsistency and variation in the maximum values of lipid content for *Nannochloropsis*, as reported in currently available literature (Suen, Hubbard et al. 1987; Emdadi and Berland 1989; Sukenik and Carmeli 1990; Hodgson, Henderson et al. 1991; Renaud, Parry et al. 1991; Sukenik 1991; Chini Zittelli, Lavista et al. 1999; Krienitz and Wirth 2006; Chiu, Kao et al. 2009; Converti, Casazza et al. 2009; Mata, Martins et al. 2010; Su, Chien et al. 2010; Doan, Sivaloganathan et al. 2011; Simionato, Sforza et al. 2011). Table 2 lists some of the available data on the lipid profile of *Nannochloropsis.* As can be seen, the two most abundant PUFAs in *N. oculata* are ***arachidonic acid*** or AA (lipid number - 20:4(5,8,11,14)) and ***eicosapentaenoic acid*** or EPA (lipid number – 20:5(5,8,11,14,17)) (Hodgson, Henderson et al. 1991; Zhukova and Aizdaicher 1995; Roncarati, Meluzzi et al. 2004). The maximum value for ***total lipid content*** noted in the literature currently is **78%** for *Nannochloropsis* sp. (Emdadi and Berland 1989) and **60%** for *N. oculata* (Hodgson, Henderson et al. 1991).

Table 2: Various PUFA profiles of Nannochloropsis from literature

PUFA – lipid number designation and *trivial name*	**Omega**	**Individual composition (as % of total lipids or fatty acids)**					
Reference		(Zhukova and Aizdaicher 1995)	(Hodgson, Henderson et al. 1991)	(Renaud, Parry et al. 1991)	(Suen, Hubbard et al. 1987)	(Roncarati, Meluzzi et al. 2004)	(Gunstone and Harwood 2007)
18:2(9,12) *Linoleic acid (LA)*	ω-6	2.2	1.9	5.2	0.1	3.61	1
18:3(9,12,15) *Alpha-linolenic acid (ALA)*	ω-3	0.2	n/a	n/a	0.6	0.65	1
18:3(6,9,12) *Gamma-linolenic acid (GLA)*	ω-6	0.7	n/a	0.2	n/a	1.87	n/a
20:2(8,11) *Eicosadienoic acid (EDA)*	ω-6	0.1	n/a	n/a	n/a	1.61	n/a
20:3(8,11,14) *Dihomo-gamma-linolenic acid (DGLA)*	ω-6	0.3	1.2	n/a	n/a	0.53	n/a
20:4(5,8,11,14) *Arachidonic acid (AA)*	ω-6	5.3	3.8	n/a	26	5.98	n/a

20:5(5,8,11,14,17) *Eicosapentaenoic acid (EPA)*	ω-3	29.7	27.8	13	n/a	21.48	27
22:6(4,7,10,13,16,19) *Docosahexaenoic acid (DHA)*	ω-3	n/a	n/a	n/a	n/a	3.23	n/a

2.4. CONVENTIONAL OIL EXTRACTION

Due to their nutritional and industrial value, the separation and analysis of oils and fats from a variety of biological materials (or *biomass*) is an important step in several industrial processes. Like most other biological molecules, oils and fats are found associated with different kinds of other bio-molecules through a variety of molecular interactions and bonding mechanisms (Shahidi and Wanasundara 2008).

While the primary property used in oil extraction is *water insolubility,* several factors affect the choice of a suitable extraction method, most significantly; the properties of the biomass (physical state, chemical composition, cost of raw material etc.) and the kind of application for the extracted oil (Williams 2005).

2.4.1. Uses of soybean and microalgal oils

Both oils are rich sources of **nutritionally important fatty acids**. As discussed earlier, industrially extracted soybean oil has been primarily used for edible/food purposes ever since the oil was first extracted. The discovery that it contains the two most important EFAs elevated this oil to nutritional significance, along with other vegetable oils rich in PUFAs (Erickson 1995). As for microalgae, the use of both whole microalgae (such as Spirulina) as well as refined microalgal products (notably different kinds of PUFAs as explained earlier) as neutraceuticals, food supplements, feed additives etc., is well-known (David, Valerie et al. 1992; Ulber, Le Gal et al. 2005; Christaki, Karatzia et al. 2010; Lordan, Ross et al. 2011). However whole (crude) microalgal oil on the other hand has little or no use as an edible oil due to its high level of pigmentation, viscosity and sometimes unpleasant ('fishy') smell. Further, it contains several other non-fatty acid components that need to be refined out properly before it could be used as an edible oil (Abuzaytoun and Shahidi 2006). Other than a recently filed Chinese patent for a natural microalgal oil capsule (Wang 2010), there is currently no information in scientific literature on the use of *whole* (crude) microalgal oil as edible oil.

The second most significant potential with these oils is in the production of **biodiesel**. Since the past few decades, there has been a tremendous interest in the use of biological oils as feedstock for the production of biodiesel. Edible (including soybean, rapeseed and corn oil among others) and non-edible (such as Jatropha, neem and castor oil) vegetable oils have already been used to produce biodiesels of varying quality, with the former garnering much of the attention until recently (Patil and Deng 2009). Some of these vegetable oil-based biodiesels are already commercially available in several parts of the globe, particularly in Europe, as a result of the attractive tax policies on biofuels in place there. Of these, soybean-based biodiesel is one of the most popular (Lang, Dalai et al. 2001; Demirbas and Demirbas 2010).

Although research on the use of algae for producing several forms of bioenergy had been progressing for more than two decades, it was only recently that work on *microalgae-based biodiesel* took on momentum. Microalgae are the fastest growing photosynthetic organisms on the planet and the lipid-rich species among them can generate oil yields that are 200 times more than that of conventional oil crops. Further, their prowess in CO_2 fixation has prompted the use of microalgae as effective *carbon traps.* In any case, the greatest strength of microalgae lies in their rapid growth rates in comparison with land-based crops (Demirbas and Demirbas 2010; Wageningen UR 2010). However, inspite of all the promise, algae-based biodiesel is yet to find mainstream commercial adoption. There are two primary roadblocks. Firstly, cultivation of microalgae is an expensive process and is not economically feasible industrially at the moment (Cheng and Timilsina 2011). Secondly, lipid-rich strains that could sustain commercial oil production are only beginning to be identified; cost-effective harvesting and extraction methods (to recover maximum quantity of oil) for these microalgal strains are still very much in the laboratory phase (Lewis and Eric 1992). Finally, microalgal lipid productivity is greatly affected by a myriad of variables, as explained in section 2.3.2. Yet, the general consensus is quite clear that microalgal oil is indeed a competitive feedstock for biodiesel production (Chisti 2007; Li, Horsman et al. 2008; Waltz 2009; Cooney, Young et al. 2011; Halim, Gladman et al. 2011; Maceiras, RodrI´guez et al. 2011).

2.4.2. Solvent extraction of oil

Both the above-mentioned applications require oils of distinctly different quality, two essential requirements common to both are high purity and high fatty acid content (Demirbas

2008; Maceiras, RodrI´guez et al. 2011). This level of quality is assured by extraction with organic solvents only; and this is often the industry standard for biological oils (Birch and Ian 2000; Wang 2004). Chemically, solvent extraction involves the selective transfer (or dissolution) of the target compound from the solid phase (within the dried biomass sample) or liquid phase (in case of liquid biomass) to the solvent, which is immiscible in the former phase(s) (Li, Pordesimo et al. 2004; Cooney, Young et al. 2009).

The choice of solvent depends on several important properties such as the good solubilisation efficiency as low or ambient temperatures, low flammability, boiling point and toxicity. In addition, the solvent must be easily recoverable and its environmental damage must be as minimal as possible. It is obviously impossible to come up with a solvent that fulfills these requirements fully. Yet, one solvent that has come very close is **hexane**. The acceptance for this solvent has been such that it somewhat enjoys a monopoly in the solvent extraction of vegetable oils. In recent times, supercritical fluids such as supercritical toluene and carbon dioxide have generated considerable interest as alternatives to hexane; especially the latter due to its non-flammable, non-toxic nature and the higher quality of extracted oil. However, they are not economically feasible at the moment and are a long way from attaining the level of commercial success that hexane enjoys (Birch 2000; Wang 2004; Hammond, Johnson et al. 2005).

2.4.3. Unit operations in industrial solvent extraction of soybean oil

Industrial extraction of soybean oil is a complex, multistep process (Fig. 8) that starts with seed preparation (or pre-treatment) followed by the actual solvent extraction and finally de-solventization (solvent removal and/or recovery) (Wang 2004; Hammond, Johnson et al. 2005; Williams 2005). Starting with dried soybeans, seed preparation is in itself a highly complicated process involving the following steps; *cleaning* (to remove all non-seed material), *cracking* (a process where the beans are broken into 6-8 pieces each), *dehulling* (removal of the carbohydrate-rich outer hulls or seed coats - carried out along with the cracking process), *moisture-conditioning* (heat-drying at 74^0C and equilibration for upto 7 days, so as to obtain a final moisture content of not more than 9.5%) and finally, *flaking* (the cracked, conditioned beans are *flaked* into particles of about 0.25 mm size – this step ensures the maximum cell disruption necessary for efficient oil extraction) (Wang 2004; Hammond, Johnson et al. 2005).

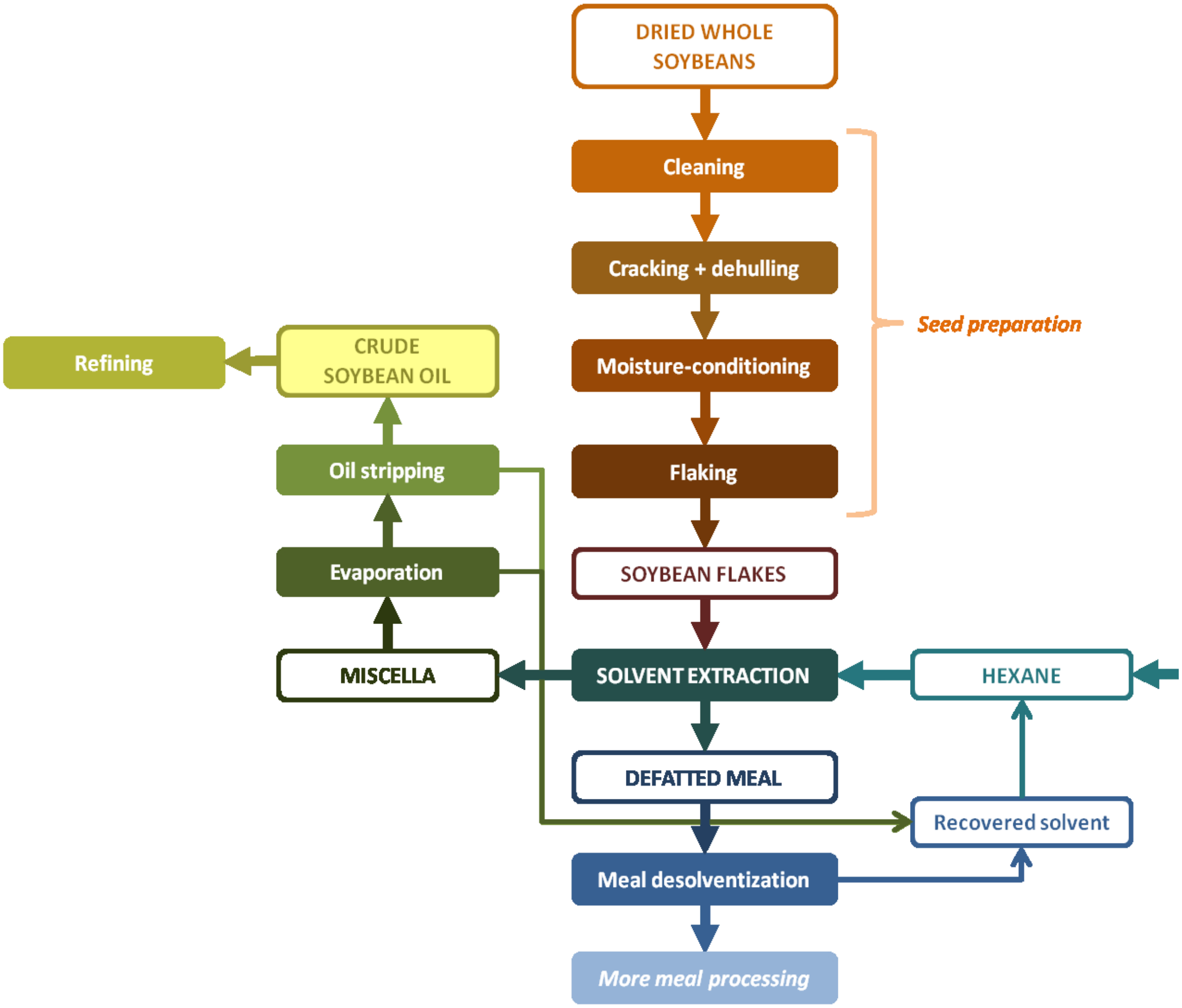

Figure 8: Industrial solvent extraction of soybean oil

Solvent extraction is mostly carried in *percolation* mode, where the solvent (n-hexane) flows through the bed (in counter-current direction to the biomass) influenced simply by gravity. Residual oil (left behind in the remaining 'meal') target is between 0.5% and 1.25%. The *miscella* (i.e., solvent + oil mixture) is then subjected to *desolventization* (solvent recovery) though two-stage (or 'double effect') evaporators followed by steam-stripping to remove the extracted oil, which is maintained at a temperature below 115^{0}C. Due to the presence of natural antioxidants like tocopherols, crude soybean oil oxidizes much slower than other vegetable oils. In the absence of air, this oil can be stored for long periods of time at room temperature before being refined (Wang 2004; Hammond, Johnson et al. 2005).

2.4.4. Microalgal oil extraction

Although hexane is still the most widely used solvent for the extraction of vegetable oils, it is surprisingly not the solvent of choice for the extraction of oil from microalgae (or most microorganisms). This is probably because, using a single non-polar solvent as hexane may not effectively extract *polar lipids* like ***phospholipids*** (PLs or *phosphoglycerides*) (Christie 1982). PLs are lipids derived from fatty acids and contain a phosphoric acid residue; the latter giving them their polar nature. Besides forming *lipid bilayers* (which are in turn, responsible for the structure and properties of all biological membranes), microalgal PLs also contain several valuable FAs within them; especially PUFAs that would normally be missed by a single non-polar solvent like hexane (Christie 1982; Fredriksson, Elwinger et al. 2006; Erickson 2008).

The most preferred way to ensure that all of the cellular FAs (or **total lipids**) are extracted is to use a **solvent mixture** comprising of a polar as well as non-polar solvent. Here, the usual extraction process is then followed by an additional partitioning step where the extraction mixture is separated into two phases – an organic phase containing the extracted lipids and an aqueous phase containing non-lipid compounds. The most popular of these solvent mixtures is chloroform-methanol (2:1, v/v), first described more than half a century ago (Folch, Lees et al. 1957; Bligh and Dyer 1959). This binary solvent system interacts with water in biological material to form a ternary system which is the real key to the extraction process. The solvent has the ability to extract both neutral lipids (like FAs) as well as most polar lipids (such as PLs). However, it may not efficiently transfer all of these lipids into the organic phase; this has prompted the modification of the original method to suit specific biological material and lipids of interest (Radin 1989; Shahidi and Wanasundara 2008; Cooney, Young et al. 2009).

This widely used solvent mixture has two severe drawbacks. The first is quite obvious; chloroform is a highly toxic chemical and poses a serious health hazard to researchers working with it. Safe disposal of the chemical into the environment is also a contentious issue (Hara and Radin 1978). Secondly, following phase separation, the organic phase (containing the lipids) is below the aqueous phase (with all other cellular material and debris) (Woertz 2007; Shahidi and Wanasundara 2008). This makes retrieval of the bottom organic phase particularly difficult and prone to error, especially when the extractions are carried out using test tubes. During preliminary extraction studies with *N. oculata* using chloroform-methanol (CM), the heavily pigmented nature of the extraction mixture often made it tiresome to identify the phase separation in the first place, let alone aspirate the bottom phase.

Several different solvent mixtures have been suggested as alternatives to CM. Some of these include hexane-isopropanol (3:2, v/v) (Hara and Radin 1978; Radin 1981), methylene chloride-methanol (2:1, v/v) (Soares, Dasilva et al. 1992) and hexane-acetone (1:1, v/v) (Johnson and Lusas 1983). Depending on the biological material and application, some of these solvents have a higher efficiency than that of CM. As for microalgae, CM continues to be the most widely used solvent mixture despite the toxicity and environmental concerns (Ibáñez González, Robles Medina et al. 1998; Lewis, Nichols et al. 2000; Fajardo, Cerdán et al. 2007; Chiu, Kao et al. 2009; Tran, Hong et al. 2009; Lee, Yoo et al. 2010; Halim, Gladman et al. 2011).

2.5. ULTRASOUND-ASSISTED SOLVENT EXTRACTION (UASE)

Whether it is soybeans or microalgae, one important task that must be undertaken before oil extraction is *cell disruption*. The presence of a rigid cell wall makes this task a difficult one for both biomaterials. For soybeans, cracking/dehulling and flaking are the standard disruption methods employed industrially. Microalgae on the other hand have so far found no single method worthy enough of being an industry standard. Numerous methods have been suggested (for oil extraction from microalgae) with varying degrees of success (Grima, Medina et al. 1994; Ibáñez González, Robles Medina et al. 1998; Lee, Yoon et al. 1998; Lewis, Nichols et al. 2000; Fajardo, Cerdán et al. 2007; Li, Horsman et al. 2008; Lavoie, Bernier et al. 2009; Tran, Hong et al. 2009; Harun, Singh et al. 2010; Lee, Yoo et al. 2010; Mata, Martins et al. 2010; Koberg, Cohen et al. 2011).

One such versatile method is *sonication-assisted* or ***ultrasound-assisted solvent extraction***. Based on the level of intensity, ultrasonic treatment could of two types; ***high intensity*** (also called *power ultrasound*) or ***low intensity***. The former makes use of low frequencies (20-100 kHz) and the latter employs much higher frequencies (2-10 MHz). Both techniques have been utilized for a variety of applications in the food processing industry and *high intensity* ultrasound has been proven to be the preferred method for cell disruption and extraction (Piyasena, Mohareb et al. 2003; Zenker, Heinz et al. 2003; Knorr, Zenker et al. 2004; Condón, Raso et al. 2005; Feng, Yang et al. 2008; Demirdöven and Baysal 2009).

The basic principle behind the creation of ultrasonic waves is *elastic deformation* in *piezoelectric* materials as a result of the application of a high frequency electric field. A power supply converts the 50/60 Hz line voltage to the electric field mentioned earlier. The deformation in the piezoelectric *transducer* is then converted to mechanical vibrations and amplified before being transmitted to a resonating *probe* or *sonotrode* which is in contact with the processing medium (Raichel 2006).

Sound waves propagate through a medium by alternating *compression* and *expansion* (or *rarefaction*) cycles longitudinally and/or transversely; hence they are in effect mechanical waves of alternating high and low pressure (from an equilibrium pressure) respectively. These vibrations (or oscillations) have a mechanical effect on the molecules or particles in the medium. When ultrasonic waves pass through a liquid medium, in the expansion cycle, the pressure becomes so low that the intermolecular forces (that keep the molecules of the medium together) are overcome and small gas-filled bubbles or *cavities* are created. During the consecutive compression cycle, these cavities contract, followed by expansion in the next cycle and so on. In course of time, the cavities expand to the limit of them imploding on themselves. This implosion is called ***cavitation*** and is the chief mechanism behind the mechanical effect of ultrasonic waves (Suslick 1990).

Low intensity ultrasound causes ***stable cavitation*** wherein the stable cavities produced oscillate and remain in the medium for extended cycles or time periods. High intensity ultrasound creates cavities that continue to increase in size over consecutive expansion cycles, eventually imploding after just a few cycles (typically over a few microseconds); this is called ***transient cavitation***. The implosion creates a *hot spot* in the immediate vicinity, where high temperature, high shear forces and free radicals work together to bring about the cell disruption of biological cells in the medium (Fig. 9). This kind of cavitation has a rapid mechanical or shear effect (as a result of temperature and pressure increase) on the molecules in its immediate vicinity. This exciting phenomenon has been very well-studied and has been manipulated to serve a plethora of purposes (Suslick 1988; Suslick 1990; Condón, Raso et al. 2005; Feng, Yang et al. 2008). For example, if the medium contains biological material, which are made up of cells, this mechanical effect is so efficient that the cell membranes are ruptured leading to cell disruption and the transfer of the intra-cellular material into the medium. When the medium is made up of solvents (i.e. liquids with specific affinities for dissolving specific compounds or molecules only), the resulting selective dissolution process becomes the principle for ***ultrasound-assisted solvent extraction*** (Wang and Weller 2006).

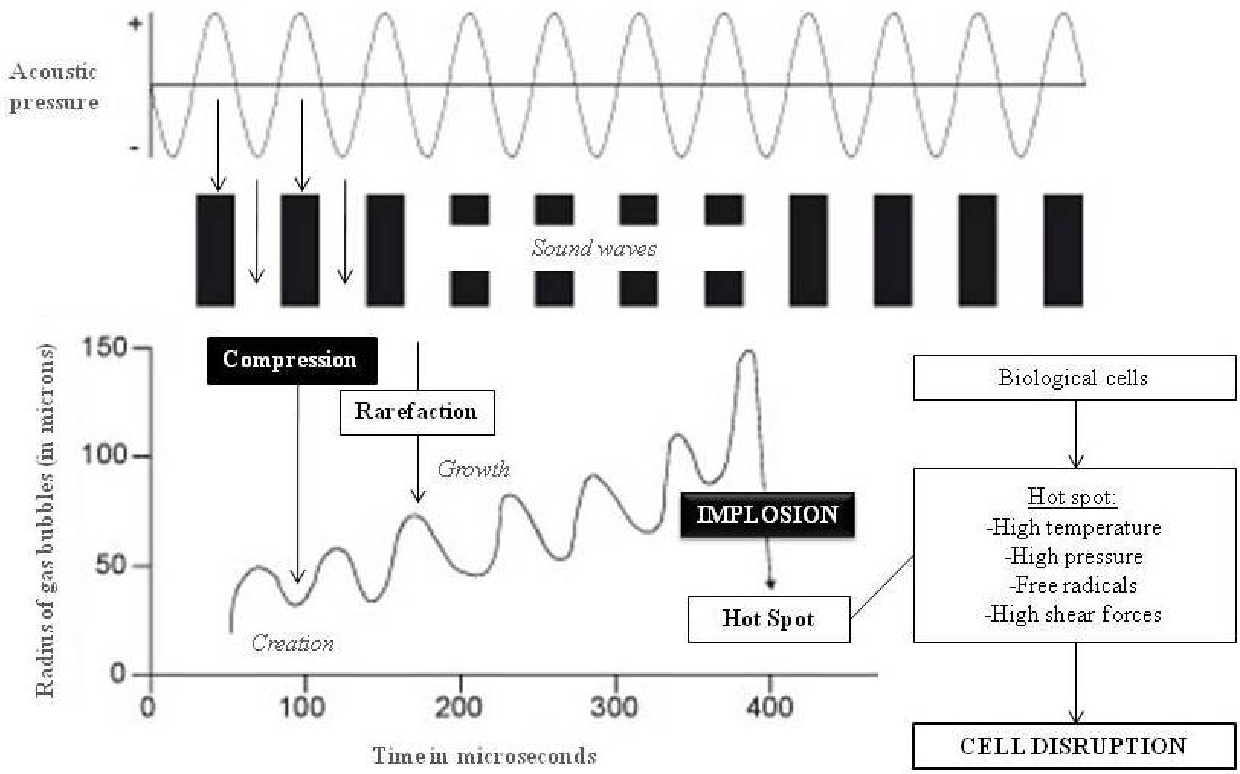

Figure 9: Principles of ultrasonic cavitation and cell disruption (Sonotronic Nagel GmbH 2011)

Of the many factors that determine the power output of an ultrasonic probe, resonance amplitude and ultrasonic wave intensity (UWI) are the most important. Resonance (or vibrational) amplitude is the vertical (up-and-down) distance (measured in microns) travelled by the probe when it vibrates. Ultrasonic wave intensity (UWI) or power intensity, on the other hand, is the amount of power transmitted to the medium (in watts), per unit cross-sectional area of the ultrasound probe. Although both variables have been used to characterize ultrasonic treatments in scientific literature, resonance amplitude has been found to be a more exact and stable parameter than power output for estimating cavitational cell disruption, especially in microbial cells. Factors that affect the ultrasonic power output include frequency, resonance amplitude, hydrostatic pressure and temperature of the processing medium (Tsukamoto, Yim et al. 2004; Bermúdez-Aguirre, Mobbs et al. 2011).

Ultrasound treatment has been utilized for the successful extraction of oils from a variety of biological materials including soybeans (Li, Pordesimo et al. 2004), sunflower and rapeseed (Luque-García and Luque de Castro 2004), rice bran (Cravotto, Binello et al. 2004), almond and apricot (Sharma and Gupta 2004) among others.

2.6. CONCLUDING REMARKS

It is clear from available literature that ultrasound-assisted processing has enormous potential as a novel processing method, especially for the extraction of biological oils, a process that has traditionally been based on the use of chemical solvents with the assistance of heat and/or agitation. The mechanical effects of high-intensity ultrasonic cavitation have been found to be ideal for the disruption of most biological cells with rigid cell walls, such as plant and microbial cells. Yet, despite the positive outlook from research on ultrasound-assisted extraction, this novel process is not an *off-the-shelf* technology, and scaled-up applications are still in their infancy. With regard to solvents used in oil extraction, scientific literature shows that n-hexane and CM continue to be the most widely used solvents for the extraction of soybean and microalgal oils, respectively. Despite their drawbacks, there are only a limited number of studies that have investigated other solvents and/or methods combining solvent extraction with other processes. HIP is one such solvent that shows promise as a safer and equivalent or superior alternative to either of these two solvents.

Clearly, the extraction of soybean and microalgal oils are two processes that share a unique level of economic importance due to their significant nutritional and biodiesel potential. The use of pulsed ultrasound-assisted solvent extraction (PUASE) using HIP in the production of these oils has the capability to offer both improved efficiency, and also yield oils of similar or superior quality, compared with conventional methods.

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CONNECTING STATEMENT

Based on the literature review (presented in chapter 2), it is clear that ultrasound-assisted solvent extraction (UASE) has significant potential in the extraction of soybean oil, and a study was carried out to test the efficiency of technique, using hexane-isopropanol (HIP) as solvent. The following chapter is a manuscript prepared from this study. A pulsed processing technique (pulsed UASE or PUASE) was used and two parameters were studied; resonance amplitude and effective treatment time. Five levels of amplitude (from 25 to 124 μm) and five levels of treatment time (from 45 to 225 min) were investigated. Extraction efficiency was measured in terms of gravimetric oil yield. The quality of the extracted soybean oils was also evaluated by FAME analysis using gas chromatography. This manuscript will be submitted for publication in the Journal of Food Research, published by the Canadian Center for Science and Education.

CHAPTER 3: **PULSED ULTRASOUND-ASSISTED SOLVENT EXTRACTION (PUASE) OF OIL FROM SOYBEANS**

ABSTRACT

Besides its dietary significance, soybean oil is a top feedstock for biodiesel production. The current study focused on pulsed ultrasound-assisted solvent extraction (PUASE) of oil from soybeans using the solvent mixture hexane-isopropanol (3:2 v/v) (HIP). A probe resonating at a maximum amplitude of 124 µm was used and two variables were studied; resonance amplitude and effective treatment time. The oil yield (assessed gravimetrically) and fatty acid profiles (from GC) of the extracted oils were analysed statistically.

The study showed that PUASE produced high oil yields (upto 19.92%), compared to regular solvent extraction by agitation (13.43%). The study also showed that high resonance amplitude and treatment time did not necessarily produce high yields. The oil extracted by PUASE was found to be comparable in fatty acid composition to that extracted by traditional methods such as Soxhlet and room temperature shaking.

Keywords: soybean, oil, pulsed ultrasound, extraction, hexane, isopropanol

3.1. INTRODUCTION

Soybean oil is one of the world's primary vegetable oils. Almost 21% (w/w dry basis) of whole dry soybeans is made up of oils and lipids (Erickson 1995; Hammond, Johnson et al. 2005; Singh 2010). It is also one of the most important dietary sources of two essential polyunsaturated fatty acids (PUFAs) namely: ***linoleic acid*** or LA (18:2(9,12)) and ***α-linolenic acid*** or ALA (18:3(9,12,15)) (Meydani, Lichtenstein et al. 1991). PUFAs have been implicated in numerous health-promoting roles such as anti-cardiovascular disease, anti-inflammation, anti-tumour and even anti-diabetes (Caygill, Charlett et al. 1996; Siguel and Lerman 1996; Zurier, Rossetti et al. 1996; Kris-Etherton, Harris et al. 2003; Abete, Testa et al. 2009).

Solvent percolation with hexane is the most widely used method for extracting soybean oil. Industrially, a series of several seed preparation steps (such as cleaning, cracking, *dehulling*, moisture conditioning and finally, *flaking*) are employed, before the actual extraction process. Besides adding to the complex nature of the process, these methods are energy-intensive and raise the costs for solvent extraction of oil significantly (Wang 2004; Hammond, Johnson et al. 2005). Further, hexane is highly volatile and easily flammable, calling for stringent safety measures (Russin, Boye et al. 2011). In addition, using a single organic solvent may not completely extract polar lipids such as phospholipids and the several fatty acids associated with them (Johnson and Lusas 1983; Erickson 2008).

Among various alternative solvents suggested, two have had significant success; supercritical carbon dioxide (SCO_2) and solvent mixtures (Johnson and Lusas 1983; Li, Pordesimo et al. 2004; Russin, Boye et al. 2011). SCO_2, although safe and environment-friendly, is currently not economically feasible due to the need for expensive and specialized equipment (Wang 2004; Hammond, Johnson et al. 2005; Wang and Weller 2006). Solvent mixtures (comprising one polar solvent and another non-polar solvent) have already proven to be much more efficient than conventional single solvent systems, due to their ability to completely extract most cellular lipids (Folch, Lees et al. 1957; Bligh and Dyer 1959; Hara and Radin 1978; Johnson and Lusas 1983; Soares, Dasilva et al. 1992). Also, solvent mixtures do not need any additional equipment than is already used for hexane extraction. Chloroform-methanol (CM) was one of the first and most efficient solvent mixtures used for oil extraction (Folch, Lees et al. 1957; Bligh and

Dyer 1959). However, the extreme health and environmental safety risks associated with chloroform have limited its applications in the extraction of edible oils (Khor and Chan 1985). Hexane-isopropanol (HIP) (3:2 v/v) has been suggested as a suitable alternative to CM and has had considerable success (Hara and Radin 1978; Radin 1981; Radin 1989).

While the solvent mixtures give improved oil yields, one significant problem with solvent extraction remains: long treatment times. This is especially relevant industrially, as most solvent extractors used for soybean oil extraction employ *percolation* (Wang 2004; Hammond, Johnson et al. 2005). Among the novel methods that have been used to assist the main solvent extraction process from oil-rich seeds, two that have had profound success are; microwaves and ultrasound waves (Cravotto, Binello et al. 2004; Li, Pordesimo et al. 2004; Li, Pordesimo et al. 2004; Luque-García and Luque de Castro 2004; Wang and Weller 2006). However, microwave-assisted extraction (MAE) has two significant drawbacks; the generation of heat (which may lead to oxidation of unsaturated fatty acids) and its low efficiency with volatile solvents (Wang and Weller 2006). Ultrasound waves on the other hand, are relatively gentler and have been found to be quite efficient for extracting bio-active compounds from a variety of plant material such as soybeans (Li, Pordesimo et al. 2004; Li, Pordesimo et al. 2004; Luque-García and Luque de Castro 2004; Cravotto, Boffa et al. 2008), sunflower and rapeseed (Luque-García and Luque de Castro 2004), rice bran (Cravotto, Binello et al. 2004), almond and apricot (Sharma and Gupta 2004) among others. When the processing medium is made up of the solvent itself, the resulting process becomes *ultrasound-assisted solvent extraction* or *UASE* (Wang and Weller 2006).

Ultrasound waves that are capable of causing cavitation are in the low frequency range (20 to 100 kHz) and are referred to as *high power* or *high intensity* ultrasound (Demirdöven and Baysal 2009). The most common parameters used to characterize high intensity ultrasonic treatment include vibrational amplitude, power intensity, frequency and temperature. Vibrational or resonance amplitude (measured in microns) is the up-and-down distance travelled by the probe during ultrasonic vibrations. Power intensity or ultrasonic wave intensity (UWI) is the amount of power transmitted to the medium (in watts), per unit cross-sectional area of the ultrasound probe. While both variables have been used to characterize ultrasonic treatments, studies on cell disruption of microorganisms had already shown that resonance amplitude is a more exact and stable parameter than power output for estimating cell disruption as a result of ultrasonic cavitation (Tsukamoto, Yim et al. 2004; Bermúdez-Aguirre, Mobbs et al. 2011).

Li et al. (2004) used continuous high-intensity UASE coupled with magnetic stirring to extract oil from soybeans. The authors observed that the oil yield increased with increasing ultrasonic power intensity and that the highest yields were obtained with the HIP solvent mixture than with hexane or isopropanol individually. In contrast to continuous treatment, the use of pulsed ultrasonic treatment has shown improved energy efficiency and a better control on processing temperature (Pan, Qu et al. 2011). Accordingly, the objectives of this study were: (1) to compare oil yields obtained from ground soybean seeds by room temperature (RT) shaking using three different solvents namely hexane, chloroform-methanol (CM) (1:2 v/v) and hexane-isopropanol (HIP) (3:2 v/v); (2) to investigate the effects of ultrasonic resonance amplitude and treatment time on oil yields during pulsed UASE (PUASE) of soybean oil with HIP; and (3) to evaluate the influence of extraction methods on fatty acid composition of extracted oils.

3.2. MATERIALS AND METHODS

3.2.1. Chemicals, sample preparation and storage

All solvents used in the study (n-hexane, isopropanol, chloroform and methanol) were of Fisher 'Optima' grade or higher (Fisher Scientific Co., ON, Canada). HIP and CM were prepared manually from the pure chemicals prior to the study. Chemicals used for FAME analysis using GC were of high resolution chromatography grade. Dried soybeans were obtained from GreenCentre Canada, Kingston, ON. The samples were ground into powder using a kitchen grinder and passed through a standard sieve to select particles smaller than 1.18 mm. The powder was stored in an airtight container under vacuum in a desiccator until used for experiments.

3.2.2. Extraction by room temperature (RT) shaking

Three solvents namely; n-hexane, CM (1:2 v/v) and HIP (3:2 v/v) were evaluated by RT shaking. CM extraction was carried out as per the method described by Bligh and Dyer (1959). Extraction with HIP and yield determination was carried out by a method modified from that of Radin (1981). An identical method was employed for n-hexane with the only difference being the solvent. Ground soybean powder was mixed with each of the solvents in capped plastic tubes and shaken at 400 rpm for 3 hours on a bench-top shaker (IKA, Schuttler MTS 4, Staufen, Germany), at room temperature (RT). A sample-solvent ratio of 1:10 w/v was maintained; each experiment was carried out in triplicates with 3 g of ground soybean powder and 30 mL of each solvent.

3.2.3. Pulsed ultrasound-assisted solvent extraction (PUASE)

A high intensity ultrasonic processor (Vibra-Cell^{TM} VCX-500, Sonics & Materials, CT, USA) equipped with an ultrasonic horn transducer and a tuned titanium alloy probe (or horn) resonating at a frequency of 20 kHz and maximum amplitude of 124 μm was employed. The processor was designed to maintain constant amplitude by continuously adjusting the power (or energy) output from the probe in response to the load (suspension). Two variables were studied; the resonance amplitude and the effective treatment time. The ultrasonic processor used in the current study was designed to automatically adjust the power output to the probe load (suspension) so as to deliver constant resonance amplitude (Sonics & Materials Inc. 2010). The effects of five levels of resonance amplitude (0, 25, 50, 87 and 124 μm which corresponded to 0, 20, 40, 70 and 100%, respectively) and five levels of effective treatment time (45 to 225 min in increments of 45 min) were investigated using a 5 X 5 two-way factorial experiment design. The 0 μm amplitude experiment was the control; basically, ultrasonics was not turned on and the sample-solvent mixture was undisturbed during the process.

Ultrasound treatment was applied to the sample by inserting the probe approximately 5 cm from the top into the sample-solvent suspension in a plastic tube. The ultrasonic treatment was applied in pulses of 10 seconds duration separated from each other by 5 seconds of resting time: so the total treatment time was 1.5 times the effective treatment time.

To avoid significant solvent evaporation that could potentially occur at long treatment periods and/or at higher amplitudes, a special spill- and evaporation-proof cap was designed and used during the ultrasonic extraction process (Fig. 10). An ice-water bath was used to maintain the temperature of the extraction medium at 25 ± 5^0C during the process. In addition, the temperature of the medium was monitored every 20 mins to ensure that there was no temperature over run.

Figure 10: The spill- and evaporation-proof cap used to prevent solvent evaporation from the extraction tube during the PUASE process

3.2.4. Determination of oil yield

After either the RT shaking or ultrasonic extraction, the sample-solvent suspension was allowed to sit at room temperature for 5-10 minutes; the supernatant was then decanted into a separate tube and the residue was washed twice with 5 mL of HIP. Next, 15 mL of 10% sodium sulphate solution was added to the decanted suspension, separating it into two phases: an upper hexane phase containing the extracted lipids and a lower isopropanol phase containing all the other cellular components. Phase separation was made more distinct by centrifugation at 4000 rpm for 10 min. The upper hexane phase (Fig. 11) was then aspirated into a pre-weighed stainless steel vessel and dried over a stream of warm air at 50 ± 5^0C in an oven (or, for GC samples, under a stream of nitrogen) for 2 to 3 hours until constant weight. The choice of drying temperature was based on preliminary studies that had shown that the drying temperature significantly affected both the yield as well as composition of extracted lipids; higher temperatures caused lower yields and lower triglyceride content. This may have been the result of the oxidation of unsaturated fatty acids due to exposure to high temperature (Widjaja, Chien et al. 2009).

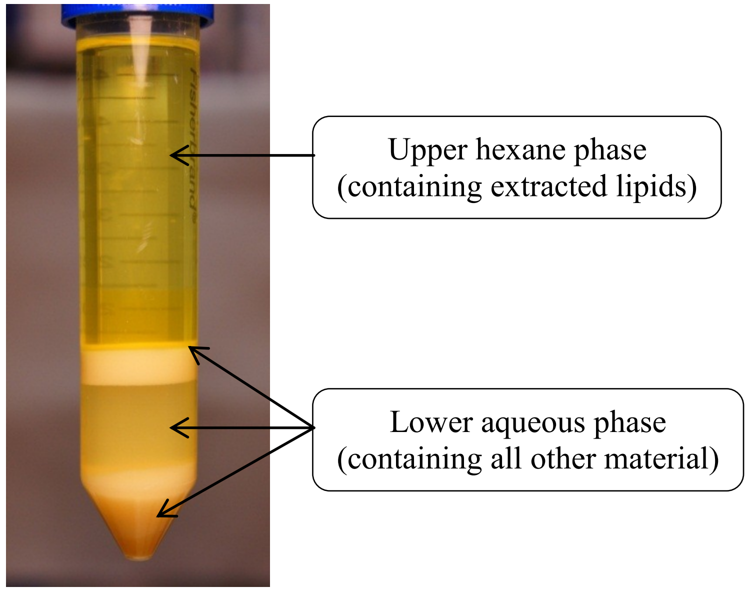

Figure 11: Soybean-solvent suspension after phase separation & centrifugation

The oil yield (Y) was determined gravimetrically:

$$Y(\%) = m_0/m_s \times 100,$$

where m_s is the mass of ground soybean (g) and m_0 the mass of extracted oil (g).

3.2.5. Fatty acid analysis using GC

Following extraction, the oil was transesterified to produce fatty acid methyl esters (FAMEs) according to a method reported earlier (Palmquist and Jenkins 2003). Samples of extracted oil (weighing 0.5 g each) were mixed with 2 mL of internal standard (nonadecanoic acid or 19:0 (Nu-Chek Prep, MN, USA), 2 mg/mL in n-hexane) and reacted with 3 mL of 10% methanolic HCl in glass test tubes with Teflon-lined screw caps, in a 90^{0}C water bath for 2 h. Following the addition of 1 mL n-hexane and 10 mL 6% K_2CO_3, the reaction mixture was vortexed and centrifuged at 4000 rpm. The upper organic phase was then transferred to a fresh tube, mixed with 10% Na_2SO_4 solution, and centrifuged again to separate the phases more distinctly. The upper phase (containing the FAMEs) was carefully aspirated into 2 mL GC auto sampler vials, capped and stored at -80^{0}C until GC analysis.

GC analysis was carried out using an HP 5890 Series II gas chromatograph equipped with a flame ionization detector (FID). Triplicate 1 μL injections of each FAME sample were injected into a ZB-WAX capillary column (Phenomenex, CA, USA). The stationary phase was polyethylene glycol and column dimensions were 30 m x 0.25 m x 0.25 μm. Helium gas flowing at a linear velocity of 45 cm/s was the carrier gas and a split ratio of 100:1 was applied. Flow rates for the FID gases were 30 mL/min and 430 mL/min for hydrogen and air, respectively. The temperatures of the injector and detector were both set to 250^0C. The temperature program employed was thus;- 60^0C (maintained for 2 min), then raised at 60^0C/min to 210^0C (kept constant for 2.5 min) and raised again at 1^0C/min to 220^0C (maintained for 3 min). Individual FAMEs in the extracted oils were identified (qualitatively) using an AOCS reference mix for vegetable oil (FAME Mix RM-1, Supelco Analytical, PA, USA) by comparing the retention times and relative peak areas. FAME profiles (showing relative composition of various FAMEs in the analysed samples) were also generated, with special attention to the PUFAs LA and ALA.

FAME profiles were obtained for oil samples extracted from three methods; (1) PUASE experiment (with HIP) that produced the highest oil yield, (2) RT shaking (with HIP at 400 rpm) and, (3) Soxhlet extraction with n-hexane (AOCS 2005; ISO 2009).

3.2.6. Statistical analysis

Each experiment was carried out in triplicates and all statistical analyses were carried out as ANOVAs using the *general linear models* procedure in SAS 9.2 (SAS Institute Inc., NC, USA). The significance level was set at 0.05.

3.3. RESULTS AND DISCUSSION

3.3.1. Evaluation of solvents by shaking at room temperature

Oil yields obtained from RT shaking of ground soybean powder with n-hexane, CM and HIP for 3 h (at room temperature and speed of 400 rpm) were 12.81, 13.08 and 13.43%, respectively. Statistical t-tests showed that while oil yield was significantly ($P \leq 0.05$) affected by the solvent used, there was no significant difference between CM and HIP. Yet, the solvent mixtures gave higher oil yields than n-hexane: this is perhaps explained by the better extraction of polar lipids (such as phospholipids) by them (Shahidi and Wanasundara 2008). Phospholipids make up 1.5-2.5% of crude soybean oil (Wang 2004).

Although not statistically significant, the oil yield obtained using HIP was 0.35% more than with CM. This small increase could be attributed to the slightly higher efficiency of HIP in extracting triglycerides (i.e., esters of three fatty acids with glycerol) which are the most abundant neutral lipids in soybeans, making up almost 95-97% of crude soybean oil (Wang 2004). This agrees with the findings of Khor and Chan (1985). They compared the oil extraction efficiencies of CM and HIP from soybeans and reported that, although CM showed a higher total lipid yield (17.8%) compared to HIP (13.9%), HIP gave a higher yield for triglycerides than CM. More than 86% of the neutral lipids extracted by HIP was found to be triglycerides, while CM extracted 81.7%. It must be noted that the authors used a chloroform-methanol (2:1 v/v) solvent; i.e., the mixture had a higher proportion of chloroform. This was in fact the original solvent mixture suggested by Folch et al. (1957). Their method was modified by Bligh and Dyer (1959) who suggested a mixture with a lesser proportion of chloroform i.e., chloroform-methanol (1:2 v/v) – their method became much more popular due to the lower volume of solvent needed, its relatively lower cost and the improved purity of the extracted lipids (Radin 1989). Other authors have reported that although CM was found to be a better solvent for the extraction of total lipids, HIP extracted slightly more triglycerides (Khor and Chan 1985; Gunnlaugsdottir and Ackman 1993; Aryee and Simpson 2009).

Li et al. (2004) used three solvents (for conventional solvent extraction of soybean oil) namely, hexane, isopropanol and HIP and obtained yields of 34.6, 20.4 and 39.8%, respectively. However, their method of yield calculation differed from this study since they reported the mass of extracted oil as a percentage of the maximum lipid mass extractable from soybeans. When the values reported by Li et al. (2004) are calculated according to the method used in this current study, the yields for hexane, isopropanol and HIP translate to 6.78, 5.57 and 7.81%, respectively. Thus, when compared on the same basis, the oil yields obtained in this study are much higher than those reported by Li et al. (2004). The higher yield obtained for HIP in this study (13.43%) could be attributed to differences between the two studies including; physical nature and particle sizes of the samples (flaked vs. ground soybeans), method of agitation (stirring vs. shaking) and, the modified extraction, washing and drying steps used in the current study.

3.3.2. PUASE with HIP – effects of amplitude and treatment time

Statistical analysis showed that ultrasonic resonance amplitude and treatment time had significant ($P \leq 0.05$) effects on oil yield. In addition, there was a statistically significant ($P \leq 0.05$) interaction between the variables. The highest oil yields at the four amplitude levels tested were 12, 19.01, 19.92 and 17.81% for 25, 50, 87 and 124 µm, respectively, as shown in Fig 11.

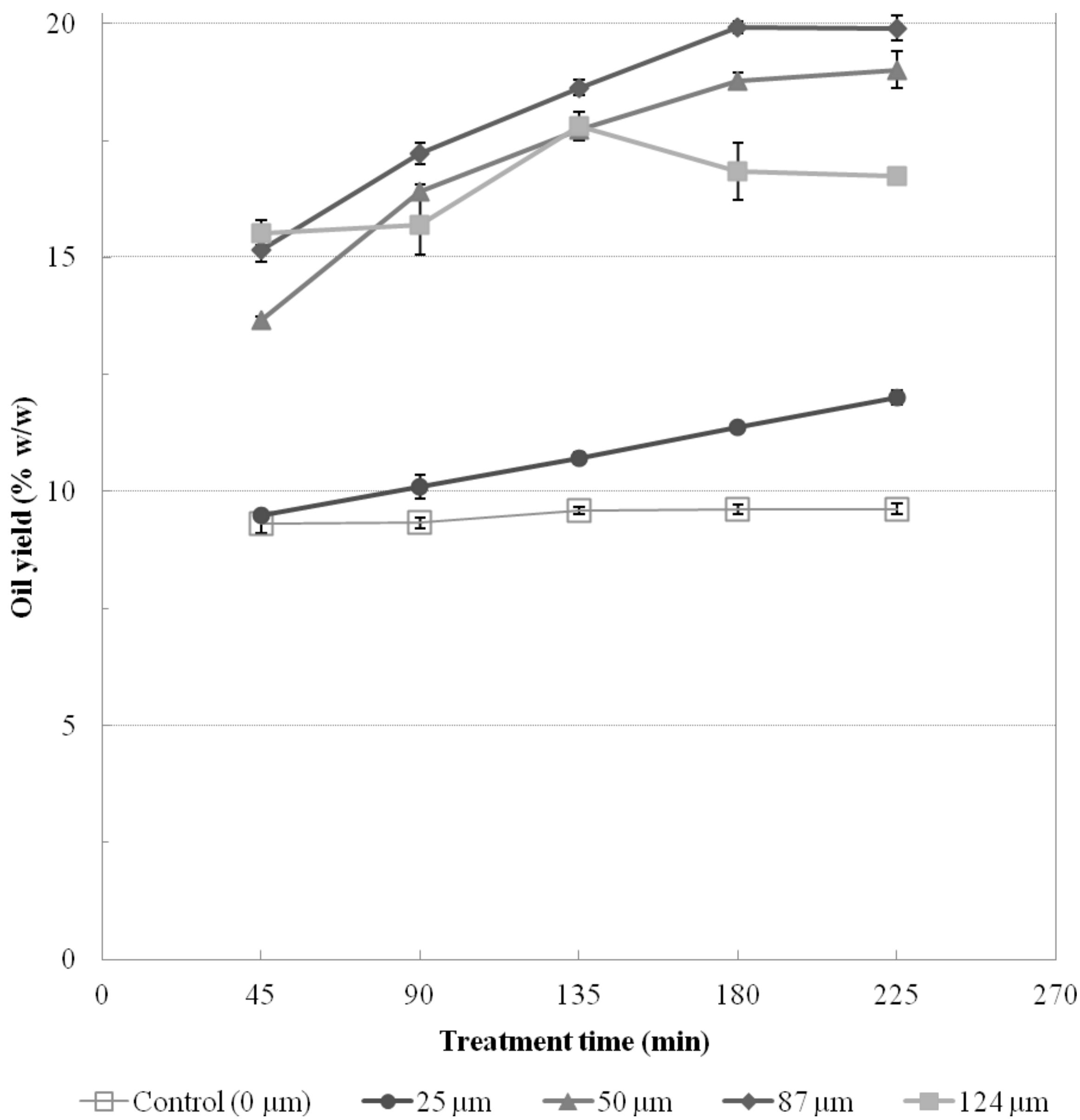

Figure 12: PUASE of soybean oil at various amplitudes and treatment times

The remarkably high oil yield of 19.92% from PUASE at 87 µm and 180 min represents an increase of more than 200% from the yield of untreated sample (9.62%) for the same treatment time. This huge increase in yield for PUASE is the result of *ultrasonic cavitation*. When ultrasound waves pass through a liquid medium, their alternating compression and expansion cycles cause the molecules of the medium to vibrate at such low pressures that the intermolecular forces (which keep them together) are overcome and microscopic gas-filled *bubbles* or *cavities* are created. These cavities contract and expand with the alternating pressure cycles, expanding in size until the point where they implode and collapse on themselves. This phenomenon is called *cavitation* and is the main principle behind ultrasound-assisted cell disruption. High intensity ultrasound (20 – 100 kHz) waves cause a more rapid form of cavitation where the cavities expand and implode after just a few cycles (usually in microseconds); this is called *transient cavitation*. The physical effects of transient cavitation produce a *hot spot* in its immediate vicinity i.e., a zone of high temperature and high shear forces where free radicals are created in the matter of a few microseconds (Suslick 1990; Condón, Raso et al. 2005; Feng, Yang et al. 2008). The mechanical effects of cavitation immediately disrupt intact biological cells in these hot spots by rupturing biological membranes and cell walls. Consequently, cellular material pours out into the processing medium made up of the solvent (HIP), and lipids are selectively dissolved in it. This process forms the basis for *ultrasound-assisted solvent extraction* (UASE) and is responsible for the much higher oil yields from ultrasonic treatment in comparison to other methods (Wang and Weller 2006).

Except for PUASE at 124 µm, the oil yield generally increased with resonance amplitude and time. The increase in oil yield with increasing treatment time was more prominent at 50 and 87 µm than at 25 and 124 µm. For the amplitude of 25 µm, the relative increase in yield from 45 to 180 min was just 1.89%. When the resonance amplitude was doubled to 50 µm, there was an almost three-fold increase in the relative yield increase (over time) – 5.12%. The increase in oil yield at the amplitude of 87 µm was 4.76%. This increase in oil yield can be attributed to greater ultrasonic cavitation at higher resonance amplitudes. Studies have found that the increased cavitational activity at higher amplitudes is the result of the increasing sizes of the bubbles (or cavities) responsible for cavitation, and also the larger zone of liquid undergoing cavitation in the vicinity of the ultrasonic probe (Raso, Mañas et al. 1999).

The oil yield obtained at 124 μm clearly followed a different pattern from that of 50 and 87 μm. At the latter two amplitudes, the yield obtained increased until 180 min but seemed to approach a plateau towards the higher treatment times. In contrast, for 124 μm, from a value of 17.81% at 135 min, the yield decreased to 16.84% at 180 min and then to 16.74% at 225 min. This may be a result of cavitational heating. Although higher treatment amplitudes may cause higher amount of cavitation (Raso, Mañas et al. 1999), a proportionally higher amount of heating occurred, and this may have raised the temperature of the suspension very rapidly during the ultrasound treatment. Apparently, at such high amplitudes and longer treatment times, the heat generated was so high that the solvent vaporized rapidly and enough of it was not available to extract oil. Although pulsed ultrasound allowed more control of processing temperature, and despite all the preventive measures used in this study (ice bath, regular temperature checks and evaporation-proof caps), evaporation of solvent was not avoided completely. Therefore, contrary to expectations, the maximum oil yield was neither the highest at the highest resonance amplitude, nor was it obtained at higher treatment times. This result proved that higher ultrasonic treatment amplitudes were not necessarily better, hence conflicting with earlier findings (Li, Pordesimo et al. 2004; Li, Pordesimo et al. 2004; Metherel, Taha et al. 2009).

Using pulsed treatment allowed the suspension to cool down temporarily between treatment pulses. This offered more control of the suspension temperature. This was also reported by a very recent study that compared pulsed and continuous ultrasonic treatments for the extraction of antioxidants from pomegranate peel (Pan, Qu et al. 2011). Besides giving comparable antioxidant yields, their study also found that pulsed treatment had 50% more energy savings than continuous treatment. Apart from this study, there are currently no other reports on the use of pulsed ultrasonic treatment for the extraction of oil or lipids.

Li et al. (2004) had investigated the effect of ultrasonic wave intensity (UWI) and treatment time on the extraction of oil from soybeans by a continuous UASE process. Three values of UWI (16.4, 20.9 and 47.6 W/cm^2) were studied against 6 values of treatment time ranging from 30 min to 180 min. Their findings indicate that oil yield increased as UWI and treatment time increased. They reported that UASE with HIP at UWI of 20.9 W/cm^2 and 3 hrs of treatment gave the highest oil yield – 62.3%. However, the method used by the authors to calculate oil yield was different from that used in this study. When calculated according to the method used in the current study, their reported yield of 62.3% corresponds to an oil yield of 12.21%. The higher oil yield obtained in this study (19.92%) could be attributed to the differences between the ultrasonic treatments used in the two studies. Li et al. (2004) used a continuous ultrasonic treatment based on UWI whereas a pulsed treatment based on ultrasonic amplitude was used in this study. Apparently, the system used in this study may have delivered a more effective treatment. Other possible reasons for the difference in yields may be due to the modified extraction method used in the current study (such as the lower solvent drying temperature of 50 ± 5^0C).

The results from the current study also show that PUASE is comparable to or better than supercritical CO_2 (SCO_2) extraction – another popular alternative to the traditional solvent extraction method (Russin, Boye et al. 2011). The highest yields for soybean oil extraction using SCO_2 from available literature are 22.6-22.9%; these figures were reported in a 1985 patent application (Friedrich and Eldridge 1985). Other high reported yields include 19.9% (Friedrich, List et al. 1982) and 19.4% (Friedrich and Pryde 1984).

3.3.3. FAME profile analysis

FAME analysis was carried out with oil extracted by PUASE at 87 µm and treatment time of 3 h; the combination that resulted in the highest oil yield. In order to compare the oil quality, oils extracted by RT shaking (400 rpm) with HIP for 3h and Soxhlet extraction with n-hexane were also analysed. As can be seen from the GC chromatogram (Fig. 13) for oil extracted by PUASE with HIP (87 µm, 180 min), soybean oil is made up of six major fatty acids namely, palmitic (16:0), stearic (18:0), oleic (18:1(9)), linoleic (18:2(9,12)), α-linolenic (18:3(9,12,15) and arachidic (20:0) acids. The relative quantities of these fatty acids (as % w/w of total FAME) as reported in refined soybean oil are 10.57, 4.09, 22.98, 54.51, 7.23 and 0.33%, respectively (Hammond, Johnson et al. 2005). Linoleic acid (LA) and α-linolenic acid (ALA) are the two most important PUFAs in soybean oil; LA is an ω-6 fatty acid and ALA is an ω-3 fatty acid. Both of these are essential fatty acids and soybeans are one of the top sources for them in human diet (Meydani, Lichtenstein et al. 1991).

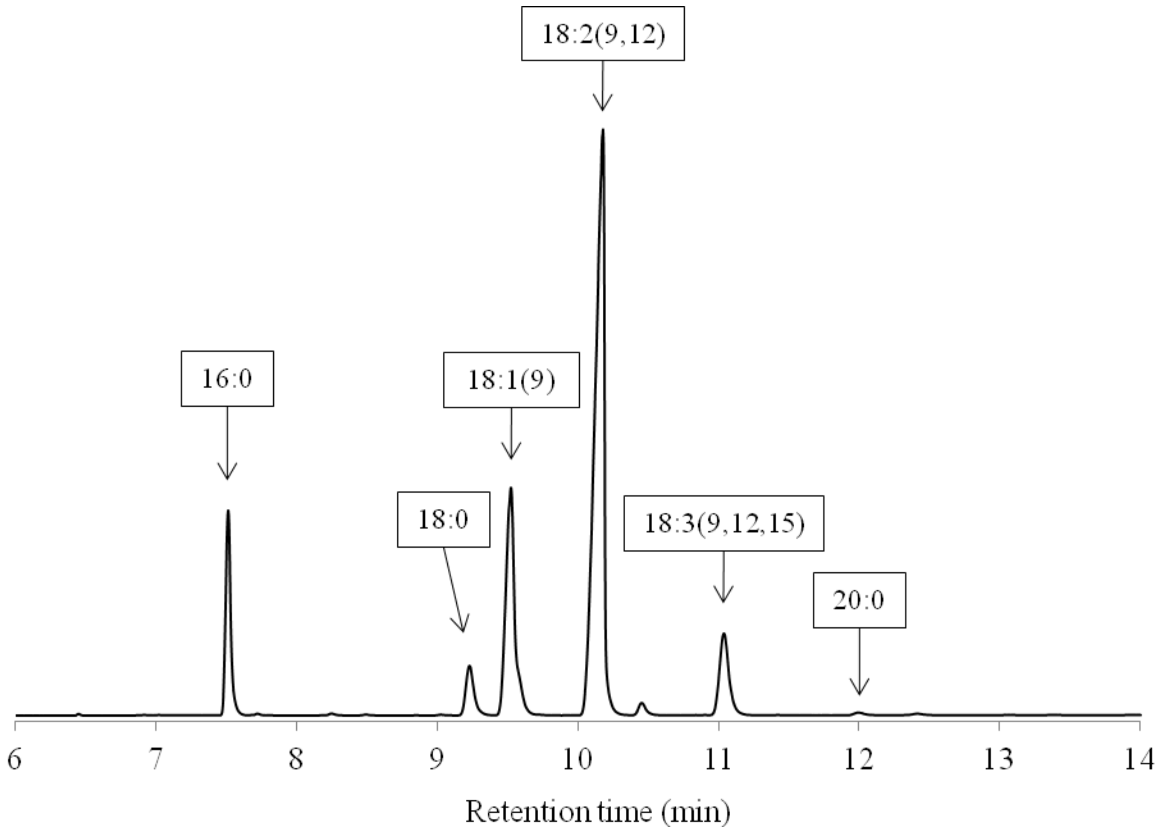

Figure 13: GC chromatogram of FAMEs in soybean oil from PUASE at 87 µm and 180 min

The FAME profiles obtained for the three extracted oils mentioned earlier are given in table 3. The results showed that all three extraction methods produced oils of very comparable quality. T-test analysis on the fatty acid profile data indicated that there were no statistically significant differences ($P \leq 0.05$) between all three extraction methods. In fact, there is a very close similarity between oils extracted using the same solvent i.e., HIP. In terms of PUFA content, PUASE produced only 0.12% more LA and 0.02% more ALA than RT shaking. Soxhlet extraction with n-hexane on the other had produced oil with 1.05% less LA and 0.22% less ALA than PUASE. The slightly lower yield for PUFAs in Soxhlet-extracted oil may be an indication of the mild thermal oxidation that happens to the PUFAs (especially ALA) during the Soxhlet extraction procedure. In general oil obtained by PUASE was found to be slightly better in terms of unsaturation, but not significantly different from the other two methods. Further, PUASE produced oil with slightly better LA and ALA content (55.41 and 7.87%, respectively) than refined oil (54.51 and 7.23%, respectively as reported by Hammond et al. (2005)).

Table 3: FAME profile obtained for soybean oils extracted using three extraction methods

Fatty acid methyl ester (FAME)		***Solvent extraction system & FAME %^{b}***		
Trivial name & lipid number	*RT^a (min)*	***Soxhlet (n-hexane)***	***RT shaking (HIP, 400 rpm, 3 h)***	***PUASE (HIP, 87 µm, 3h)***
Palmitic acid – 16:0	7.5	10.60	11.52	11.06
Stearic acid – 18:0	9.2	4.05	4.02	4.05
Oleic acid – 18:1(9)	9.5	20.59	20.64	20.64
Linoleic acid or LA – 18:2(9,12)	**10.1**	**54.36**	**55.29**	**55.41**
α-Linolenic acid or ALA – 18:3(9,12,15)	**11.0**	**7.65**	**7.85**	**7.87**
Arachidic acid – 20:0	12.0	0.28	0.35	0.28
Total saturated FAME		14.93	15.89	15.39
Total unsaturated FAME		82.60	83.78	83.92
Polyunsaturated (PUFA) FAME		62.02	63.14	63.28

^{a} retention time, $^{\mathbf{b}}$ as % w/w of total FAME

Even so, the slightly higher PUFA profile in soybean oil from PUASE differs from the results obtained by Cravotto et al. (2008) with soybean germ oil. Using hexane as solvent, they reported that application of ultrasound actually caused a slight decrease in both LA (-0.5%) and ALA (-0.3%) content, when compared with Soxhlet extraction. They attributed this decrease to the slight oxidation caused by ultrasonic treatment. However, it must be noted that the authors used a very high-intensity ultrasound treatment involving a combination of a cavitating tube at 19 kHz and an immersion horn at 25 kHz. In contrast, the current study used only an immersion horn (or probe) at 20 kHz. Even Li et al. (2004) reported a small decrease in the percentage of unsaturated fatty acids in oil extracted through PUASE with HIP as solvent. However, Li et al. only studied oleic acid and LA; they did not study the ALA content in their work.

3.4. CONCLUSIONS

The findings of the current study clearly re-ascertain that PUASE with hexane-isopropanol (3:2 v/v) is one of the most efficient methods available currently for the extraction of soybean oil. It was shown that higher resonance amplitude and/or longer treatment time do not necessarily give higher oil yields; this could be attributed to high rate of solvent evaporation as a result of cavitational heating. Choosing an ideal combination of amplitude and treatment time resulted in high oil yields. GC analysis of the FAMES in the extracted oils indicated that PUASE with HIP produced soybean oil that was similar in quality to those extracted by conventional RT shaking with HIP and by Soxhlet extraction using hexane. These results indicate that PUASE could have immense potential in the extraction of oil from soybeans and other oilseeds or oil-rich plant materials. A more thorough knowledge of the kinetics of PUASE would be beneficial in suggesting improvements that could make this process more attractive commercially and industrially.

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CONNECTING STATEMENT

This first manuscript (chapter 3) investigated the extraction of soybean oil using pulsed ultrasound-assisted solvent extraction (PUASE). A study of three popular solvents (used in soybean oil extraction) showed that HIP was a superior solvent than both n-hexane and petroleum ether. PUASE with HIP was found to have a high extraction efficiency; the highest oil yield obtained was 19.92%, at resonance amplitude of 87 µm and treatment time of 180 min. Additionally, FAME analysis showed that soybean oil obtained from PUASE was similar in quality to that extracted by conventional methods such as shaking with HIP at room temperature, and Soxhlet extraction with n-hexane.

The second manuscript (chapter 4) reports the use of PUASE for extracting oil from the marine microalga *Nannochloropsis oculata*. Based on the results of the study with soybeans, the amplitude levels studied have been narrowed to a range from 50 to 105 µm (compared to 25-124 µm in the first study). Also, the difference between consecutive levels of treatment time has been increased to 60 mins (instead of 45 min in the soybean study) and the number of levels have been reduced to three (60, 120, 180 min). This manuscript will be submitted for publication in the Bioresource Technology Journal, published by Elsevier Journals.

CHAPTER 4: **PULSED ULTRASOUND-ASSISTED SOLVENT EXTRACTION (PUASE) OF OIL FROM THE MARINE MICROALGA *Nannochloropsis oculata***

ABSTRACT

Nannochloropsis oculata is a species of marine microalgae which is well-known both for its high PUFA-rich oil content as well as its biodiesel potential. The current study investigated pulsed ultrasound-assisted solvent extraction (PUASE) of oil from *N. oculata* using the solvent hexane-isopropanol (3:2 v/v) (HIP). Ultrasonication was carried out using an immersed sonotrode resonating at constant amplitude; values were varied between 50 and 105 µm. The effective treatment time (varied between 60 and 180 min) was also studied. The oil yield was assessed gravimetrically and fatty acid profiles of the extracted oils were analysed.

The results show that PUASE with HIP extracted up to 69.53% oil from *N. oculata*, compared to 35.19% yield obtained from regular solvent extraction by agitation. Both amplitude and treatment time were found to have significant effects ($P \leq 0.05$) on oil yield. In addition, the extracted oil had a superior fatty acid profile compared to that extracted by traditional methods such as Soxhlet and room temperature shaking.

Keywords: microalgae, *Nannochloropsis oculata*, oil, pulsed ultrasound, hexane, isopropanol

4.1. INTRODUCTION

Much of the research on microalgal lipids has focused primarily on PUFAs and has spawned two distinct areas of interest; nutrition and biodiesel. In terms of nutrition, microalgae are a major source of polyunsaturated fatty acids (PUFAs). Besides possessing important health effects against several health disorders (most notably coronary heart disease, atherogenesis, cancer and diabetes), PUFAs have also been implicated in several other vital functions such as cellular metabolism and maintenance of membrane fluidity (Spolaore, Joannis-Cassan et al. 2006; Teale 2006).

Nannochloropsis oculata is a marine microalga that is abundant in PUFAs, especially ***eicosapentaenoic acid*** or EPA (20:5(5,8,11,14,17)) and ***arachidonic acid*** or AA (20:4(5,8,11,14)) (Hodgson, Henderson et al. 1991; Seto, Kumasaka et al. 1992; Zhukova and Aizdaicher 1995; Roncarati, Meluzzi et al. 2004; Patil, Kallqvist et al. 2007; Yu, Chen et al. 2007). The maximum value for total lipid content of this microalga reported in the literature is **78%** for *Nannochloropsis* sp. (Emdadi and Berland 1989) and **60%** for *N. oculata* (Hodgson, Henderson et al. 1991).

From currently available reports, it is clear that chloroform-methanol (CM) is the most widely used solvent for laboratory-scale extraction of lipids from microalgae (Bligh and Dyer 1959; Whyte 1987; Volkman, Jeffrey et al. 1989; Sukenik and Carmeli 1990; Sukenik, Zmora et al. 1993; Zhukova and Aizdaicher 1995; Rebolloso-Fuentes, Navarro-Perez et al. 2001; Roncarati, Meluzzi et al. 2004; Mansour, Frampton et al. 2005; Patil, Kallqvist et al. 2007; Chiu, Kao et al. 2009; Lee, Yoo et al. 2010; Koberg, Cohen et al. 2011; Moazami, Ranjbar et al. 2011; Prabakaran and Ravindran 2011; Zheng, Yin et al. 2011). However, this solvent has significant drawbacks (such as the serious health and environmental safety concerns arising out of the high toxicity and volatility of chloroform) that are roadblocks to successful scale-up (Halim, Gladman et al. 2011). Yet, in spite of these drawbacks, CM continues to be the most popular solvent used for microalgal lipid extraction, and very few studies have investigated solvents other than CM (Guckert, Cooksey et al. 1988; Pernet and Tremblay 2003; Andrich, Nesti et al. 2005; Fredriksson, Elwinger et al. 2006; Dayananda, Sarada et al. 2007; Li, Min et al. 2009; Mulbry, Kondrad et al. 2009; Halim, Gladman et al. 2011). Among alternative solvents suggested, hexane-isopropanol (HIP) has had significant success in oil extraction from different biological materials including oilseeds (Radin 1981; Radin 1989; Li, Pordesimo et al. 2004; Metherel, Taha et al. 2009). Yet, very few studies have investigated its efficacy in extracting microalgal lipids and there are no reports of it with *N. oculata* (Guckert, Cooksey et al. 1988; Mulbry, Kondrad et al. 2009; Halim, Gladman et al. 2011).

At 2-5 µm, *N. oculata* is a very small microalga. In addition, the presence of a rigid cell wall makes its cell disruption an exceptionally difficult process (Rodolfi, Zittelli et al. 2009; Harun, Singh et al. 2010). Consequently, several novel extraction methods have been suggested, where the main solvent extraction process is 'assisted' by specialized techniques. The application of ultrasonic waves has already been found to be sufficiently efficient for extracting oils from different kinds of microalgae (Pernet and Tremblay 2003; Cravotto, Boffa et al. 2008; Ranjan, Patil et al. 2010; Prabakaran and Ravindran 2011). The basic principle of ultrasound-assisted extraction is cell disruption as a result of *transient cavitation*. This is the process by which the implosion of microscopic gas-filled bubbles or *cavities* (created in a liquid medium due to the passage of high-intensity ultrasound waves of low frequency, i.e., 20 to 100 kHz) leads to the rapid (in a matter of few microseconds) production of *hot spots* in the medium; these are regions of high temperature, shear forces and free radicals. The mechanical effects of these hot spots and the shockwaves created by them lead to the rapid rupturing of microalgal cell walls in their vicinity and their subsequent cell disruption (Suslick 1990; Condón, Raso et al. 2005; Feng, Yang et al. 2008; Harun, Singh et al. 2010). When the medium itself is made up of the solvent, the resulting process becomes *ultrasound-assisted solvent extraction* or *UASE* (Wang and Weller 2006).

High power or high intensity ultrasound waves are characterized by their vibrational amplitude, power intensity, frequency and temperature of medium. Vibrational or resonance amplitude is the maximum displacement of the ultrasonic probe or horn (also called sonotrode) tip during vibrations. Power intensity or ultrasonic wave intensity (UWI) is the power output from the ultrasonic horn into the medium, per unit cross-sectional area of the horn. Studies on microbial cell disruption had shown that resonance amplitude is a more exact parameter for estimating the extent of ultrasound-assisted cell disruption (Tsukamoto, Yim et al. 2004; Bermúdez-Aguirre, Mobbs et al. 2011).

The objectives of this study were; (1) to compare oil yields obtained from freeze-dried and ground *N. oculata* using room temperature (RT) shaking with four solvents namely n-hexane, petroleum ether, CM and HIP; (2) to investigate the effects of resonance amplitude and treatment time during PUASE of microalgal oil with HIP; and (3) to evaluate the influence of extraction methods on the fatty acid composition of extracted oils.

4.2. MATERIALS AND METHODS

4.2.1. Chemicals, sample preparation and storage

All solvents used in the study (n-hexane, petroleum ether, isopropanol, chloroform and methanol) were of Fisher 'Optima' grade or higher (Fisher Scientific Co., ON, Canada). Chemicals used for FAME analysis using GC were of high resolution chromatography grade. Dewatered and frozen samples of *N. oculata* were obtained from NutrOcean Co. (Rimouski, QC, Canada) and stored at -80^{0}C. A few days prior to experiments, the samples were freeze-dried and ground into a fine powder immediately afterwards. In order to limit oxidation, the dark green coloured powder was stored in airtight amber glass bottles and used within 2 weeks of grinding.

4.2.2. Extraction by room temperature (RT) shaking

Prior to studies on PUASE, four solvents (n-hexane, petroleum ether, CM (3:1 v/v) and HIP (3:2 v/v)) were evaluated for their effectiveness in extracting microalgal oil by RT shaking. Extraction with CM was carried out as per a method described recently for microalgal oil extraction (Ranjan, Patil et al. 2010). HIP extraction was carried out by a method modified from that of Radin (1981). Extraction with n-hexane and petroleum ether was carried out identical to HIP, with the only difference being the solvent. *N. oculata* powder was mixed with each of the above four solvents in capped plastic tubes and shaken at 400 rpm for 120 min on a bench-top shaker (IKA, Schuttler MTS 4, Staufen, Germany), at room temperature (RT). A sample-solvent ratio of 1:10 w/v was used; each experiment was carried out in triplicates with 3 g of sample and 30 mL of each solvent.

4.2.3. Pulsed ultrasound-assisted solvent extraction (PUASE)

A high intensity ultrasonic processor (Vibra-Cell^{TM} VCX-500, Sonics & Materials, CT, USA) equipped with an ultrasonic horn transducer and a tuned titanium alloy probe resonating at a frequency of 20 kHz was used for this purpose. The probe was 5.5 inches long, 13 mm wide and resonated at maximum resonance amplitude of 124 µm. The processor had the ability to continuously change the power output to the probe load (i.e., the microalgae-solvent suspension), so as to maintain constant resonance amplitude (Sonics & Materials Inc. 2010). Five levels of resonance amplitudes (0, 50, 68, 87 and 105 µm, respectively) were studied at treatment times of 1, 2 and 3 h. The experimental design was that of a 5 X 3 two-way factorial experiment with three replicates per trial. The 0 µm amplitude experiment was basically 'untreated' extraction i.e., instead of ultrasound treatment, the suspension was kept undisturbed at room temperature.

The sample-solvent suspension was taken in a plastic 50 mL tube and sonicated by inserting the probe tip approximately 5 cm from the top of the liquid. The treatment was pulsed; treatment pulses of 10 seconds were followed by 5 seconds of rest. This cycle repeated until the required treatment time was attained; so the total treatment time was 1.5 times the effective treatment time.

In order to limit solvent evaporation at longer treatment time and/or higher amplitudes, the temperature of the sample-solvent suspension was maintained at ambient conditions (25 ± 5^0C) using an ice-water bath. Also, the temperature of the suspension was monitored every 20 mins to ensure that there was no over-heating. In addition, a specially designed spill- and evaporation-proof cap was used to prevent spillage and evaporation during the application of ultrasonic treatment.

4.2.4. Determination of oil yield

After the RT shaking (or ultrasonic treatment), the sample-solvent suspension was kept undisturbed at room temperature for 5-10 min; the supernatant was then decanted into a separate tube. The remaining residue was washed twice with 5 mL of HIP and the supernatant combined with that from the original extraction. Next, 15 mL of 10% sodium sulphate solution was added to the decanted suspension, separating it into two phases: an upper organic (hexane) phase containing the extracted lipids and a lower inorganic phase (isopropanol) phase composed of all the other cellular components. Centrifugation at 4000 rpm made the phase separation more distinct and the heavily pigmented upper hexane phase (Fig. 14) was then decanted into a pre-weighed stainless steel vessel. Due to the easily oxidizable nature of microalgal oil, the hexane phase was then dried slowly over a stream of warm air at 50±5^{0}C in an oven (or, for GC samples, under a stream of nitrogen) for 2 to 3 hours until constant weight. This drying condition was maintained consistent throughout the experiment since solvent drying temperature could affect both yield and composition of lipids extracted from microalgae, especially those rich in PUFAs (Widjaja, Chien et al. 2009).

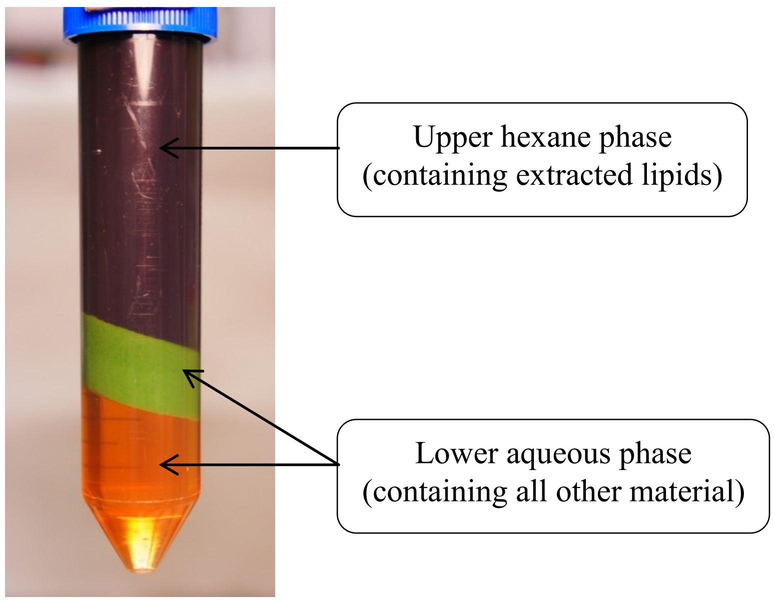

Figure 14: N. oculata-solvent suspension after phase separation & centrifugation

The oil yield (Y) was determined gravimetrically:

$$Y(\%) = m_0/m_s \times 100,$$

where m_s is the mass of *N. oculata* powder taken (g) and m_0 the mass of the extracted oil (g).

4.2.5. FAME analysis using GC

Following extraction, the oil was transesterified to produce fatty acid methyl esters (FAMEs) according to a method reported earlier (Palmquist and Jenkins 2003). Samples of extracted oil (weighing 0.5 g each) were mixed with 2 mL of internal standard (nonadecanoic acid or 19:0 (Nu-Chek Prep, MN, USA), 2 mg/mL in n-hexane) and reacted with 3 mL of 10% methanolic HCl in glass test tubes with Teflon-lined screw caps, in a 90^0C water bath for 2 h. Following the addition of 1 mL n-hexane and 10 mL 6% K_2CO_3, the reaction mixture was vortexed and centrifuged at 4000 rpm. The upper organic phase was then transferred to a fresh tube, mixed with 10% Na_2SO_4 solution, and centrifuged again to separate the phases more distinctly. The upper phase (containing the FAMEs) was carefully aspirated into 2 mL GC auto sampler vials, capped and stored at -80^0C until GC analysis.

The FAME composition of the transesterified oil was analysed using an HP 5890 Series II gas chromatograph equipped with a flame ionization detector (FID). Triplicate 1 µL injections of each FAME sample (dissolved in n-hexane) were injected into a ZB-WAX capillary column (Phenomenex, CA, USA). The column stationary phase was polyethylene glycol and column dimensions were 30 m x 0.25 m x 0.25 µm. Helium gas was the carrier gas used; a linear velocity of 45 cm/s and a split ratio of 100:1 were used. The detector (FID) gases were hydrogen (30 mL/min) and air (430 mL/min). The temperatures of the injector and detector were both set to 250^0C. The temperature program employed was as thus; 60^0C (maintained for 2 min), then raised at 60^0C/min to 210^0C (2.5 min) and raised again at 1^0C/min to 220^0C (3 min). Individual FAMEs in the extracted oils were identified using a standard FAME mix by comparing the retention times and relative peak areas. Since the FAME profiles of microalgae (and microorganisms in general) are more complex and comprise several rare fatty acids, GLC-68D (Nu-Chek Prep, MN, USA) – a more diverse FAME mix comprising several popular and less popular FAMEs – was used to create a FAME profile for oil extracted from *N. oculata*. FAME profiles for analysed samples were then generated using the individual FAME quantities (% of total FAME), calculated as ratios of individual peak areas and total area of all FAME peaks.

A comparison was made between the FAME profiles obtained for oil samples extracted from three methods; (1) PUASE experiment (with HIP) that produced the highest oil yield, (2) RT shaking (with HIP at 400 rpm) and, (3) Soxhlet extraction with n-hexane (AOCS 2005; ISO

2009). Soxhlet extraction was carried out on an automated solvent extractor (SER 148/6, Velp Scientifica srl, Usmate, Italy).

4.2.6. Statistical analysis

Each experiment was carried out in triplicates and all statistical analyses were carried out as ANOVAs using the *general linear models* procedure in SAS 9.2 (SAS Institute Inc., NC, USA). The significance level was set at 0.05.

4.3. RESULTS AND DISCUSSION

4.3.1. Evaluation of solvent systems by shaking at room temperature

Shaking samples at room temperature (400 rpm for 120 min) was used to evaluate four solvents namely n-hexane, petroleum ether, CM (3:1 v/v) and HIP (3:2 v/v) for their capacity to extract total lipids from microalgae (freeze-dried and ground *N. oculata*). The total lipid yields obtained were 21.38, 20.33, 32.92 and 35.19%, for n-hexane, petroleum ether, CM (3:1 v/v) and HIP (3:2 v/v), respectively. Petroleum ether and n-hexane, due to their non-polar nature, extract mostly neutral lipids (such as triglycerides) and are poor extractants of polar lipids. Earlier studies have already proved that polar lipids (phospholipids, glycolipids and cholesterols) make up a significant portion of marine microalgal lipids (Volkman, Jeffrey et al. 1989; Seto, Kumasaka et al. 1992; Fredriksson, Elwinger et al. 2006). The higher oil yields for solvent mixtures (CM & HIP) over single solvents could be attributed to additional extraction of polar lipids by the polar solvent in the mixture (methanol and isopropanol, respectively). Hence, solvent mixtures are clearly superior in the extraction of total lipids (Halim, Gladman et al. 2011).

Although HIP was found to have a slightly higher yield (2.27% more) than CM, statistical analysis showed that there is no significant difference ($P \leq 0.05$) between HIP and CM. Yet, HIP offers numerous advantages over CM; hexane is much safer, cheaper and easier to handle than chloroform (Radin 1981; Radin 1989). Also, since hexane is less volatile than chloroform, solvent evaporation in PUASE (as a result of cavitational heating) will be lesser than with CM. In addition, the extraction method with CM has a significant procedural limitation. The phase separation step results in an upper inorganic phase and a lower organic phase – the latter is the lipid-rich chloroform phase. Aspirating this phase is problematic in the case of highly pigmented microalgae (like *N. oculata*); the extremely dark colour of the two phases makes the process tedious and prone to errors (Woertz 2007; Shahidi and Wanasundara 2008).

4.3.2. PUASE with HIP – effects of amplitude and treatment time

Statistical analysis showed that both variables affected the oil yield significantly ($P \leq 0.05$). There was also a statistically significant interaction between them, as seen from Fig. 15. The highest oil yield of 69.53% was obtained using the resonance amplitude of 87 µm and effective treatment time of 120 min. The highest oil yields obtained at the other amplitude levels were 46.86, 61.83 and 53.99% at 50, 68 and 105 µm, respectively. Hodgson et al. (1991) had reported an oil yield of approximately 60% from *N. oculata* using a method modified from that of Bligh and Dyer (1959); the authors used isopropanol instead of methanol. As in the original Bligh & Dyer method, homogenization was used as the method of agitation. The maximum oil yield obtained in the current study was about 10% higher than that reported by Hodgson et al. (1991). This could be attributed to the use of HIP as main extraction solvent rather than chloroform-isopropanol, and also to the application of pulsed ultrasound treatment to assist to solvent extraction process.

The yield obtained at 87 µm (69.53%) is almost 250 times the value obtained from untreated sample (28.11%) for the same treatment time (120 min). This huge increase in yield demonstrates the effectiveness of pulsed ultrasound treatment in disrupting microalgal cells, especially those with tough cell walls and small sizes such as *N. oculata*. Other than for 87 and 68 µm, all other amplitudes demonstrated a directly proportionate relationship between treatment time and oil yield; i.e., the highest oil yields were obtained at the highest levels of treatment time – 180 min. Treatment at 87 and 68 µm showed a trend where the yield first increased, reached the highest value at the middle level (120 min) and then decreased. This is a result of the rapid evaporation for the solvent (as a result of cavitational heating) at higher treatment times. Hence, as the treatment time increased, the amount of solvent available to extract oil from the disrupted cells reduced relatively. At the highest amplitude (105 µm), the solvent evaporation was so rapid that there was almost no difference between 120 or 180 min of treatment. It must be noted that inspite of the several measures taken to prevent this (temperature monitoring and regulation using pulsed treatment and ice bath, and evaporation control using a specially designed cap), the phenomenon could not be avoided. From the graph it is clear that the only solution to this problem is to choose an ideal combination of treatment time and resonance amplitude.

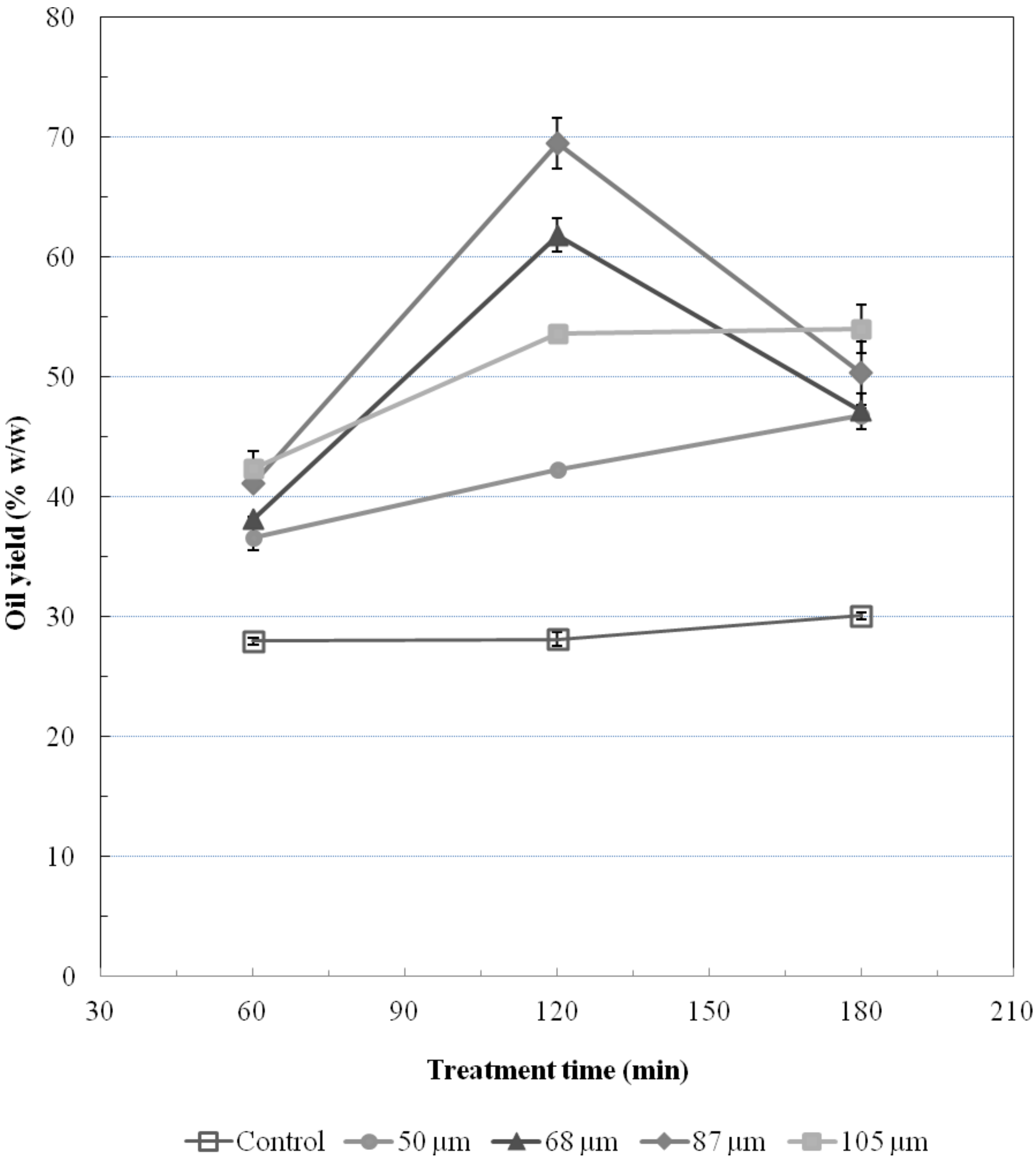

Figure 15*: PUASE of N. oculata oil at various resonance amplitudes and treatment times*

Since sonication is a well-established method of cell disruption, there have been several studies where it was used as a pre-treatment to solvent extraction of oil from microalgae

(Volkman, Jeffrey et al. 1989; Lee, Yoon et al. 1998; Li, Min et al. 2009; Lee, Yoo et al. 2010; Prabakaran and Ravindran 2011; Zheng, Yin et al. 2011). However, there are only a few reports on the use of UASE (i.e., simultaneous sonication and solvent extraction) for extracting oil from microalgae, and none on *N. oculata.* In one of the earliest reports, sonication using a sonication bath was compared with grinding and a combination of both for the extraction of oil from microalgae harvested on filters; the combined method was found to be most effective (Pernet and Tremblay 2003). A study on the microalga *Crypthecodinium cohinii* found that UASE (with n-hexane) was the most efficient method out of UASE, microwave-assisted solvent extraction and a combination of both. Ultrasonic treatment was carried out using a specially developed high intensity system combining a cavitating tube at 19 kHz frequency and an immersion horn (sonotrode) at 25 kHz (Cravotto, Boffa et al. 2008). In a more recent study with *Scenedesmus* sp., UASE with CM gave the highest oil yield, when compared to Soxhlet extraction with n-hexane, conventional CM extraction and UASE with n-hexane. However, the authors suggested that the cell disruption was incomplete. This may have been due to the low intensity treatment used namely 30 min of continuous UASE at 20% amplitude (Ranjan, Patil et al. 2010).

4.3.3. FAME profile analysis

The fatty acids present in *N. oculata* were identified from FAME analysis using the GLC-68D FAME standard mix. Oil samples extracted from three methods were compared; the PUASE (with HIP) combination that produced the highest oil yield (i.e., 87 µm for 120 min), Soxhlet extraction with n-hexane (AOCS & ISO standard) and RT shaking extraction with HIP (400 rpm, 120 min).

As can be seen from the GC chromatogram (Fig. 16) for *N. oculata* oil extracted by PUASE with HIP (87 µm, 120 min), the seven most abundant FAMEs in *N. oculata* were; myristate (14:0), palmitate (16:0), palmitoleate (16:1(9)), oleate (18:1(9)), linoleate (18:2(9,12)), arachidonate (20:4(5,8,11,14)) and eicosapentaenoate (20:5(5,8,11,14,17)). Eicosapentaenoic acid (EPA) is an ω-3 PUFA while arachidonic acid (AA) and linoleic acid (LA) are both ω-6 PUFAs (Zhukova and Aizdaicher 1995; Roncarati, Meluzzi et al. 2004).

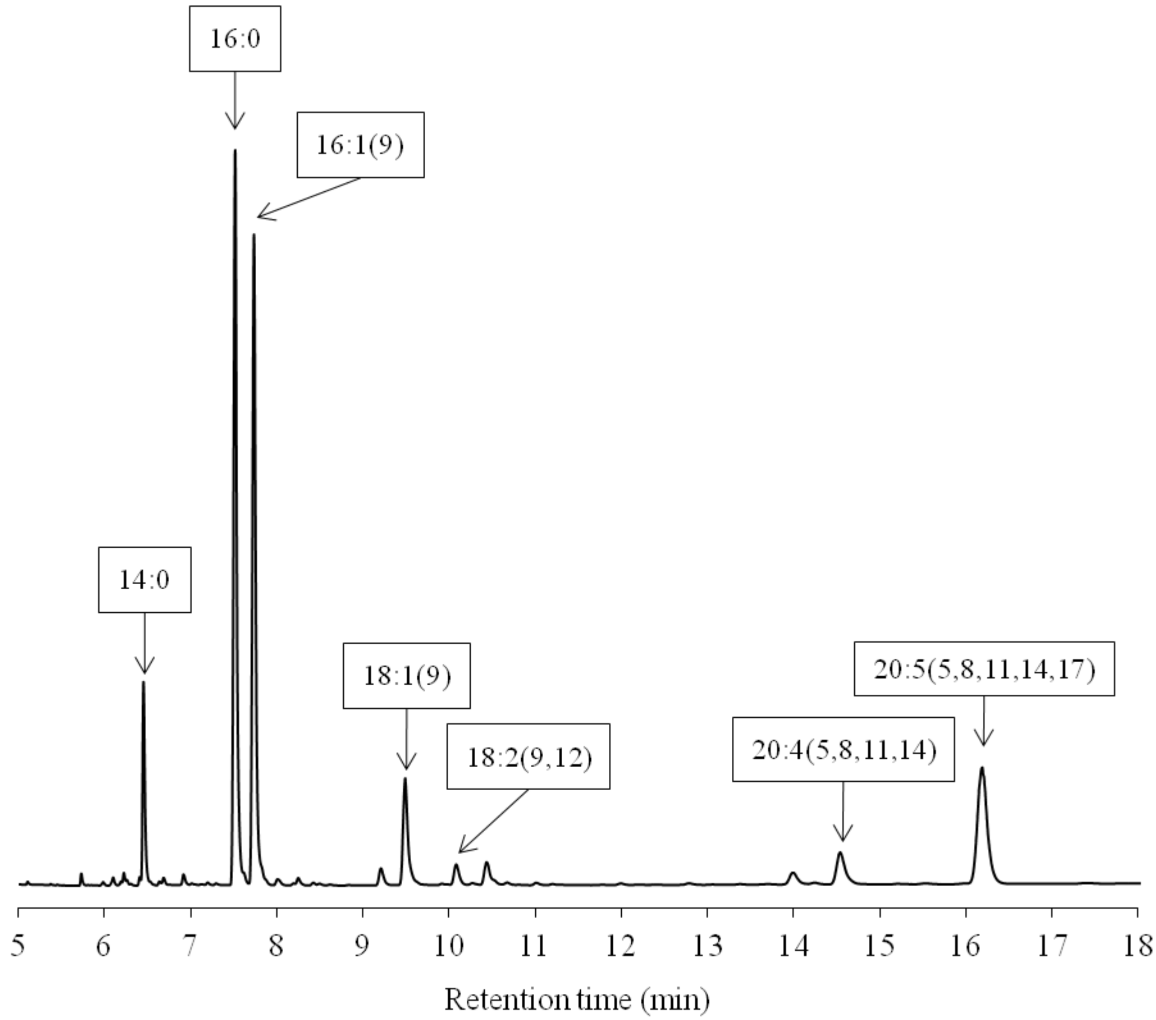

Figure 16: GC chromatogram of FAMEs in N. oculata oil from PUASE at 87 μm and 120 min showing the seven most abundant FAMEs

The FAME yields for the three methods mentioned above are provided in Table 4. The highest PUFA yield (20.38%) was obtained from PUASE with HIP. In comparison, shaking at RT gave a lower PUFA yield (18.49%). The Soxhlet extraction (traditionally considered to be an intense method) gave the lowest PUFA yield (15.38%). A similar order was seen for total unsaturated fatty acids (i.e., mono- and polyunsaturated fatty acids combined). However, the trend is reversed for saturated fatty acids for which Soxhlet method gave the highest yield of 40.46%.

N. oculata is renowned for its rich PUFA content, most notably EPA and AA. The current study showed that PUASE was able to extract the highest quantity of EPA (15.28%) among the three methods. Both the Soxhlet method with n-hexane and RT shaking with HIP produced lower EPA yields; 11.30 and 14.13%, respectively. A similar increase is seen with AA, with PUASE producing the highest yield (3.66%). However, as for LA, both PUASE and Soxhlet extracted slightly higher quantity of LA (1.45% each) than RT shaking (1.34%). In addition to the higher yields of PUFAs from HIP and PUASE, statistical t-tests confirmed that there were significant differences ($P \leq 0.05$) between all three methods in the EPA and AA contents.

Table 4: FAME profiles of N. oculata oil extracted using three extraction methods

Fatty acid methyl ester (FAME)		***Solvent extraction system & FAME %[b]***		
Trivial name & lipid number	*RT[a] (min)*	***Soxhlet extraction (n-hexane)***	***RT shaking (HIP, 400 rpm, 2h)***	***PUASE (HIP, 87 µm, 3h)***
Myristic acid – 14:0	6.4	7.21	7.16	6.10
Palmitic acid – 16:0	7.5	33.26	31.50	30.66
Palmitoleic acid – 16:1(9)	7.7	30.68	29.52	28.25
Oleic acid – 18:1(9)	9.5	7.01	6.72	6.89
Linoleic acid or LA – 18:2(9,12)	**10.4**	**1.45**	**1.34**	**1.45**
Arachidonic acid or AA – 20:4(5,8,11,14)	**14.5**	**2.63**	**3.02**	**3.66**
Eicosapentaenoic acid or EPA – 20:5(5,8,11,14,17)	**16.2**	**11.30**	**14.13**	**15.28**
Total saturated FAME		40.46	38.66	36.76
Total unsaturated FAME		53.07	54.72	55.53
Polyunsaturated (PUFA) FAME		15.38	18.49	20.38

[a]retention time, [b]% w/w total FAME

Although there have been suggestions to exploit *Nannochloropsis* sp. for the large-scale production of EPA (Sukenik 1991), commercial systems for growing microalgae and extracting valuable substances from them are not currently economically feasible (Spolaore, Joannis-Cassan et al. 2006; Chisti 2007). Hence, the ability to extract the maximum amount of PUFAs is extremely crucial for any microalgal disruption and/or extraction method used. The current study clearly demonstrates that PUASE has enormous potential in this regard.

These results markedly differ from previous studies which reported that the fatty acid composition of oil extracted with UASE was comparable to other conventional methods. For example, Cravotto et al. (2008) found that UASE produced a higher oil yield, but the FAME profiles of oil from UASE and Soxhlet extraction were almost comparable. According to their work, application of ultrasound had no significant effect on FAME profile. However, one important point to note is that the solvent they used was n-hexane, which is less efficient than solvent mixtures (like HIP or CM) in extracting triglycerides. In addition, even though the Soxhlet method is thermally vigorous, it is not recommended for the extraction of oils that are rich in unsaturated lipids (such as microalgal oils) – these are susceptible to degradation at high temperatures and could lead to the formation of artefacts (Guckert, Cooksey et al. 1988).

4.4. CONCLUSIONS

The findings of this study showed that HIP was comparable to or more efficient than popular solvents like n-hexane and CM, for extracting total lipids from the marine microalga *N. oculata*. The used of pulsed ultrasonic processing in combination with solvent extraction (PUASE) was found to drastically improve microalgal lipid extraction; oil yields as high as 69.5% were obtained with this method. However, lower yields were obtained at higher treatment time and resonance amplitude. This could be attributed to increased solvent evaporation as a result of greater cavitational heating.

FAME analysis of microalgal oil extracted using PUASE with HIP showed that it was superior in its PUFA content, when compared with oils extracted by the Soxhlet method (with n-hexane) and RT shaking (with HIP). PUASE produced oil with substantially higher quantities of important PUFAs like eicospentaenoic acid (EPA) and arachidonic acid (AA). Hence, the current study shows that ultrasonic processing has enormous potential for the extraction of such valuable substances from microorganisms; even marine microalgae like *N. oculata* that normally pose several disruption challenges.

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CHAPTER 5: **SUMMARY AND CONCLUSIONS**

5.1. SUMMARY

The current study investigated the capability of pulsed ultrasound-assisted solvent extraction (PUASE) in improving the extraction of biological oils. The two oils chosen for the study were soybean and microalgal oils, the latter from a marine microalga *Nannochloropsis oculata*. Conventionally, these oils are extracted by standalone solvent extraction using n-hexane and chloroform-methanol (CM), respectively. However, in light of numerous drawbacks with these solvents, an alternative solvent mixture hexane-isopropanol (HIP) was chosen for the study. An initial study comparing this solvent with two other popular solvents was carried out prior to ultrasonic processing. Two parameters that influence the efficiency of PUASE, namely resonance amplitude and effective treatment time were studied. Extraction efficiency was evaluated both quantitatively (through calculation of gravimetric oil yield) and qualitatively (through analysis of fatty acid profiles generated using gas chromatography).

5.2. CONCLUSIONS

i. Hexane-isopropanol (HIP) is a relatively safer and more efficient solvent for the extraction of vegetable oils, compared to conventional solvents like n-hexane, petroleum ether, and chloroform-methanol (CM). It has already been proven that solvent mixtures are more efficient than standalone solvents at extracting total lipids; this was clearly seen in the current study as well, where both HIP & CM produced much higher oil yields than n-hexane and petroleum ether. Studies with solvent extraction assisted by shaking at room temperature showed that HIP extracted slightly more oil from both soybeans (HIP – 13.43%, CM – 13.08%) and *N. oculata* (HIP 35.19%, CM – 32.92%). Although there was no statistical difference ($P \leq 0.05$) between these values, the similar or slightly higher yields, and the better safety associated with HIP make it an ideal alternative to CM and other conventional solvents.

ii. PUASE with HIP is several times more efficient than conventional solvent extraction methods. For example, PUASE at resonance amplitude of 87 µm and effective treatment time of 180 min gave a yield of 19.92% for soybean oil. This is an increase of almost 200%

compared to the yield from untreated solvent extraction (9.62%), and almost 50% more than that obtained from solvent extraction assisted by shaking at room temperature for the same time period (13.43%). It must be noted that the maximum oil content of soybeans is approximately 21%; hence PUASE was able to extract almost 95% of this oil content. A similar high efficiency was obtained for *N. oculata* as well: PUASE at resonance amplitude of 87 µm and treatment time of 120 min resulted in a remarkably high oil yield of 69.53%. Besides being the highest reported value of total lipid yield from *N. oculata*, this value is almost 2.5 times the yield obtained from untreated solvent extraction (28.11%) and almost double of that obtained from solvent extraction assisted by shaking at room temperature (35.19%).

iii. Although both resonance amplitude and effective treatment time had statistically significant effects on the oil yield, the relationship between each parameter and the yield was not directly proportionate. Yield values dropped dramatically at higher treatment times and/or resonance amplitudes. This drop could be due to the rapid solvent evaporation caused by increased cavitational heating at high amplitudes. Also, at higher treatment times, a substantial amount of solvent had already been lost due to evaporation and there wasn't enough of it left to extract oil from the disrupted cells. So, it was found that an ideal combination of intermediate resonance amplitude and treatment time was necessary to reduce solvent evaporation and produce high yields.

iv. Fatty acid profiles of oils extracted by PUASE (with HIP) were compared to those extracted by conventional methods such as Soxhlet extraction (with n-hexane) and solvent extraction assisted by shaking at room temperature. For soybean oil, all three methods produced oils of very similar fatty acids profiles. For *N. oculata*, oil extracted by PUASE was found to be superior in terms of PUFAs.

These findings clearly prove that PUASE has enormous potential as a novel method of oil extraction, especially from commercially important sources like oilseeds and microalgae. More research on the effects of other parameters affecting the efficiency of PUASE, and the development of more evaporation-proof reactor systems could help make this technique more economically feasible for industrial applications.