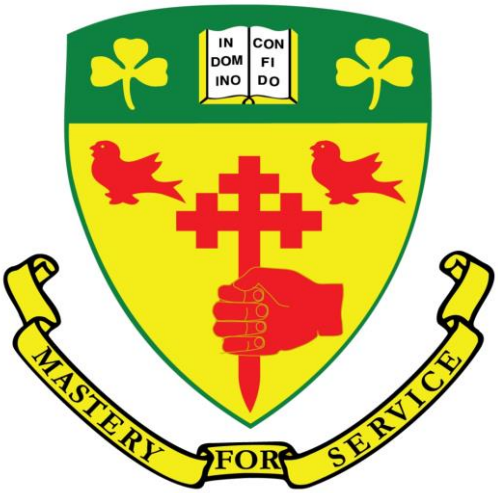
Rapid and minimally invasive lipid analysis of Pea seeds using HRMAS-NMR



Ashutosh Kushwaha

Degree of Master of Science

Department of Bioresource Engineering

McGill University

Montreal, Quebec, Canada

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Master of Science

Abstract

Demand for alternative lipid resources, such as vegetable oil, has been ever-increasing. To fulfil this increase in popularity, alternative oilseed crops are under investigation. This research investigates the development of field pea (*Pisum sativum*) as an alternative oilseed crop in Western Canada, as it is a major producer of field pea because of its climate. The objective was to adapt a minimally invasive and rapid methodology for lipid analysis in dried seeds of pea to facilitate a breeding program. This technique enabled post-analysed seeds to germinate as only a tiny mass of tissue is required (7 mg) for lipid analysis. The HRMAS (high-resolution magic angle spin) NMR spectroscopy was used to quantify lipid content in genetically modified and wild accession pea seeds. The lipid content in wild-type ranged from 2.37 % to 5.89 %. The lipid content of the germinated seed was significantly correlated with the 100 seed mass (r= 0.56) and stem diameter (r= 0.85). This newly adapted methodology will be used in the selection of individuals in the next phase of the breeding program.

Résumé

La demande pour des ressources lipidiques alternatives, comme l'huile végétale, connaît une augmentation constante. Afin de répondre à cette popularité accrue, des cultures oléagineuses alternatives sont à l'étude. Cette recherche porte sur le développement du pois fourrager (Pisum sativum) comme plante oléagineuse alternative dans l'Ouest canadien, cette région étant un important producteur de pois fourragers en raison de son climat. L'objectif était d'adapter une méthodologie rapide et non invasive d'analyse des lipides dans les pois séchés pour faciliter le programme de sélection d'individus. Cette technique peu intrusive n'a pas empêché la germination des graines analysées à cause de la masse de tissue requise (7 mg) pour l'analyse des lipides. La spectroscopie haute résolution par RMN et RAM a été utilisée pour analyser le contenu lipidique des graines de pois génétiquement modifiés et de pois sauvages. La teneur en lipides des pois sauvages allait de 2,37 % à 5,89 %. Le contenu lipidique des graines germées était significativement corrélé à sa masse de 100 graines (r= 0.56) et le diamètre de la tige (r= 0.85). La méthodologie développée sera utilisée pour la sélection d'individu lors de la prochaine phase du programme de sélection.

Acknowledgements

I would like to acknowledge the valuable advice and guidance of my supervisor Dr. Mark Lefsrud and of my committee member Dr. Marie-Josée Dumont, without whose dedication, knowledge and assistance I would not have finished this degree. I would also like to thank Dr. Valerio Hoyos-Villegas and Dr. Jaswinder Singh from the Plant Science Department.

I would like to thank Dr. Andrée Gravel for guiding and helping me in conducting the experiments. I would like to thank Harbin Seed Farm for their generous financial supports, in-kind support from Lefsrud Seed & Processor, and NSERC for the CRD Grants for this project. I want to extent a large thank you to Dr. Michael Ngadi for granting us the permission to use their laboratories and equipment. I would like to thank Mr. Michael Bleho and Mr. Marc Samoisette for accessing farm and equipment.

I would like mention Ehsan Khodapanahi, Dr. Rajvinder Kaur and Philip Wiredu Addo whose data has contributed to the research. I would like to thank Chelsea Scheske, Natalie Wu and Intisar Syed Mahmood and all of my lab mates. Special thanks to Dr. Sarah MacPherson for helping in editing and Princy Agnihotri for helping in analytical troubleshooting.

Finally, I would like to thank my parents and my brother, their combined efforts have made all this possible for me.

Authorship and manuscript

The contributions of the authors are:

1. Reviewing the literature, executing laboratory experiments, performing calculations, data analysis and writing of manuscripts (student).
2. Supervising the research and reviewing of manuscripts (supervisor)
3. Advising throughout the research and reviewing of manuscripts (advisor)

This thesis is written in three manuscript-based format papers. The authorship of preparing papers is as follow: A. Kushwaha; M. Lefsrud; M. J. Dumont.

Table of Contents

List of figures

List of tables

List of acronyms

μ_0	Permeability of free space
B	Effective magnetic field
CAGR	Compound annual growth rate
DNA	Deoxyribonucleic acid
F	Filial generation
FAO	Food and agricultural organisation
FT-IR	Fourier transform infrared
H	Hydrogen
H_0	External magnetic field
HRMAS	High resolution magic angle spin
Kel-F	Polychlorotrifluoroethylene
LCT	Low chain triglycerides
M	Magnetic dipole moment
MCT	Medium chain triglycerides
MIR	Mid infrared
Mt	Metric tonne
NIR	Near infrared
NMR	Nuclear magnetic resonance
P	Parental generation
PC	Phosphatidylcholine
PLS	Partial least squares
PRESS	Predicted residual error sum of squares
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acids
w/v	Weight/volume
ΔB	Magnetic field gradient
χ	Susceptibility factor

Chapter 1: Literature Review

1.1 Introduction

Plants produce different components from which humans can derive energy in the form of carbohydrates, lipids, and proteins. A variety of compounds can be assigned to lipids, such as oils and fats. Lipids are glycerides that have a glycerol backbone and three fatty acids. Fatty acids are hydrocarbon chains that can be saturated and unsaturated. Oils primarily constitute saturated fatty acids; these are in liquid form at room temperature. Fats consist of saturated fatty acids that are solid at room temperature due to a chain-like structure allowing them to stack well at room temperature (Akoh, 2017). The plant-based sources of these lipids can be legumes, grains, fruit, and vegetable oil extracted from seeds and other parts of the plant. Some important plants that are grown for lipids include soybean (*Glycine max*), canola (*Brassica napus* and *Brassica oleracea*), and olives (the fruit of *Olea europaea*) ((Tarranum, Chauhan, Kumar, & Chauhan, 2015). Industries utilise these compounds to produce edible as well as non-edible products. Most of the oils and fats produced are used in the food industry, followed by the oleochemical industry and animal feed.

The primary composition of pea (*Pisum sativum*) seeds consists of starch and protein (Munro & Small, 1997; Shand, Ya, Pietrasik, & Wanasundara, 2007; Sosulski & McCurdy, 1987). There has been research that assessed the lipid content in peas and back the idea of field pea as a lipid producing crop (Bastianelli, Grosjean, Peyronnet, Duparque, & Regnier, 1998; Letzelter, Wilson, Jones, & Sinnaeve, 1995). This thesis will review the value and application of lipids in various industries, methods of oil quantification, development of a spectroscopic method for oil quantification in peas for rapid analysis, and conventional breeding in peas to aid in alternative oilseed development.

Research objectives

The research done in this thesis is aimed to aid in the development of field pea as an alternative oilseed crop. The objectives here are;

1. To develop a methodology for determination of total lipid content in pea seeds using HRMAS-NMR spectroscopy.
2. To determine the total lipid content in wild type and genetically modified varieties.
3. To initialize the breeding program using classical interbreeding.

1.2 Lipids

Lipid is a term used for both fat and oil. In the field of biophysical chemistry, the terms are used interchangeably. Lipids consist of macromolecules made of polar compounds such as phospholipids, fatty acids, sphingolipids, and nonpolar compounds such as sterols and acylglycerols (Frankel, 2014; F. D. Gunstone & Norris, 2013).

Fatty acids

Fats and edible oils are primarily esterified glycerol and fatty acids (Frankel, 2014). The fatty acids are the major components of lipids, and they have a carboxylic acid attached as a functional group on one end and aliphatic chains on the other. There are 14-24 carbon atoms in most fatty acids. Naturally obtained fatty acids go through the biological process of fatty acid elongation, in which carbon atoms are added, making an even number of carbon atoms. Some fats have short-chain fatty acids, usually found in dairy fats and tropical oils, which have <14 carbon atoms (Damodaran, Parkin, & Fennema, 2007). Fats and oils are a mixture of unsaturated and saturated fatty acids (F. D. Gunstone & Norris, 2013; Haas, 2005). Unsaturation is the presence of a double bond in a carbon chain of a molecule, as seen in Figure 1.1. A monounsaturated fat consists of one double bond, and polyunsaturated has more than one double bond.

Phospholipids

The phospholipids, also known as phosphoglycerides, are modified triacylglycerol with a phosphate group attached and two fatty acids forming diacylglycerol. Phospholipids are present in the outermost layer of the cell (plasma membrane). Plasma membranes have two essential phospholipids, phosphatidylserine (PS) and phosphatidylcholine (PC) (Damodaran et al., 2007). The property of surface activity in phospholipids can be attributed to its polar nature. The phospholipid surface activity implies that modification of physical properties of lipids is possible by modifying the crystallization behaviour of lipids and by emulsification action (Fahy et al., 2005).

Sphingolipids

Lipids that commonly constitute a sphingosine base are known as sphingolipids. Sphingosine is a structure with an unsaturated hydrocarbon chain and 18-carbon amino alcohol, and these are naturally occurring base parts of sphingolipids (Pruett et al., 2008). Sphingomyelin, gangliosides, cerebrosides, and ceramides are some common examples of

sphingolipids. These lipids are usually related to cell membranes, such as in nervous tissue. In food lipids, they do not account for a major portion (Crivelli et al., 2020).

Sterols

Sterols are nonpolar lipids and are derived from steroids. They have one five-carbon ring attached to an aliphatic chain on one side and three six-carbon rings on the other side. A hydroxyl group is attached to the third carbon of the terminal six-carbon ring(Damodaran et al., 2007). Sterols can be found in both animals and plants. Phytosterols are plant sterols, where stigmasterol and sitosterols are the majority major sterols in plants (Aboobucker & Suza, 2019). Cholesterol dominates the sterols present in animals while found in low quantities in plants (Shahzad et al., 2017). There is an increased risk of cardiovascular disease associated with high blood cholesterol (Control & Prevention, 2012). Phytosterols have been observed to reduce cholesterol synthesis and absorption and are added to food products to maintain blood cholesterol levels (Jesch & Carr, 2017).

Acyglycerols

Most fatty acids present in animals and plants are in esterified form (Muanruksa & Kaewkannetra, 2020). Free fatty acids comprise a minor portion because of their cytotoxic nature that disrupts the cell membrane's organization in living tissues. Esterification with glycol decreases surface activity and reduces cytotoxicity ((Petropoulos et al., 2019). Acylglycerols can be further categorized into monoacylglycerols, diacylglycerols, and triacylglycerols. Figure 1.1 shows an example of triacylglycerol (also known as triglyceride or triacylglyceride) with α-linolenic acid at the bottom, oleic acid in the middle, palmitic acid on the top part, and a glycerol unit as the backbone (Litchfield, 2012). Mono- and diglycerides can be incorporated as food additives; triglycerides are generally present in food products in significant amounts (Msagati, 2013; Srivastava & Prasad, 2000).

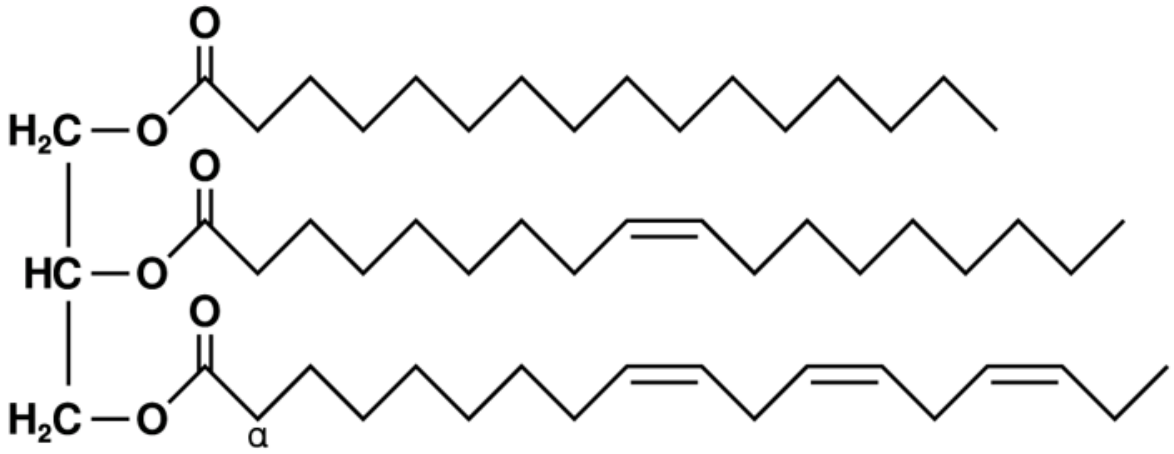


Figure 1.1. Structure of an unsaturated fat triglyceride. The glycerol unit is present on the left, α-linolenic acid at the bottom, oleic acid in the middle, and palmitic acid on the top part.

Waxes

Wax can be chemically defined as an esterified compound with long-chain alcohol and a long chain acid. Waxes can be categorized based on their origins, such as carnauba wax (plant), petroleum wax (mineral), and beeswax (animal) (Tinto, Elufioye, & Roach, 2017). Waxes are hydrophobic and inhibit water loss, and they are present on the outer layers and surfaces of animal and plant tissues. They are used in slowing transpiration losses during postharvest storage of fruits (Damodaran et al., 2007). Waxes used in foods and industries are usually a combination of chemicals, including sterols, aldehydes, ketones, alcohols, esters, and wax esters (Mandua, Barrera-Arellano, Santana, & Fernandes, 2020).

1.3 Lipid value

Lipids are naturally found in vegetable oil, dairy products (butter, whole milk, cream, cheese), and foods such as corn, nut, avocado, and olives. Vegetable oil is mainly used for human consumption (80%), followed by oleochemical industry (14%) and animal feed (6%) (F. D. Gunstone & Norris, 2013).

Lipids act as carriers of minor compounds like phytosterols and vitamins (Smith & Charter, 2011). They can add flavour and texture to food products (Brown, 2018) and are a major source of energy in an average human diet, accounting for 25-30% of total calories (Jones, 1974). Lipids are sources of essential fatty acids that are not bio-synthesized by the human body (Rosdahl & Kowalski, 2008). They act as dissolving medium and carriers for vitamin A, D, E, and K and phytosterols (Smith & Charter, 2011). Lipids are involved in living

organisms' cells, acting as energy storage, contributing to cell structure and other biological processes (Shyu, Wong, Crasta, & Thibault, 2018). They are essential constituents of the plasma membrane and cellular components. Lipids can perform signalling or structural roles, for example, stabilization of different membrane curvatures (Kusumi et al., 2012). Non-edible vegetable oil is used in the oleochemical industry to produce consumer products; surfactants, biofuels, cosmetics, and lubricants (Hayes, 2021).

1.3.1 Food and medicinal applications

The nutritional quality of the foods and the caloric value is determined by analysing the cholesterol, unsaturated and saturated fatty acid content. In testing food products, lipid content quantification is a mandatory practice (Valdés, Beltrán, Mellinas, Jiménez, & Garrigós, 2018). The lipid degradation caused due to frying and oxidative instability can be checked against the PUFA nutritional value, which is determined by the degree of unsaturation of the fatty acids (Johnson, White, & Galloway, 2015).

The medicinal applications of lipids in drug delivery are primarily because of the solubility properties, lipid-based drug delivery system can be used for poorly water-soluble drugs (Müllertz, Ogbonna, Ren, & Rades, 2010). The medium and low chain triglycerides (MCT and LCT) obtained from oil from crops like coconut (*Cocos nucifera*), soybean, and safflower (*Carthamus tinctorius*) are used in the processing of insoluble drugs and oral lipid-based formulations that use oleic acid (Chen, Qi, & Liu, 2008).

1.3.2 Oleochemical industry

Oleochemical industries use fats and oils for manufacturing soaps, esters, amines, glycerols, and alcohols: personal care products, cosmetics, surfactants, lubricants, and biofuels (Hayes, 2021).

Surface-active molecules that reduce the surface tension of liquids, lower interfacial tension, and permit easier spreading, are known as surfactants (Goodwin, 2009). This property of surfactants arises because one end has hydrophilic groups (polar heads), and the other has a hydrophobic group that attracts lipids (Baran, Chivot, Shalita, Lewis, & Wechsler, 2005). Surfactants are made from petroleum raw materials (Kjellin & Johansson, 2010). Petroleum products have negative environmental impacts, have increased in price, and the non-compliance to strict legislative rules set under sustainable usage of resources have made researchers look for alternative organic approaches. Bio-surfactants show promise over petroleum-based products as they are biodegradable and have lower toxicity (Patel et al., 2015).

The physiochemical properties and bio-activity of lipids are the two characteristics of lipids that add value for cosmetic formulations. The bioactive components in lipids aid in improving skin health because of their anti-inflammatory and antioxidant properties. These components can be phytosterols, tocopherols, and essential fatty acids. The physicochemical properties usually account for the consistency in the products, and lipids can function as emulsifiers, moisturizers, or texturizers (Duprat-de-Paule, Guilbot, Roso, Cambos, & Pierre, 2018). Vitamin F (alpha-linolenic acid and linoleic acid) and arachidonic acid are important for healthy skin, and their deficiency can cause skin-related problems (T.-H. Huang, Wang, Yang, Chou, & Fang, 2018).

The physicochemical properties are responsible for imparting smoothness in movements by lowering the friction between surfaces, and these lipids are known as lubricants (Walker & Wood, 2010). The lubricants are majorly used in hydraulic fluids or automotive engines. The lubricant can be a liquid, solid, gas or grease (Almasi, Ghobadian, Najafi, & Soufi, 2021). Lubrication reduces wear and tear in moving mechanical parts by reducing friction and preventing corrosion by acting as a protective layer (Yunus, Rasheed, & Zulkifli, 2020).Vegetable oil can be used in lubrication, directly in liquid form or grease, and additives (Erhan, Sharma, Liu, & Adhvaryu, 2008; Liao, 2009). The effectiveness of bio-based lubricants falls short compared to mineral oils (Erhan et al., 2008). However, there have been efforts to modify oilseed varieties to achieve comparable performance, and partial success has been observed in oilseed varieties with high oleic oil content (3 to 6 times oxidative stability) (Schmidt, Dietrich, & Cahoon, 2005).

Animal fat and vegetable oil have been used as alternative biodiesel resources (usually methyl esters derived from oils and fats) and bioethanol. Biodiesel is defined as the mono-alkyl esters of oils and fats from animals and vegetables. Biodiesel has a similar combustion property as petroleum diesel as it constitutes regulated pollutants (Demirbas, 2008). There are benefits to using biodiesels such as higher biodegradability and cetane number (Gülüm, Yesilyurt, & Bilgin, 2019), low sulphur and aromatic content, renewability, and portability (Knothe, Krahl, & Van Gerpen, 2015). The significant attribute is that they do not contribute significantly to the total atmospheric carbon dioxide. The carbon dioxide gas released from fossil fuels is carbon dioxide trapped millions of years ago, while carbon dioxide released from biofuels comes from carbon dioxide taken up by the plant over the plant's lifespan (Hanaki & Portugal-Pereira, 2018). This carbon is released as carbon dioxide during the combustion of plant material and absorbed by new plants completing the cycle (Yi et al., 2018).

1.4 Lipid Analysis

The composition of fatty acids is used to characterize the lipids (F. D. Gunstone & Norris, 2013). The method for determining lipid composition depends on the analysis and form of the material. Lipid molecules present in bio-materials experience electrostatic forces, van der Waals forces, hydrogen, and covalent bonding (Vance & Vance, 2008). These interactions lead to the formation of an intertwined network as present in lipoproteins. Various methods have been developed over the years in accordance with the research objectives (Khodapanahi, Lefsrud, Orsat, Singh, & Warkentin, 2012). There are two approaches for lipid analysis; one is the extraction method, and the other is the non-extraction method. In the extraction method, lipids are detached from the cells using chemical reagents or mechanical forces. Various methods are developed for performing extraction at an industrial (large scale) scale or laboratory scale (Rawat, Kumar, Mutanda, & Bux, 2013). The non-extraction methods can be spectroscopic methods or density measurement, ultrasonic, and X-ray absorption methods (Akoh, 2017). The extraction methods use a large amount of sample, and the sample cannot be reused, which is why they are also known as destructive techniques. Non-extraction methods do not destroy the sample, and the sample can be used further as desired.

1.4.1 Non-destructive techniques for lipid assessment of seeds

Spectroscopy is the interaction of electromagnetic radiation with matter. The interaction can result because of absorption, transmission, or reflection of electromagnetic radiation in the electromagnetic spectrum (Figure 1.2). This interaction produces a spectrum subjected to qualitative and quantitative analysis (M. Huang, Wang, Zhu, Qin, & Huang, 2015). There are various spectroscopic techniques such as infrared spectroscopy, Raman spectroscopy, Fourier transform infrared (FT-IR), near-infrared (NIR), and mid-infrared (MIR). The infrared spectroscopies (MIR and NIR) exploit the information obtained from molecular vibrations and overtones. The FT-IR is an information-rich analytical technique that uses the mathematical function Fourier transform, which provides a greater amount of chemical information about the scanned sample (Lohumi, Lee, Lee, & Cho, 2015). Another spectroscopic technique is nuclear magnetic resonance (NMR) spectroscopy, and this technique can be classified based on the sample matrix; liquid, solid and semi-solid. NMR spectroscopy works on the interaction between the nuclei's spin with the change in the external magnetic field. The sample with a semi-solid state is analysed using high-resolution magic angle spin (HR-MAS) NMR. Fluorescence and Raman spectroscopy have been used successfully for the qualitative authentication of agricultural commodities. Raman

spectroscopy can provide the information required to identify matrices of samples based on the model compounds, for example, proteins, carbohydrates, and lipids, and it is sensitive to minor components (Seo et al., 2016).

Hyperspectral imaging and machine vision are emerging analytical tools and can have a variety of applications (Rahman & Cho, 2016). Machine vision, known as computer image processing or computer vision, is a technique based on artificial intelligence that simulates human vision (M. Huang et al., 2015). Hyperspectral imaging is a tool for qualitative and authentication analysis of food and agricultural products. The technique acquires spatial and spectral information from the sample (Wu & Sun, 2013). Analysis using machine vision is quick, consistent, and non-destructive. The technique has been proven to be robust and very effective for quality assessment of agricultural products, especially seeds (Hornberg, 2006).

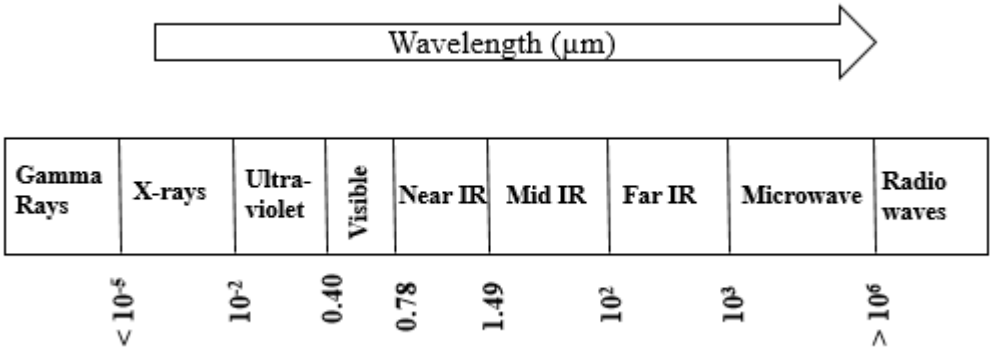


Figure 1.2. The electromagnetic spectrum. This spectrum covers a continuous range of wavelengths, from low-energy radio waves to gamma (γ) rays at the high-energy end.

1.4.2. Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance, a branch of spectroscopy, explores the use of the magnetic properties of an atomic nucleus (Kruk et al., 2017). It has applications in a wide range of disciplines such as chemical and petroleum industries, agriculture, food industry, pharmaceutical, and disease control sectors. In agriculture and the food industry, NMR is mostly used for quantitative and qualitative analyses, such as structure determination, chemical composition, product purity, and adulterations, kinetic reaction monitoring, in situ tests of ripening, etc. (Corsaro et al., 2016; Laghi, Picone, & Capozzi, 2014). NMR can compare the different profiles of genetically modified foods to their organic foods (Sobolev et al., 2010). Since agricultural commodities such as seeds have a heterogeneous matrix, new NMR techniques are used to analyse these samples. Developed in the mid-1990s, this technique is known as high-resolution magic angle spin (HR-MAS) (Wong & Lucas-Torres, 2018).

There are two highly successful NMR techniques and the first is liquid state NMR which produces a high-resolution spectrum, and the second is solid-state NMR. HR-MAS selects the primary characteristic from the two techniques to produce a spectrum for a semi-solid sample by averaging the residual bipolar coupling and magnetic susceptibility characteristic attributes of heterogeneous samples. The spectrum obtained is comparable to the liquid state NMR spectrum (Wong & Lucas-Torres, 2018). HR-MAS NMR has been used for the determination of the chemical composition of semi-solid samples such as wheat (Maria Antonietta Brescia, Di Martino, et al., 2002), bread and flour (Maria A Brescia, Sgaramella, Ghelli, & Sacco, 2003), and meat (Maria Antonietta Brescia, Jambrenghi, et al., 2002). An extensive range of biomedical applications, including metabolic profiling of whole cells and tissues (W. Li, 2006; Lindon, Beckonert, Holmes, & Nicholson, 2009), use the HR-MAS technique. Lipid composition and deposition during grain filling in intact barley (*Hordeum vulgare*) mutant grains was studied by ^{1}H HR-MAS NMR (Seefeldt, Larsen, Viereck, Petersen, & Engelsen, 2011).

In NMR, the effective magnetic field B induced in a sample can be expressed as:

$$B = \mu_0 H_0 + M \quad (1)$$

where H0 is the external magnetic field, μ0 is the permeability of free space ($4\pi \times 10{-}7$ H A−1), and M is the sample magnetization (or magnetic dipole moment) per unit volume. If the magnetic susceptibility of the sample is isotropic, then M can be defined as χB_0, where χ is a scalar component of the susceptibility, and $B_0 = \mu_0 H_0$ is the magnetic field induction. Thus, B can be defined as:

$$B = \mu_0 H_0(1 + \chi) = \mu H_0 \quad (2)$$

where $\mu = \mu_0(1 + \chi)$ is the permeability of the sample. The above equations, the effective field B experienced in a sample depends directly on the susceptibility factor χ, which arises from the local dipole moment M. For example, if a sample is immersed in a homogeneous liquid, the susceptibility of the liquid medium induces an additional homogeneous magnetic field in the sample that results in a shift of the sample NMR signals. Consider the simple case of lipids in muscle tissues, consisting of two distinct lipid compartments: one intra-myocellular and one extra-myocellular. As a result, the related spectrum contains two distinct ^{1}H NMR signals with a chemical shift difference of about 0.2 ppm, attributed to the different susceptibility between the two lipid compartments (Schick et al., 1993). However, metabolites in tissues are usually merged in complex heterogeneous media with multiple different cellular boundaries, each of which has a different susceptibility. As a result, a magnetic field gradient ΔB is induced on the metabolites, resulting in a chemical shift gradient and broad ^{1}H NMR signals. With HR-MAS

spectroscopy, samples spun at the "magic angle" (54.7°) to the static magnetic field remove unwanted line broadening effects.

The HR-MAS NMR instrument can be seen in Figure 1.3a) is the HR-MAS NMR machine at McGill University Health Centre situated in Montreal and Figure 1.3b) shows the zirconia rotor from Bruker in which the sample is placed, and then the rotor goes into the probe surrounded by the cylindrical superconducting magnet that is being cooled by liquid nitrogen. There is another form of disposable insert available that can be used to hold the sample and further inserted into the rotor. Just a tiny part of the seed is required (7 mg in case of pea seeds, the volume of the insert is about 25 µl) to make a sample and quantify. This tiny sample size causes minimal intrusion in the pea seed, and lipid analysis can be performed.

1.3a) 1.3b)

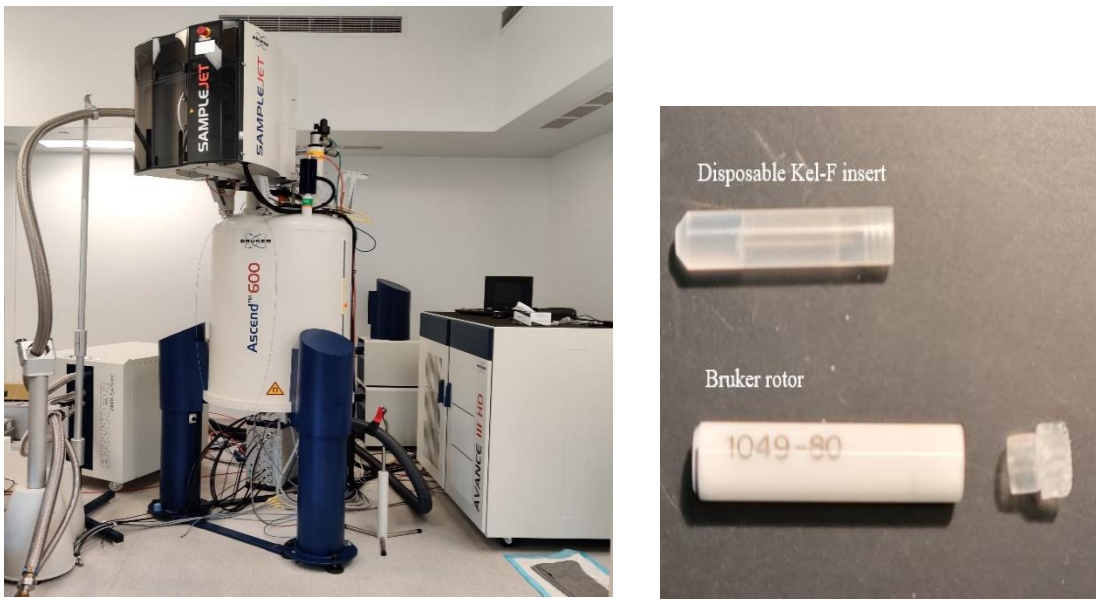


Figure 1.3. NMR equipment at MUHC Vendome. (a) HRMAS-NMR machine. (b) Standard Bruker disposable Kel-F insert and Bruker rotor.

1.5 Natural sources of lipid

The major crops cultivated for extracting oil are soybean, corn (*Zea mays*), mustard (*Brassica nigra*), cotton (*Gossypium*), olives, coconut, palm oil (*Elaeis guineensis*), safflower, sunflower (*Helianthus anuus*), rapeseed (*Brassica napus*) and peanuts (*Arachis hypogaea*). The oil content can range up to 40% in canola (M. Sarwar, Ahmad, Siddiqui, Rajput, & Tofique, 2003; M. F. Sarwar, Sarwar, Sarwar, Qadri, & Moghal, 2013), and other major oilseed crops

oil content can be seen in Table 1.1. The vegetable oil commodities can be classified into three groups based on kind of crop (F. Gunstone, 2011); The oil extracted while processing of crops like corn and cotton as a side product, there is oil obtained from trees such as olive and coconut, and vegetable oil is extracted from annual crops such as sunflower and canola.

1.5.1 Trends in oil production

According to a report from FAO, the world production of oils and fats (includes oils and fats of vegetable, animal, and marine origin) is approximately 203.3 million tonnes (Food and Agricultural Organisation of United Nations, 2014), where 75% of the oil and fats produced are derived from plants (Keneni & Marchetti, 2017). The global oilseed farming market is expected to decline from $366.1 billion in 2019 to $365.8 billion in 2020 at a compound annual growth rate (CAGR) of -0.1%. The decline is mainly due to economic slowdown across countries owing to the COVID-19 outbreak and the measures to contain it. The market is then expected to recover and grow at a CAGR of 7% from 2021 and reach $447.6 billion in 2023 (The Business Research Company, 2020). Growing demand for vegetable oil and fat is predicted almost everywhere in the world. The increase would be mainly toward edible uses in East Asia and Latin America and biofuel in Europe and North America.

Table 1.1 Major oilseed crops grown around the world and percentage of oil content present (Sharma, Gupta, & Mondal, 2012).

Oilseed crop	**Oil content (%)**
Canola	43-45
Cotton	15-20
Peanut	43-55
Olive	10-25
Palm	17-20
Rapeseed	38-39
Soybean	18-23
Sunflower	35-40
Safflower	28-30

There are other plants such as pennycress (*Thlaspi arvense*) and Lesquerella (*Lesquerella fendleri)* that have potential as an oilseed crop to support biodiesel development (Farm energy, 2019). At bio-resource engineering department of McGill University has been researching field pea as a potential oilseed crop. The lipid content of 151 wild accessions was evaluated, reported to have a lipid content between 0.9% - 5% (Khodapanahi et al., 2012). The pea seed samples reported a fatty acid profile of unsaturated fatty acid profile that primarily includes linoleic, oleic, and linolenic acids. The saturated fatty acids present are majorly stearic and palmitic, and a small portion consists of other long-chain fatty acids (Solis, Patel, Orsat, Singh, & Lefsrud, 2013). With this unique fatty acid profile, field pea could be used to create a new oilseed crop. Field pea can be used for human consumption, biofuel production, and other industrial applications and could compete with other traditional oilseeds such as canola and soybean (Cahoon, 2003).

1.6 Field pea

Pea belongs to the *Leguminosae* family (Genus: *Pisum*, subfamily: *Faboideae* tribe: *Fabeae*). There are 18,000 species and 650 genera of legumes, making them the third-largest family of flowering plants (Lewis, Schrire, Mackinder, & Lock, 2005). Pea is one of the oldest domesticated crops in the world (Warkentin et al., 2015). The field pea is a vital cool-season

crop that has an important role in sustainable cropping systems (Duc et al., 2010; Jensen et al., 2012; Nemecek et al., 2008).

The dry pea production in 2019 accounted for 15.6 Mt worldwide (FAOSTAT, 2019). The temperate regions of the world primarily cultivate pea owing to fertile and well-drained soils. Canada produced 4.2 Mt and is the main producer of dry peas, followed by the Russian Federation at 2.36 Mt, China at 1.46 Mt, India at 0.81 Mt, USA at 1.01 Mt, and Australia 0.21 Mt (FAOSTAT, 2019).

Nitrogen is abundantly present in the Earth's atmosphere (78%) and is an essential component in protein and DNA (Ohyama, 2010). The legume crops such as peas and beans use their root nodules to foster bacteria that are able to transform atmospheric nitrogen into inorganic compounds viable to plants, also known as fixed nitrogen (Herridge, Peoples, & Boddey, 2008). There has been a resurgence of sustainable farming practices such as crop rotation and cover crops using legumes (Kaye & Quemada, 2017). The ecological services associated with practices of pea cultivation are significant, including the ability to act as an alternative crop to reduce and break pathogen and pest pressure and the development of interdependent nitrogen fixation (Hayer et al., 2010; MacWilliam, Wismer, & Kulshreshtha, 2014; Nemecek, Kägi, & Blaser, 2007). The crops are dependent on expensive external fertilizers for obtaining nitrogen as they lack any mechanism to use atmospheric nitrogen can benefit by adding pea cultivation in existing crop rotation (Sainju, Lenssen, Allen, Jabro, & Stevens, 2019). Peas can fix nitrogen at rates varying from 31 to 107 (Kg Nitrogen)/ha annually with steady precipitation (Carranca, De Varennes, & Rolston, 1999). There has been research that shows pea, if grown in rotation with wheat, can fix organic nitrogen in the soil and reducing the usage of external fertilizers with the greatest average four-year economic returns (Miller et al., 2015).

1.6.1 Field pea composition

Pea seeds have a remarkable and diverse nutrient profile. The primary components being proteins (15.8–32.1%), starch (18.6 - 54.1%), fibres (5.9–12.7%), oil (0.6–5.5%) and sucrose (1.3–2.1%) (J Burstin, Gallardo, Mir, Varshney, & Duc, 2011; Khodapanahi et al., 2012). There are vitamins, minerals, and micro-nutrients that are tested for their beneficial effects on health, such as saponins, polyphenols, phytic acids (Arnoldi, Zanoni, Lammi, & Boschin, 2015; Dahl, Foster, & Tyler, 2012; Marles, Warkentin, & Bett, 2013; Mitchell, Lawrence, Hartman, & Curran, 2009). Peas are consumed in different forms that allow them entry into human nutrition. Most peas are consumed in the form of green vegetables and dry

seeds used in the cooking of various dishes. They can be consumed as immature pods and fresh seedlings. Protein and starch of high quality are extracted from dry pea seeds, and functional and structural characteristics are assessed for improvement in food ((Brummer, Kaviani, & Tosh, 2015). The dried plants from the pea crops can be used as fodder for ruminant animals (Bastida, González-Ronquillo, Dominguez Vara, Romero-Bernal, & Castelan Ortega, 2011).

The field pea seed's endosperm consists of lipids comprised of fatty acids (Solis et al., 2013). Traditional methods for extraction, quantification, and fatty acid profiling of lipids have been employed in various studies over the years (Caprioli et al., 2016; Khodapanahi et al., 2012; Ryan, Galvin, O'Connor, Maguire, & O'Brien, 2007). The traditional methods determine fatty acids using the following steps; first triacylglycerol hydrolysis, second is the esterification of the liberated fatty acyl groups using methanol or directly by transesterification and the third step is using gas chromatography for separation, identification and quantification of the fatty acids present. The required process is necessary because a minor part of the fatty acids are present as free fatty acids, whereas a major part of the fatty acids is present in the form of amides and esters. The traditional methods to analyse the fatty acid profile of plants result in the exposure of the oils to various chemicals, leading to oxidation of the lipids (Mazurek, Chmiel, & Górecka, 2017). Therefore, a need arises to develop a new method that can replace the traditional methods. With the advancement in spectroscopic methods, chemical composition and structural analysis is possible. In spectroscopic methods, the interaction between electromagnetic radiation and the sample is exploited to obtain data. The data is further processed for qualitative analysis as well as quantification. The modern methods reduce labour, time, and chemical consumption compared to chemical methods. This review focuses primarily on non-destructive techniques that have already been employed and the techniques that can be potentially employed on peas (and other oilseeds) for lipid analysis. The techniques are infrared spectroscopy, nuclear magnetic resonance spectroscopy, Raman spectroscopy, and hyperspectral imaging.

Over the years, various approaches are used to increase crop production and include increased farming area, increasing yield per unit area, reducing stresses, and reducing diseases (Vollmann & Rajcan, 2009). Conventional plant breeding and genetics have played an important role in enhancing oilseed crops (Maheshwari & Kovalchuk, 2014; Murphy, 2014; Seyis, Friedt, & Luhs, 2005). There has been various transgenic modification research recorded about the characteristics of the crops. Research has investigated the modification of fatty acids, enhanced lipid content, and improved biomass yield (F. Gunstone, 2011). Plants have been

modified for pest resistance, herbicide resistance, drought tolerance, abiotic stress resistance, salt, cold tolerance, improved processing ability, and nutrient enrichment (Kausch et al., 2010).

1.7 Conclusion

The ever-growing demand for vegetable oil is directly proportional to the consumption of edible oil. The non-edible applications are beginning to replace petroleum products as climate change awareness has led to stricter regulations for sustainable usage of resources. Field pea has been chosen as an alternative oilseed crop. Peas are having a favourable lipid profile and an environmental benefit through their ability to fix nitrogen. Fulfilling the increasing demand for peas has increased the production and cultivation area of field peas, genetic research and modification, and increased breeding practices. The breeding practices have proven effective in improving nutritional composition and other agronomic factors. The development of an alternative oilseed crop requires reliable analysis methods. A fast, non-destructive method development would be very beneficial to the development of an oilseed field pea. Spectroscopic methods are being used as quick analytical tools for qualitative and quantitative analysis. HRMAS NMR spectroscopic method for quantifying lipids at early stages for breeding or genetic modification results can speed up the process of development of pea as an oilseed crop.

Connecting statement

The HRMAS-NMR spectroscopy can be used as an analytical method for lipid analysis in pea seeds. Method development was done using a statistical calibration model. Lipid analysis was done in wild varieties and genetically modified varieties. There was a germination test done on the same individual seeds analysed with NMR. The correlation between lipid content and other parameters was checked.

Chapter 2: A rapid and minimally invasive method for estimating total lipid content in field pea (*Pisum sativum*) using ^{1}HRMAS-NMR spectroscopy

2.1 Abstract

The field pea (*Pisum sativum*) is being considered as an alternate oilseed crop. Genetic modification and conventional breeding techniques have been employed to improve lipid content in this plant species. However, current chemical extraction methods for quantifying lipid content are time-consuming and require a large sample size. The purpose of the study was to develop a rapid, minimally invasive, and precise spectroscopic method for quantifying lipid content in pea seeds using high-resolution magic angle spinning-nuclear magnetic resonance (HRMAS-NMR). Small sample sizes (7 mg to 8 mg) were cut from whole seeds and used to obtain high-resolution spectra in 7 min. A methodology was developed using calibrated spectroscopic data and PLS regression to form a predictive model. The PLS method identified maximum variation of the predictor variables, which yielded a correlation coefficient of 0.992 between predictor and response variables. Total lipid content determined with HR-MAS NMR was compared to traditional extraction methods with different solvents. This methodology was used to determine total lipid content in a large germplasm collection, with seeds collected from 108 wild pea accessions and genetically modified plants. Total lipid content ranged from 2.37 % to 5.89 %. Correlation coefficients for oil content with 100 seed mass and with stem diameter were 0.56 and 0.85, respectively. The remaining seed fractions were successfully germinated, ascertaining that this method is non–destructive. Proof-of-concept testing with soybean demonstrates its potential for use with other oilseed crops. Findings indicate that HRMAS-NMR is an effective tool for assessing oil content in pea seeds that can propel research and the development of improved crops forward at an unprecedented speed.

2.2 Introduction

Legumes belong to the Fabaceae (or Leguminosae) family, and these cultivated plants' fruit or seeds are often referred to as pulse crops when harvested as mature and dry grain. Legumes are primarily cultivated for human consumption, feed for livestock, and green manure for soil enhancement. Soybeans (*Glycine max*), peanuts (*Arachis hypogaea*), peas, chickpeas (*Cicer arietinum*), and beans (*Phaseolus vulgaris*) are some of the most commonly known legumes. Lipid content is generally high in legumes such as peanuts and soybean, containing

approximately 50 % and 20 % lipid, respectively (Sipeniece et al., 2021). As an important of essential fatty acids, proteins, and calories, *P. sativum*, known as field pea or pea, is one of the most commonly cultivated legumes worldwide and is ranked fourth with respect to world legume production (Daryanto, Wang, & Jacinthe, 2015; Maphosa & Jideani, 2017). Canada is the largest producing country in the cultivation of field pea (dry), with a production of 4.236 million tonnes in 2019 (FAOSTAT, 2019).

Seeds obtained from legumes are generally high in lipid content. Lipids are extracted from the seeds, pods, or other parts of the plant to produce vegetable oil. The demand for edible vegetable oil accounts for approximately 80% of the total vegetable oil produced, whereas 14% of total vegetable oil produced is used in oleochemical industries that require bio-lipids (F. Gunstone, 2011). The ever-increasing demand for bio-lipids provides a motivation to search for new oilseed crops. While field pea is a major source of starch and protein, with an average concentration of 55% and 23%, respectively (Munro & Small, 1997; Sosulski, Hoover, Tyler, Murray, & Arntfield, 1985; Sosulski & McCurdy, 1987), it has the potential to become a source of ω-6 and ω-3 polyunsaturated fatty acids that are beneficial for human health (Ryan et al., 2007). Previous research has shown that lipid content in field pea ranges from 1% to 5% (Khodapanahi et al., 2012). New value can be added to the crop by increasing lipid content in field peas using genetic engineering and traditional breeding techniques.

The field pea seed's endosperm contains lipids that are comprised of fatty acids (Solis et al., 2013). Traditional methods for extraction, quantification, and fatty acid profiling of lipids have been employed in various studies (Caprioli et al., 2016; Khodapanahi et al., 2012; Ryan et al., 2007). These methods for determining fatty acids use the following three time-consuming steps: triacylglycerol hydrolysis, esterification of the liberated fatty acyl groups using methanol or by direct transesterification, and gas chromatography for separation identification and quantification of fatty acids present. This process is necessary because a minority of the fatty acids present are free fatty acids, whereas the majority present in the form of amides and esters. Using traditional methods, the process requires fatty acid standards, and the extracted oil is exposed to various chemicals that lead to lipid oxidation (Mazurek et al., 2017). With the advancement in spectroscopic methods, chemical composition and structural analysis is possible by exploiting the interaction between electromagnetic radiation and the plant material sample to determine lipid content. Data are further subjected to qualitative and quantitative analyses. Using innovative spectroscopic technologies, it may be possible to reduce labour, time, and chemical consumption when compared to traditional methods.

Nuclear magnetic resonance (NMR), a branch of spectroscopy, explores the use of the magnetic properties of an atomic nucleus (J. Li, Vosegaard, & Guo, 2017). It has applications in a wide range of disciplines, including the chemical and petroleum industries, the agricultural and food industry, the pharmaceutical industry, and the disease control sectors. For the agricultural and food industry, NMR is mostly used for quantitative and qualitative analyses, such as structure determination, chemical composition, product purity, and adulterations, kinetic reaction monitoring, in situ tests for ripening, etc. NMR can be used to compare the different profiles of genetically modified foods to their unmodified or heirloom relatives (Sobolev et al., 2010). Since agricultural commodities such as seeds are a heterogeneous matrix, high-resolution magic angle spin (HR-MAS) NMR, developed in the mid-1990s (Wong & Lucas-Torres, 2018), may be used to analyse different seed samples in a minimally invasive manner.

The advantages of using two well established NMR techniques, liquid and solid state NMR, are widely reported (Corsaro et al., 2016; D'Imperio et al., 2007). Samples in a liquid state are homogenous (i.e., biofluids), allowing for non-variable magnetic susceptibility that can produce a high-resolution spectrum. When the sample matrix is heterogeneous (such as a seed or other plant tissue), variable magnetic susceptibility obstructs the signal, causing low resolution and broadening in the spectrum. This broadening can be resolved using solid state NMR, which accounts for line narrowing in solid samples. A semi-solid HR-MAS uses primary qualities from both techniques to produce a spectrum for a semi-solid sample by averaging the residual bipolar coupling and magnetic susceptibility that are characteristic attributes of heterogeneous samples. The spectrum obtained is comparable to the liquid state NMR spectrum (Wong & Lucas-Torres, 2018). HR-MAS NMR has been successfully reported for chemical composition determination of semi-solid samples such as wheat (Maria Antonietta Brescia, Di Martino, et al., 2002), bread and flour (Maria A Brescia et al., 2003), and meat (Maria Antonietta Brescia, Jambrenghi, et al., 2002)..

The aim of this study was to investigate how NMR could be used to study fatty acids in pea, while developing calibration models to predict pea lipid content in this non-traditional oilseed. Using this approach to analyse different wild pea varieties and modified seeds, high resolution spectroscopic information provided a rich and complex data set that was used to develop a low-cost, rapid, and minimally invasive method for lipid profiling and determining lipid content in pea that could be applied to other leguminous crops.

2.3 Materials and methods

Plant material and extraction

Field pea seeds were obtained from three different sources for this study: a 2018 harvest from McGill University's Macdonald Campus (Sainte-Anne-de-Bellevue, QC, Canada), Plant Gene Resources of Canada (Saskatoon, SK, Canada), and a pea collection belonging to the US Department of Agriculture (USDA; Pullman, WA, US). Genetically modified pea samples obtained from Dr. Kaur (McGill University, Plant Science Department) included accessions CN 43016 (P10-4 and P13-6) seeds from plants generated using particle bombardment, in addition to Greenfeast (P276a-5-2 and P276a-4-1) and CN 43016 (P333-4-3, P283a-2-6, P283 2-3), which were generated using agro-transformation (Kaur and Lefsrud, unpublished data). Numbers associated with each accession represent different trials. Pure pea oil was obtained using a Soxhlet extraction unit (VELP Scientifica, SER-148, Usmate Velate (MB) - Italy) accessed at Macdonald campus, McGill University. Briefly, a 5-g ground pea sample of CN 43016 was added to a cellulose thimble. Soxhlet extraction was performed with a petroleum ether solvent with 30 min immersion, 45 min washing, and 15 min recovery at 130 °C. This pure pea oil was used to make samples with known lipid content. For precise measurement of the oil collected, a stock solution (w/v) of pea oil was obtained using deuterated chloroform as a solvent. The solution was pipetted into the inserts used to store samples for HR-MAS NMR analysis. Sample volume varied according to the mass of the pure oil required. Inserts were then left in a fume hood to allow the solvent to vaporize, leaving the oil in the insert for HR-MAS NMR analysis. Soybean seeds were obtained from plants grown in 2015 at the Belcan Agro Centre (Sainte-Marthe, QC, Canada).

HR-MAS NMR

The qualitative and quantitative analysis of lipids in pea oil and pea seed samples were performed using the HR-MAS NMR spectrometer (ADVANCE III HD 600, Bruker Cooperation, Billerica, Massachusetts, US) located at the McGill University Health Centre (Montreal, Canada) and operating at 600.17 MHz for 1H. A 4-mm triple-resonance 1H/31P/13C high-resolution magic-angle-spinning probe with Z-gradient directed along the magic angle axis was used. Small portions of seed (7 mg) were cut from whole seeds (Figure 2.1a) with a scalpel, weighed in the insert, and 15 μL D_2O (Deuterium oxide) were added to hydrate the sample (Figure 2.1b). The insert was placed in a 1.5 microfuge tube and centrifuged at 2000 RPM for 15 s to remove bubbles. The insert was then plugged and screwed to seal the

insert (Figure 2.1b). To produce a reference peak in the spectrum, D2O containing 5 mM trimethylsilylpropanoic acid (TMSP or TSP; Sigma-Aldrich, St. Louis, Missouri, US), which formed 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid or TMSP-d4 (TSP + D2O), was used as an internal standard. This internal standard (15 μL TSP + D2O) was pipetted into the zirconia rotor. The rotor was then centrifuged at 2000 RPM for 15 s. Inserts containing samples were placed in the rotor and spun at 5 kHz at 20°C (room temperature). For pure pea oil, samples with known lipid content were prepared and pipetted into the Bruker KelF rotor insert (Bruker Corporation, Billerica, Massachusetts, US) without adding D2O, and similar steps were followed. Proton NMR spectra were obtained with a 1D pulse sequence and pre-saturated water. For each spectrum acquired, there was a 1-s relaxation delay with a spectral width of 8417.5 Hz, an acquisition time of 0.97 s, and a 90° pulse of 6 μs; each data set comprised 16k data points obtained using 128 scans.

2.1a) 2.1b)

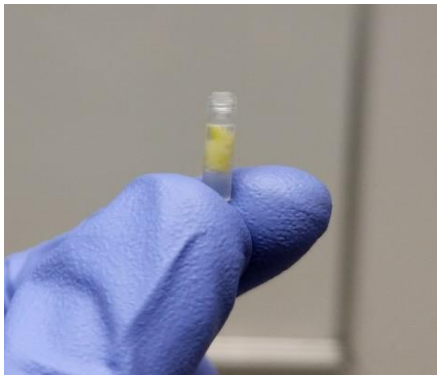


Figure 2.1. Dried pea seeds and in the form of a sample. (a) Representative image of pea (*P. sativum*) seeds used in this study (accession CN 112351); b) Pea seed sample loaded in an insert for HR-MAS NMR analysis.

The 1H nuclear magnetic resonance (NMR) spectrum of the pea seed oil contains 11 distinct signals of variable intensity. The various signals observed in the spectrum are the result of protons in different chemical environments, and the neighbouring protons cause the peak splitting. The area under each signal in the proton NMR spectrum was proportional to the number of hydrogen atoms of each type of fatty acid in the oilseed. The area was obtained by integration of the peaks using Topspin 4.0.3 software (Bruker Corporation, Billerica, Massachusetts, US). A multiple integration function in the software integrated a list of spectra, and an integral value for each signal was obtained for the respective sample.

Total lipid content estimation

Lipid content estimation was performed using a calibration model based on spectra from 11 pea oil samples with known lipid content (Figure 2.2). Each pea oil sample produced 11 peaks that were associated to protons present in different fatty acids. Peaks were integrated to obtain a corresponding integral value and set of calibration data. Based on this data, partial least squares (PLS) regression was used to model and predict the total lipid content with SAS studio 3.8 (SAS Institute Inc., Cary, NC, USA). Out of 11 integral values, 8 integral values were taken as predictor parameters, and mass was taken as the response variable. Using this method, successive linear combinations of predictors known as factors (also known as latent variables, latent vectors, or components) were extracted and cross-validated. The optimal

number of factors was calculated by minimizing the predicted residual error sum of squares (PRESS) and a statistical model comparison described by van der Voet (van der Voet, 1994). After obtaining the calibration model, 108 accessions of pea seed samples with unknown lipid content (Table 2.3) were analysed in triplicate using HR-MAS NMR. The spectrum for each pea seed sample was compared to pure pea oil, and manual phasing and baseline corrections were performed. Peaks associated with fatty acids in each pea seed sample were chosen, and using the multiple integration of NMR spectra function, integral values were obtained. These integral values were used in the statistical model to obtain the lipid mass in each respective sample.

Germination tests

The remaining portions of whole seeds from seven accessions (CN 29579, CN 112351, CN 42819, CN 29600, CN 43016, CN 29526, CN 45760) were concurrently subjected to germination tests. Germination was carried out in sterile Petri dishes (90 mm) lined with Whatman No. 1 filter papers and 10 ml dH20 at room temperature (20 °C). Seeds were considered germinated when hypocotyl and epicotyl systems emerged. For each accession, three seeds were tested.

Statistical analysis

The PLS regression model was used for estimating lipid content of pea seeds, using proc pls procedure in SAS studio 3.8 (SAS Institute Inc., Cary, NC, USA). Analysis of variance (ANOVA) and correlation coefficients were calculated using Microsoft Excel (2016) Redmond, Washington, US. Analysis of variance (ANOVA) was done using paired t-test for comparing data obtained from NMR with solvent extraction methods. P (0.05) value was calculated to determine the significant difference in the data. The correlation was calculated between lipid content with 100 seed mass and with stem diameter.

2.4 Results

Qualitative analysis of pea lipids

To qualitatively analyse lipids, present in pea samples, an HR-MAS NIR spectrum was obtained with pea oil Soxhlet-extracted from *P. sativum* (Figure 2.2). A total of 11 unique signals were observed. These signals were generated due to hydrogen atoms present in different fatty acid molecules in the sample (Table 2.1), and the assignment of these signals has been described previously (Barison et al., 2010; Guillén & Ruiz, 2008; Salinero et al., 2012). Table 2.1 lists observed signals, their chemical shifts, and associated functional groups compared with data from available studies reporting the same signal peaks.

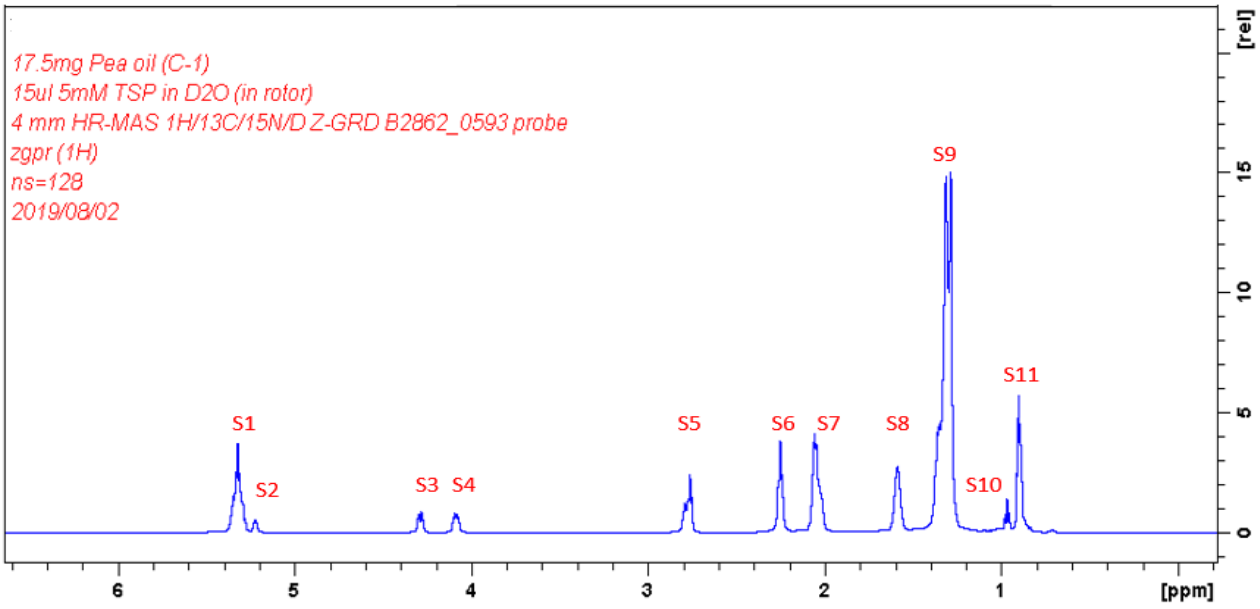


Figure 2.2. ^{1}H HRMAS-NMR spectrum of pea oil extracted from *P. sativum*. The peaks are influenced by unsaturation (S1, S5 and S7) in the vicinity, by glycerol units (S2, S3 and S4), by carbonyl group (S6 and S8) and by presence of methyl protons (S9, S10 and S11).

Table 2.1. Assignment of signals observed in a pea (*P. sativum*) oil spectrum obtained with HR-MAS NMR.

Signal	Functional group	Observations recorded	(Salinero et al., 2012)	(Barison et al., 2010)	(Guillén & Ruiz, 2003)
S1	[-C**H**=C**H**-]	5.44 – 5.259	5.37–5.30	N/A	5.40 – 5.26
S2	[-C**H**OCOR]	5.254 - 5.095	5.27 – 5.24	N/A	5.26 - 5.20
S3	[-C**H$_2$**OCOR]	4.38 – 4.239	4.30 – 4.26	4.27	4.32 - 4.10
S4	[-C**H$_2$**OCOR]	4.176 – 4.024	4.15 – 4.06	4.12	4.32 - 4.10
S5	[=HC-C**H$_2$**-CH=]	2.894 - 2.709	2.81 – 2.74	2.74	2.84 - 2.70
S6	[-OCO-C**H$_2$**-]	2.346 - 2.188	2.33 – 2.28	2.28	2.36 – 2.23
S7	[-C**H$_2$**-CH=CH-]	2.163 - 1.973	2.06 – 1.98	2.02	2.14 - 1.94
S8	[-OCO-CH$_2$-C**H$_2$**-]	1.671 – 1.524	1.64 – 1.57	1.59	1.70 - 1.52
S9	[-(C**H$_2$**) n-]	1.462 - 1.223	1.35–1.23	N/A	1.42 – 1.22
S10	[-C**H$_3$**]	1.007 - 0.942	N/A	0.98	1.03 - 0.93
S11	[-C**H$_3$**]	0.942 – 0.817	0.89 – 0.86	N/A	0.93- 0.83

Signal S1 represents hydrogen (-CH=CH-) present in unsaturated fatty acids and is caused by the olefinic hydrogen atoms in the triacylglycerol groups. This peak slightly overlaps the S2 signal, which is due to the β-hydrogen atom on carbon 2 of the glycerol backbone. The α-hydrogens of the glycerol backbone on carbons 1 and 3 caused signals S3 and S4. Divinyl methylene protons or bis-allylic protons of linoleic and linolenic fatty acids are represented by signal S5. The α- and β- positions of hydrogen atoms along the acyl chain with respect to the carbonyl group are represented by signals S6 and S8, respectively. The allylic hydrogen atoms at the α position represent the only double bond in all unsaturated fatty acids, which are represented by the S7 signal. All methylene protons on saturated carbon atoms present at the γ position or further along the chain with respect to the carboxylic groups in the triglyceride are represented by the S9 signal. The S10 signal represents terminal methyl protons of the linolenic acid, and the S11 signal represents the terminal methyl protons of linoleic, oleic, and saturated fatty acids (Miyake, Yokomizo, & Matsuzaki, 1998; Sacchi, Addeo, & Paolillo, 1997).

With another HR_MAS NMR spectrum obtained with a 7-mg pea seed sample (cut from a whole seed) to confirm the position of peaks and the chemical shift (Figure 2.3). Overlaid spectra show that there is no significant difference in chemical shifts in fatty acids,

indicating that relevant peaks corresponding to fatty acids can be identified in pea seed spectra for further qualitative lipid analysis.

Quantitative analysis

Quantitative lipid analysis of the 7-mg pea seed samples was performed using the integration function in Topspin 4.0.3 software. The integral values of the distinct peaks obtained increased linearly with an increase in oil mass of analysed samples with known lipid content. This linear trend shows sample mass (ranging from 0.035 mg to 17.5 mg) plotted with their respective integral values in Figure 2.4. This generated a predictive model using calibration data to determine the total lipid content in pea seed samples from accessions of wild field peas.

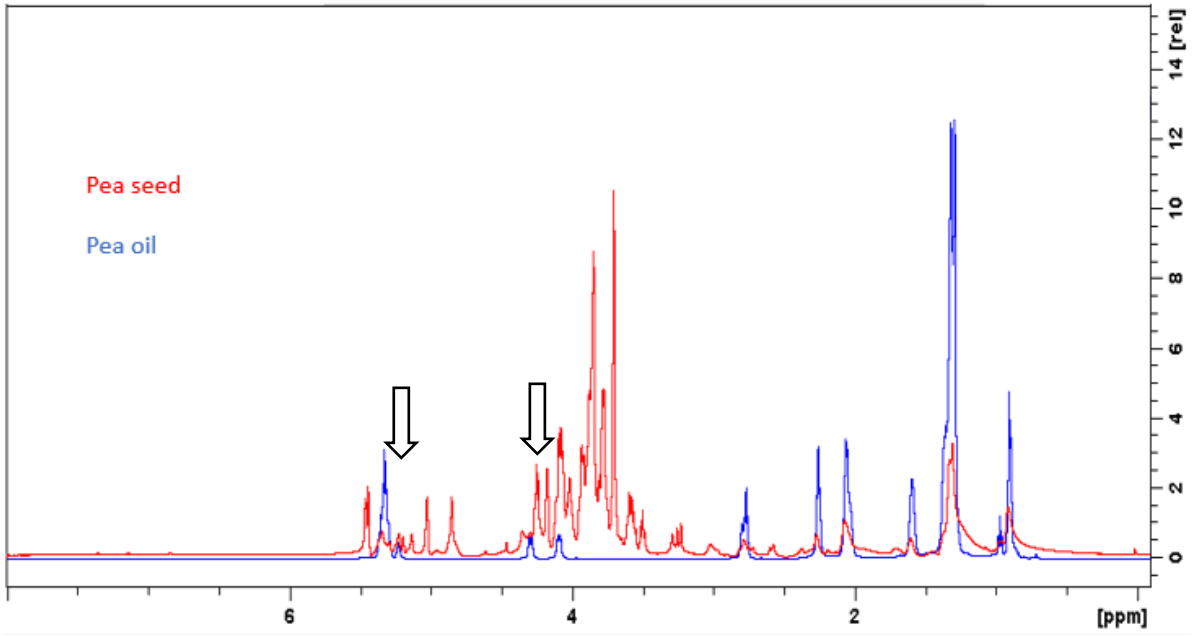


Figure 2.3. Overlapping spectra obtained with Soxhlet-extracted pea oil (blue) and a 7-mg pea seed sample (red) cut from a whole pea seed. The pea oil spectrum is reduced in scale for comparison with the pea seed sample spectrum. Arrows correspond to overlapping signals that were not associated with fatty acids but were interfering with the fatty acid related peaks.

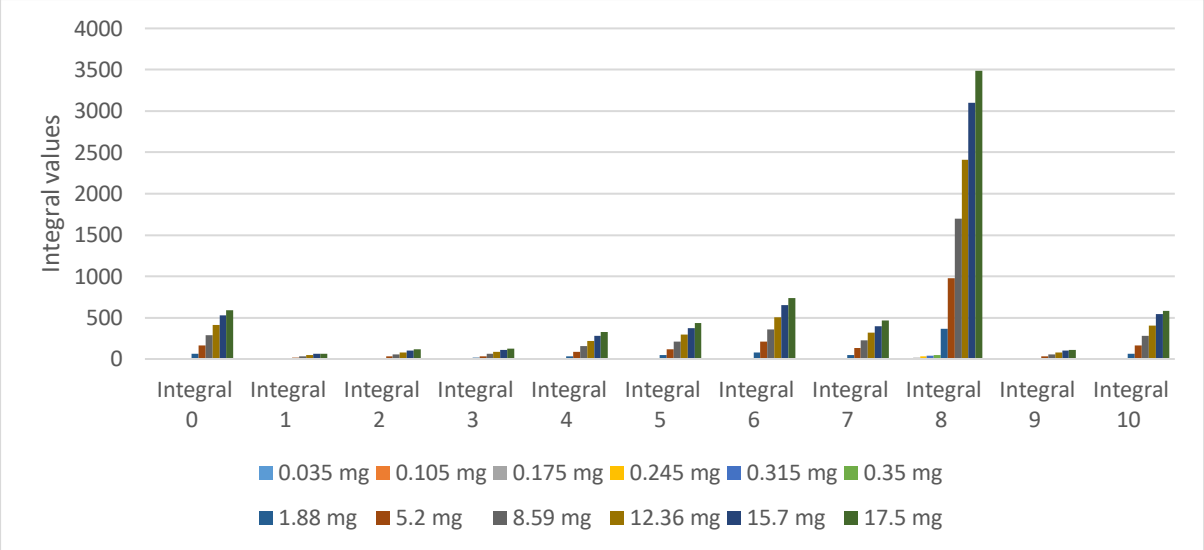


Figure 2.4. Integral values of unique peaks plotted with pea oil samples containing known lipid content (ranging from 0.035 mg to 17.5 mg), as shown in the legend for each series.

A PLS method with principal components regression was used to develop the predictive model, whereby extracted factors explain predictor sample variation. Signals S1, S5, S6, S7, S8, S9, S10, and S11 were integrated, and the integral values were then used as predictors corresponding to lipid (oil) mass present. Signals S2, S3 and S4 were not incorporated into the model as there are other overlapping signals that were not associated with fatty acids (Figure 2.3, arrows), yet they were observed when the pea seed sample spectrum was compared to the spectrum obtained with pea oil. A variation summary indicates that 99.96% of the predictor variation and 98.41% of the response variation are accounted for by one factor. X scores and Y scores correlation can be used to evaluate the quality of the PLS model. Response scores and predictor scores yielded a correlation coefficient of 0.992. The developed model was used to analyse lipid content in X pea seed samples acquired from reported accessions and genetically modified seeds. As a first step, estimated lipid content using this minimally invasive HR-MAS NMR method was compared to lipid content estimated after extraction with traditional methods, including Soxhlet extraction with two different solvents (Table 2.2), reported previously by (Kalia, 2016; Khodapanahi et al., 2012). Analysis of variance using a paired t-test resulted in $p < 0.05$ when both solvent extraction methods were compared with the NMR method (Table 2.2). The correlation coefficient of the NMR method with butanol extraction method was 0.65, and with hexane-isopropanol was 0.37. The total lipid content determined with HRMAS-NMR was higher than lipid content determined with the solvent extraction methods.

Table 2.2. Total lipid content determined for pea seed samples using HRMAS-NMR and two Soxhlet extraction methods with different solvents.

Method	**HRMAS-NMR**	**Soxhlet extraction (butanol)[1]**	**Soxhlet extraction (hexane-isopropanol)[1]**
Sample preparation	Minimally invasive; requiring 7-mg sample cut from whole seed	Destructive; requiring 2-g ground pea seed sample	Destructive; requiring 2-g ground pea seed sample
Accession no./ID	**Lipid content (% ± SD)**	**Lipid content**	**Lipid content**
Pea (CN 112351)	2.7 ± 0.16	1.6	1.4
Pea (CN 42819)	3.4 ± 0.02	2.5	1.7
Pea (CN 43016)	4.0 ± 0.15	3.6	2.2
Pea (CN 29600)	4.1 ± 0.16	2.6	1.7
Pea (CN 45760)	4.1 ± 0.13	3.5	2.1
Pea (CN 29579)	3.4 ± 0.29	3.7	2.6
Pea (CN 29526)	3.5 ± 0.10	3.1	1.3
Greenfeast	4.02 ± 0.43	NR	NR
Soybean	17.5 ± 1.7	13.9	NR
Genetically modified seeds			
CN 43016 (P333-4-3)	4.04 ± 0.31	NR	NR
CN 43016 (P10-4)	4.03 ± 0.53	NR	NR
CN 43016 (P282-a-2-6)	4.59 ± 0.62	NR	NR
CN 43016 (P13-6)	4.68 ± 0.79	NR	NR
CN 43016 (P283-a-2-3)	3.97 ± 0.15	NR	NR
Greenfeast (P276-a-5-2)	4.3 ± 0.13	NR	NR
Greenfeast (P276-a-4-1)	4.95 ± 0.78	NR	NR

1.(Khodapanahi et al., 2012)

This HRMAS-NMR and calibration model methodology was similarly used to evaluate total lipid content in 108 commercial and wild pea accessions (Table 2.3). Total lipid content ranged from 2.37 % to 5.89 %. Whole seed appearance was noted to determine if differences in physical traits correlated to total lipid content; the wrinkled seed surface was found to have higher lipid content than smooth surface seeds. Other physical trait data were scrutinized to identify any correlation between total lipid content and other physical attributes compiled by the USDA (United States Department of Agriculture, 2021). Correlation coefficients for oil content with 100 seed mass (29 accessions) and stem diameter (23 accessions) were 0.56 and 0.85, respectively. The correlation with 100 seed mass is weak, while with stem diameter is considerably higher.

Table 2.3. Total lipid content values recorded for wild accessions using HRMAS-NMR.

Accession no./ID	Lipid content (% ± SD)	Seed surface colour	Seed surface
PI 347518	3.6 ± 0.17	green/white	wrinkled
PI 347490	4.43 ± 0.57	green/white	wrinkled
PI 343257	3.9 ± 0.31	Green/white	wrinkled
PI 288263	4.08 ± 0.34	green/white	wrinkled
PI 341889	4.01 ± 1.11	green/white	wrinkled
PI 285740	3.9 ± 0.23	green/white	wrinkled
PI 358637	3.17 ± 0.23	mixed	wrinkled
PI 285732	3.84 ± 0.04	green/white	wrinkled
PI 280626	4.2 ± 0.08	green/white	wrinkled
PI 411143	4.36 ± 0.64	green/white	wrinkled
PI 512082	3.47 ± 0.05	green/white	wrinkled
PI 365420	5.34 ± 0.62	green/white	wrinkled
PI 601058	4.27 ± 0.18	green/white	wrinkled
PI 471406	3.63 ± 0.10	green/white	wrinkled
PI 600915	3.95 ± 0.27	green/white	wrinkled
PI 600964	4.17 ± 0.37	green/white	wrinkled
PI 595580	4.4 ± 0.05	green/white	wrinkled
PI 413684	4.38 ± 0.34	green/white	wrinkled

PI 206794	3.01 ± 0.35	green/white	wrinkled
PI 197990	2.55 ± 0.07	green	wrinkled
PI 173840	3.77 ± 0.49	green	wrinkled
PI 642175	3.99 ± 0.65	green/white	wrinkled
PI 210627	4.1 ± 0.36	green/white	wrinkled
PI 141966	3.61 ± 0.54	green	wrinkled
PI 210582	3.69 ± 0.57	green/white	wrinkled
PI 210682	3.88 ± 0.43	green/white	wrinkled
PI 244139	3.61 ± 0.12	green/white	wrinkled
PI 210670	3.78 ± 0.27	green/white	wrinkled
PI 210608	3.44 ± 0.29	green/white	wrinkled
PI 210643	4.01 ± 0.24	green	wrinkled
PI 240517	3.48 ± 0.25	green	wrinkled
PI 244107	3.83 ± 0.40	green/white	wrinkled
PI 210561	3.39 ± 0.55	green	wrinkled
PI 404226	3.75 ± 0.20	green/white	wrinkled
PI 411142	4.54 ± 0.27	green/white	wrinkled
PI 365423	5.89 ± 1.36	green/white	wrinkled
PI 385981	3.89 ± 0.17	green/white	wrinkled
PI 381333	3.17 ± 0.18	Brown pigmented	wrinkled
PI 413690	4.03 ± 0.53	green/white	wrinkled
PI 413685	4.38 ± 0.14	green/white	wrinkled
PI 411144	3.89 ± 0.48	green/white	wrinkled
PI 413679	4.46 ± 0.26	green/white	wrinkled
PI 404218	3.72 ± 0.12	green/white	wrinkled
PI 206861	3.84 ± 0.38	green/white	wrinkled
PI 347506	4.33 ± 0.59	green/white	wrinkled
PI 347502	3.38 ± 0.33	green/white	wrinkled
PI 347497	3.56 ± 0.06	green/white	wrinkled
PI 347498	4.09 ± 0.10	green/white	wrinkled
PI 341890	3.67 ± 0.22	green/white	wrinkled
PI 343077	3.89 ± 0.20	green/white	wrinkled

PI 343250	3.32 ± 0.38	green/white	wrinkled
PI 343251	3.81 ± 0.15	green/white	wrinkled
PI 343253	3.71 ± 0.40	green/white	wrinkled
PI 343255	3.83 ± 0.02	green/white	wrinkled
PI 343256	3.71 ± 0.05	green/white	wrinkled
PI 343282	2.38 ± 0.22	green/white	wrinkled
PI 343258	3.12 ± 0.07	green/white	wrinkled
PI 343260	3.02 ± 0.23	green/white	wrinkled
PI 347277	3.5 ± 0.34	green/white	wrinkled
PI 347484	2.57 ± 0.18	green/white	wrinkled
PI 347489	2.99 ± 0.33	green/white	wrinkled
PI 343333	2.97 ± 0.33	Brown pigmented	wrinkled
PI 244160	3.1 ± 0.14	green	wrinkled
PI 512084	2.99 ± 0.10	green	wrinkled
PI 600939	3.32 ± 0.36	green/white	wrinkled
PI 471179	3.95 ± 0.37	green/white	wrinkled
PI 471089	3.93 ± 0.48	green/white	wrinkled
PI 413698	3.93 ± 0.30	green/white	wrinkled
PI 365421	4.71 ± 0.49	green/white	wrinkled
PI 471375	3.88 ± 0.04	green/white	wrinkled
PI 505085	3.84 ± 0.50	green/white	wrinkled
PI 280064	3.64 ± 0.55	green	wrinkled
PI 356983	3.57 ± 0.27	green pigmented	wrinkled
PI 357296	2.41 ± 0.04	green/white	wrinkled
PI 269821	3.35 ± 0.16	green/white	wrinkled
PI 272194	2.6 ± 0.42	green/white	wrinkled
PI 279742	3.14 ± 0.05	green/white	wrinkled
PI 358703	2.57 ± 0.16	green pigmented	wrinkled
PI 365416	3.76 ± 1.59	green/white	wrinkled
PI 365417	3.61 ± 0.53	green/white	wrinkled
PI 365419	5.21 ± 1.04	green/white	wrinkled
PI 347528	3.22 ± 0.33	green/white	wrinkled

PI 358654	2.37 ± 0.04	green pigmented	wrinkled
PI 314795	4.81 ± 0.41	green/white	wrinkled
PI 324693	3.35 ± 0.13	green/white	wrinkled
PI 505146	2.76 ± 0.28	green/white	wrinkled
PI 505122	2.43 ± 0.35	green/white	wrinkled
PI 341888	4.11 ± 0.20	green	wrinkled
PI 302992	3.41 ± 0.43	green/white	wrinkled
PI 505127	2.85 ± 0.14	green/white	wrinkled
PI 343261	3.23 ± 0.08	green/white	wrinkled
PI 261633	4.31 ± 0.09	green/white	wrinkled
PI 347515	4.02 ± 0.04	green/white	wrinkled
PI 244239	3.59 ± 0.51	green	wrinkled
PI 244217	3.12 ± 0.07	green/white	wrinkled
PI 347509	3.35 ± 0.42	green/white	wrinkled
PI 347510	3.62 ± 0.68	green/white	wrinkled
PI 244188	3.19 ± 0.31	green/white	wrinkled
PI 269544	4.21 ± 0.31	green/white	wrinkled
PI 347513	3.52 ± 0.13	green	wrinkled
PI 250446	4.14 ± 0.56	Green/White	wrinkled
CN 112351	2.74 ± 0.16	green	smooth
CN 42819	3.36 ± 0.02	yellow	smooth
CN 43016	4.25 ± 0.37	green	wrinkled
CN 29600	4.06 ± 0.16	yellow	wrinkled
CN 45760	4.1 ± 0.13	green	wrinkled
CN 29579	3.36 ± 0.29	brown	wrinkled
CN 29526	3.52 ± 0.10	mixed	smooth

The usefulness of the predictive model for lipid content estimation in other oilseed crops

The model developed for pea seeds in this study was applied to soybeans to determine the feasibility of using this minimally invasive method for qualitative and quantitative lipid analyses in other oilseed crops. Soybean samples were similarly prepared prior to HRMAS-NMR analysis, and the estimated total lipid content for soybean is presented in Table 2.2. As expected, soybean lipid content was higher than pea, and total lipid content was higher than total lipid content determined through solvent (butanol) extraction. This supports the trend seen in the majority of the pea seed samples.

Minimally invasive methodology verified by seed germination

While 7-mg pea seed samples cut from whole seeds underwent qualitative and quantitative lipid analyses, remaining portions of pea seeds from seven accessions were subjected to concurrent germination tests to verify that this method was minimally invasive. With the addition of water, seed swelling occurred on Day 2, and seed coat shedding and seed splitting was observed on day 3. Radicle emergence occurred on Day 4. On Day 5, the plumule emerged, and on the following days, shoot and root systems continued to elongate. Foliage leaves were observed on Day 7, and secondary roots were observed on Day 8. A 100% germination rate was observed for all accessions tested (Figure 2.5).

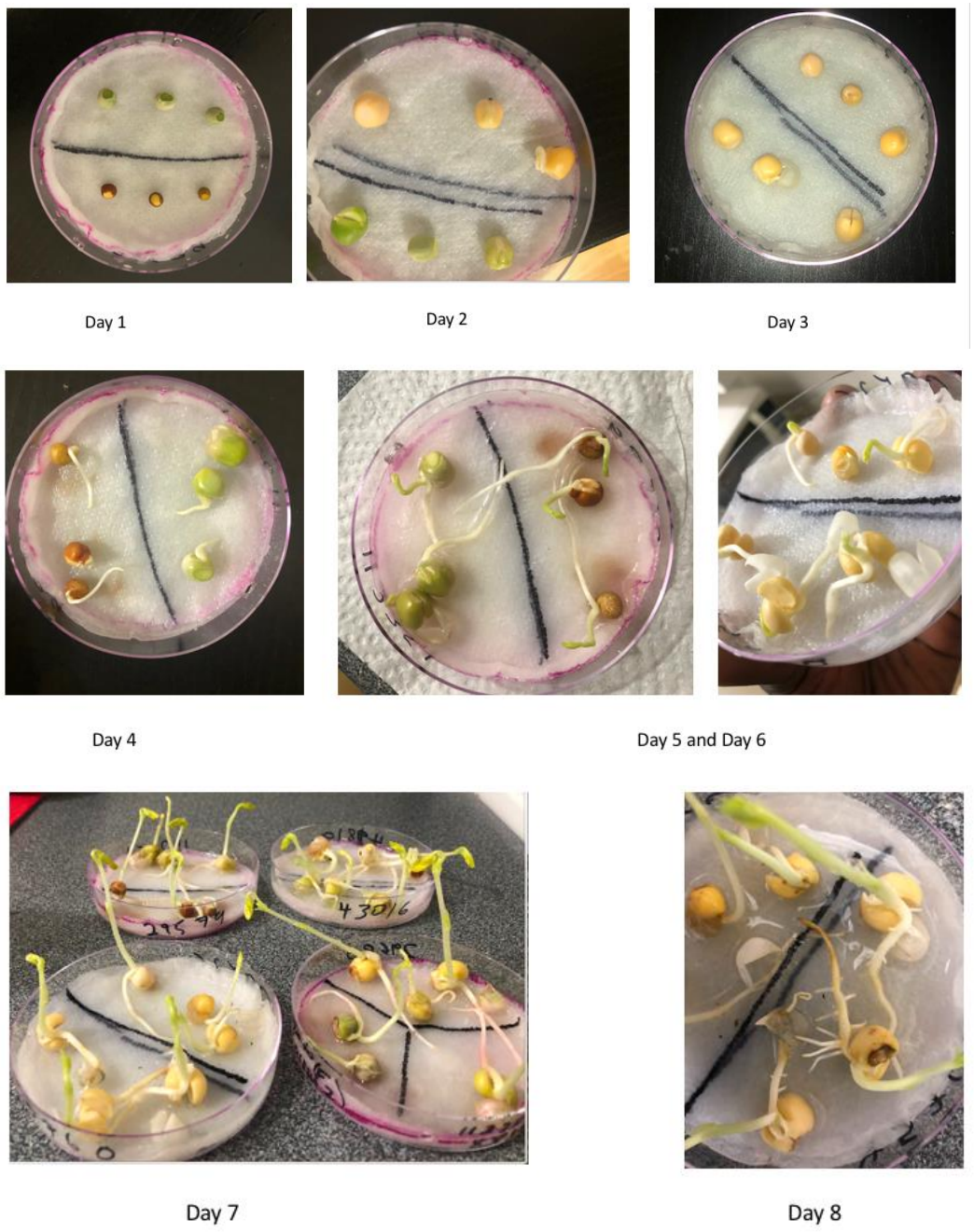


Figure 2.5. Representative images of germination tests performed on remaining pea seed samples from Days 1 to 8. Three seeds from each accession were tested.

2.5 Discussion

The growing global demand for vegetable oil has fuelled renewed interest in research and the development of novel oilseed crops with improved desirable traits, including higher lipid content. While investigating the potential of *P. sativum* as a cold weather oilseed, we developed a rapid and minimally invasive method for qualitatively and quantitatively analysing lipids that could be used to screen whole seeds genetically modified for higher lipid content. Using pea oil and pea seed spectra acquired with HRMAS-NMR, we have developed a minimally invasive methodology to estimate the lipid content of pea seeds. When this minimally invasive HRMAS-NMR method was compared to traditional methods for total lipid content estimation using Soxhlet extraction with two solvents, some notable differences were observed. These can be attributed to polar and non-polar solvent's ability to extract bound, neutral, and unbound lipids (Osagie, 1987). It has been previously reported that the total lipid content in pea ranges from 1% to 4% (Daveby, Abrahamsson, & Åman, 1993; Davies, Coxon, Gavrel, & Wright, 1985; Welch & Wynne Griffiths, 1984; Yoshida, Tomiyama, Tanaka, & Mizushina, 2007). NMR analysis and total lipid content of wild pea accessions in this work were found to be higher than solvent extraction methods reported previously. This infers that the extractable amount of oil is likely less than the oil actually present in a given analysed sample. This should be considered when evaluating lipid content and comparing different methods. The same methodology was used to estimate the total lipid content in soybean, which showed lipid content to be higher than the solvent extraction method. This suggests method may prove valuable for estimating lipid content in other oilseed crops or food products. The method has a high prospect of utilizing this technique for qualitative and quantitative analysis of lipids in oilseeds and other metabolic studies (Ritota, Marini, Sequi, & Valentini, 2010).

Seeds with a smooth surface have, on average, a lower amount of oil content as compared to the wrinkled surfaced seeds. The relationship between lipid concentration and seed shape or size has been previously reported, and the lipid content can be up to 68% higher in wrinkled seeds when compared to smooth seeds (Kosson, Czuchajowska, & Pomeranz, 1994; Murcia & Rincón, 1992). The data in this study also observes higher lipid content in wrinkled varieties. Correlations with seed dry mass and stem diameter were evaluated; these parameters can be useful in the initial selection of varieties in the breeding program.

With this newly developed method, a small fraction of a whole pea seed can be sliced off, and the remaining seed can be germinated for plant propagation, as all pea accessions and samples analysed were successfully germinated. Genetically modified Greenfeast (P276a-5-2, P276a-4-1) seeds and CN 43016 progeny (P333-4-3, P10-4, P283a-2-6, P13-6, P283 2-3) were

readily analysed using this method, and remaining pea seed samples proved viable. This verifies that this method is rapid and minimally invasive and could be appealing to researchers when genetically modified seeds are generated in insufficient numbers. Importantly, HRMAS-NMR could be used to analyse seeds at every stage of transformation for generation advancement. This study further demonstrates that NMR can be successfully employed to quantify oil in whole pea seeds, and sample preparation is relatively effortless when compared to solvent extraction methods. There is no sample pre-treatment, and one run takes approximately 7 minutes to produce a spectrum. Being minimally invasive and using a relatively small sample size, it is possible that other analyses could be considered for genetically modified plant species, and NMR is confirmed as a powerful tool for determining oil content present and identifying modifications that other extraction methods might not detect.

2.6 Conclusion

HR-MAS NMR produced lipid profiles for 7-mg field pea seed samples taken from whole seeds without any pre-processing steps. Considerable research time may be saved as seeds generated via genetic modification, or conventional breeding may be assessed for desirable traits at an earlier stage, and remaining seed portions can be planted for further study. When PLS regression was combined with this HR-MAS NMR technique, the correlation coefficient of the model (predictor and response scores) is 0.992, assuring the quality of the prediction model and highlighting its potential to evaluate the total lipid content in peas or other crops. Findings suggest that this method can be effectively used to screen large collections of wild accessions, and that HRMAS-NMR is an effective tool for lipid characterization and quantification of lipids that can aid in the development of novel oilseed crops.

Appendix (i): Integration on multiple NMR spectra in Bruker Topspin 4.1.0

Example: Multiple integration of spectra obtained by analysing varying concentration of pea oil.

1. Collect the spectrum data and put it in the folder and name it for e.g. Pea_data and then make another folder Pea_oil_20201007. Paste the data into Pea_oil_20201007.

2. Open Bruker Topspin 4.1.0 and right click in the section shown by the green arrow in the Figure (i).

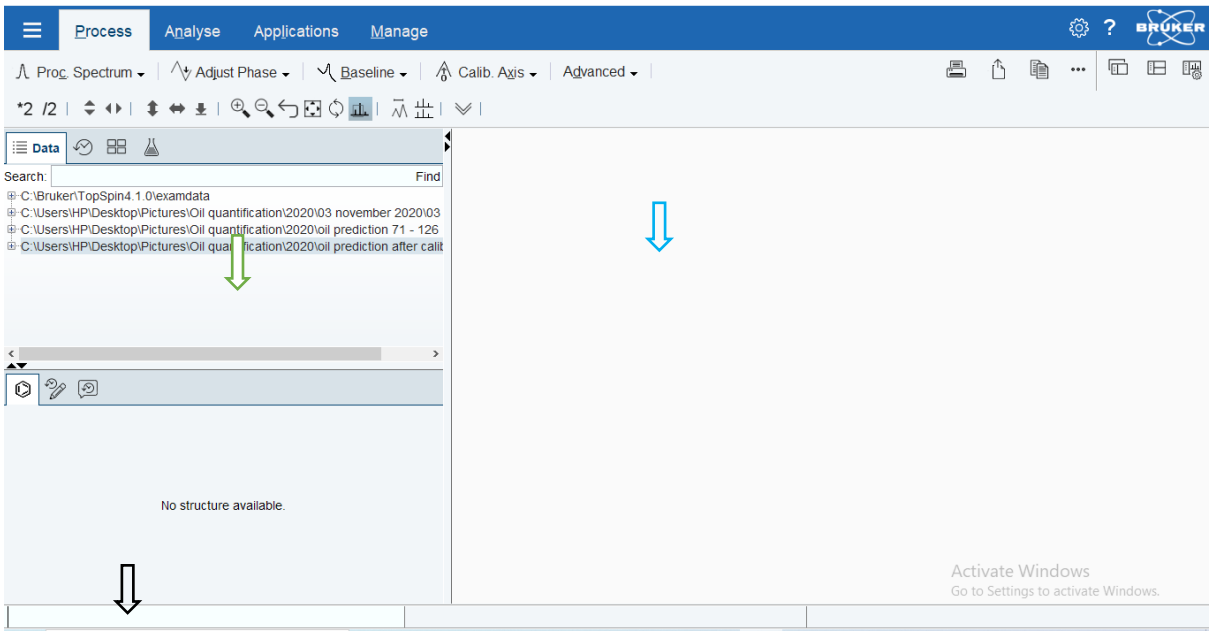


Figure (i). View of Topspin application on a windows desktop.

3. After right click, select add new directory and browse for Pea_data (in step 1).

4. Load the first spectrum by clicking and dragging to the right window (blue arrow). Go into the *Process* tab use *adjust phase* and *baseline.* Automatic phasing and baseline correction can be done by using commands *apk* and *absn* respectively (black arrow). It can also be done manually. Pick the peaks for integration using Figure 2.2 and Figure 2.4 for identification and comparison.

5. To integrate, go in *Analyse* tab, click the Integration button and pick the relevant peaks and integrate.

6. For integration of a list of spectra, go into *Integrate* tab select Show more options...(int)* a new menu bar will appear (Define List/Define Parameters/•Execute).

7. *Define List - Build dataset list using "find"*

   a. NAME: *Pea_oil_20201007* (name of the directory with all spectra to integrate)

   b. EXPNO: * (will list all experiments in the directory)

   c. PROCNO: 1

d. Data directories:(select the directory path where the spectra are found)

* all other fields I left blank (except Dimension and Data type = *Any*)

8. Click *Ok*, a list will be generated with all the spectra found under these defined parameters.
9. Press *Ok*.
10. Click *Define Parameters*.

Integration on Multiple NMR spectra in Topspin

a. Options: *normalize sum of integrals*

b. Normalization value: *100*

c. Global scaling: *yes*

11. Click *Execute* (will generate a file in a new window with the integration values)
12. Copy the data from the new window and paste it in an excel sheet.
13. In excel file go to *data* tab, click on *Text to Columns.*

a. Choose file type: *Delimited*, click *Next >*

b. Delimiters: *semicolon* (look in *data preview* to make sure all the integration values at the bottom of the file are separated in different columns)

c. Column data format: *General*, click *Finish*.

Connecting statement

Breeding is an important technique in the modification of traits. Classical interbreeding was used for initiating the breeding program for elevation lipid content in peas. The crossing was performed, and the crosses were advanced for segregation. The methodology developed in the previous chapter can be used to analyse the lipid content in advanced generations. The data obtained in Chapter 2 with lipid content and the correlation values can be used in the selection of varieties for the next phase of the breeding program. Wild varieties of known lipid content were planted in the field for physical parameter analysis.

Chapter 3: Pea breeding for lipid improvement

3.1 Abstract

The increasing demand for vegetable oil needs to be addressed, and new alternative oilseed crops should be developed. Plant breeding has been practiced for a long time for improving various traits in the crops. The objective here is to initialize the breeding program, which includes crossing and studying the parameters of various wild accessions in the field experiment in summer. Classical interbreeding was used to make crosses, and the obtained crosses were advanced to their subsequent filial generations. Wild accessions were planted in the field to increase the sample size, and their physical parameters observations were recorded. The crossed varieties with generations advanced till F4 should be evaluated for the lipid content using the HRMAS NMR method (Chapter 2). The next phase of breeding can be initiated by selecting the varieties from wild accessions. The parameters 100 seed mass, wrinkled seeds, and stem diameter were correlated with lipid content (Chapter 2) and can be used in selection of accessions for breeding.

3.2 Introduction

Pea (*Pisum sativum*) has been cultivated for 10,000 years, making it one of the oldest crops to be domesticated (Smýkal et al., 2015; Weiss & Zohary, 2011; Willcox, Buxo, & Herveux, 2009). The cultivation of pea then expanded quickly to Europe, Asia, and the Mediterranean (Zohary, Hopf, & Weiss, 2012). The wide range of use of peas as feed, food, and fodder and extensive cultivation in different parts of the world has resulted in adaptation to various end uses, different cropping systems, and diverse environments (Judith Burstin et al., 2015). The vast variety act as a collection with different traits to pick from for improving the crops. There is great variation between plant accessions resulting in a wide range of traits and genes associated; this has allowed for the development of modern pea varieties. This range of traits was the reason that Mendel used them as his model plant for the earliest genetic studies (Hallauer, 2011). For example, seeds with the wrinkled surface can significantly differ in seed composition that is linked with modification in starch synthesis (Wang, Domoney, Hedley, Casey, & Grusak, 2003). The dry peas are observed to vary with respect to plant architecture, flowering time, or cotyledon colour. Apart from having a diverse collection of cultivated peas, cultivated peas and wild peas can be interbred using plant breeding practices to create different genetic combinations for new varieties (Smýkal et al., 2015).

Plant breeding is considered one of the longest ongoing activities conducted by human beings and has evolved over human civilization (Acquaah, 2009). Plant selection of wild species and subsequent breeding has been assumed to have begun at the first stages of human civilization to maintain plant production and quality (Schlegel, 2017). Plant breeders have been domesticating wild species into cultivable species and selecting wild plants for transference into commercial cultivars. Major traits that have been selected include drought tolerance, pest and disease resistance, and other traits that have benefits for human production (Ansari, 2021). The traits that breeders target can be related to biomass and grain yield, abiotic and biotic stresses, and quality or quantity of biological molecules such as fibers, proteins, sugars, vitamins, and lipids (Verheye, 2010).

There are various methods for successful plant breeding. It can be as simple as propagating plants with desirable traits and crossing these traits into breeder plants which comes under classical plant breeding, or it can be more complex, which involves the knowledge of chromosomes, genetics, and gene placement to obtain the selected traits (Hartung & Schiemann, 2014). In classical plant breeding, the selection of plants with specific traits is considered standard plant breeding. The plants dominating desirable traits are selected for propagation. The other option is crossing between different varieties of crops (interbreeding) to produce lines with better survival and desirable traits (Ceballos, Kawuki, Gracen, Yencho, & Hershey, 2015). One variety's genetic code is combined with the other variety's genetic code to produce a crossbred or hybrid plant. For example, a high-yielding pea with lower disease tolerance can be crossed with a wild pea with high disease tolerance. The aim would be to retain the high yield from the former variety and high disease tolerance from the latter. The crossed lines produced can be tested for high yield and high disease resistance plants for further development. The traits that are usually incorporated into plants are the longer shelf life of postharvest crops, improved quality, and quantity (lipids, proteins, carbohydrates, vitamins, etc.), tolerance of environmental factors (temperature, drought), resistance towards microorganisms (bacteria, fungi, and viruses), tolerance of herbicides and pests (Deppe, 2000).

Breeding has played an important role in improving oil content and raising the quality of oil present in several oilseed crops. The modification of fatty (oleic, linolenic acid) acids has been observed in brassica genotypes (Oram, Salisbury, Kirk, & Burton, 1999). The oil content in local sunflower (*Helianthus*) varieties of Russia have improved from 30-33% to 43-46% (Fick & Miller, 1997). The effect of breeding practices has been seen in soybeans (*Glycine max*), where the oil content has been reported up to 21% in modern soybean varieties. There have been elite lines that have been developed with even higher oil percentages (up to 24%)

via conventional breeding and early generation composition analysis (Yadava, Vasudev, Singh, Mohapatra, & Prabhu, 2012). Over the years, research programs on the breeding of oilseed crops have found success in the improvement of the oil content in the present oilseed crops such as sunflower, sesame (*Sesamum indicum*), safflower (*Carthamus tinctorius*), oil palm (*Elaeis*), coconut (*Cocos nucifera*), cotton (*Gossypium hirsutum*), castor (*Ricinus communis*), olive (*Olea europaea*), soybean and groundnut (*Arachis hypogaea*) (Yadava et al., 2012). Breeding of pea can be practiced for producing high oil-yielding cultivars. There is a potential for developing an alternative oilseed crop that can supplement the increasing demand for edible oil for food and feed.

The aim of the study is the initialization of a breeding program for lipid content enhancement in peas. Here we use the classical interbreeding approach for lipid content improvement.

3.3 Material and methods

In this study, field-pea seeds were obtained from three different sources, 2018 harvest from Macdonald Campus of McGill University (Ste-Anne-de-Bellevue, QC, Canada), Plant Gene Resources of Canada (Saskatoon, SK, Canada), and the pea collection of the U.S. Department of Agriculture (Pullman, WA, USA).

Greenhouse

The research greenhouse at Macdonald Campus was used for plant cultivation. It is glass-covered and with environmental control (temperature, humidity, and electrical lighting). The pea seeds were grown in the greenhouse in pots using G6 soil mix (agro mix, greenhouse growing medium PV20). There were 16 accessions sowed in pots (3 gallons), and the pots were placed on the bench in the greenhouse, as shown in Figure 3.1. The air temperature in the greenhouse was maintained at 24 ℃. A fertilizer 20-20-20 (Green flag fertilizer, Derco Horticulture Inc., Quebec, CA) was used every 20 days and was added to the pot after mixing along with irrigation water. A metallic cage was set for the pea plant to climb (Figure 3.2). Once the flowering begins, the breeding between two varieties was done manually by selecting two plants at a time. Peas were cross-pollinated by hand by opening the flower buds to remove their pollen-producing stamen (and prevent self-pollination) and dusting pollen from one plant onto the stigma of another (Figure 3.2).

Figure 3.1. Pea plants, after ten days sown in pots, are placed on a bench in the greenhouse.

Figure 3.2. Fully grown plants are climbing up the cage installed into the soil. The yellow tag is used for the identification of the crossed pod.

The plants used in the crossing are known as the parental generation and denoted as P. Potential crosses were monitored carefully and harvested when the pods had dried. The progeny generation is called the first filial generation (F1 plants). The subsequent generations produced by self-pollination are symbolized as F2, F3, and so forth. The advancement of generation was done in the green house during winter and in the field during summer.

Field experiment

A plot located at the Macdonald Campus of McGill University, Sainte Anne de Bellevue, was used for the field experiments. The field was tilled twice before planting, and the soil is loamy clay. The average temperature was 19 ℃ in the summer season. A total of 21 pea varieties were grown at the field site to bulk up the sample size and measure the physical traits (Figure 3.3). These varieties have relatively higher oil content and have the potential to be used in the breeding program. Physical traits were observed and noted for the varieties, such as plant height, number of branches, number of pod per plant, green pod length and width, number of seeds per pot, and 100 seed dry mass. Weeds were removed by hands every other day. The pods after the harvest were put into paper bags and dried in an oven at 60 ℃ for 48 hours and stored for future trials.

Figure 3.3. Progress of field pea growth at the Mac Market location.

3.4 Results and discussion

The crossing was performed under greenhouse conditions. The generation advancement was performed in the greenhouse in September 2019, and a second advancement was started in mid-June 2020. The harvest was done in December 2019 and September 2020, respectively. The current stages of crossed generations can be seen in Table 3.1, along with the 100 seed

mass in grams and the number of days of flowering in 50% of each variety's plants. The field experiment was conducted to increase the sample size and observe physical traits (Table 3.2).

Table 3.1. The crosses made by manual interbreeding and present seed generation.

Internal name	Generation	Pedigree	100 Seed mass (g)	Days of flowering
APS190002	F1	CN 112351/CN 36165	24	48
APS190003	F1	CN 25969/CN 43016	17	37
APS190004	F1	CN 29579/CN 112351	12	37
APS190005	F1	CN 36165/CN 112351	21	41
APS190006	F1	CN 36165/CN 29579	18	37
APS190007	F1	CN 43016/CN 112351	19	37
APS190009	F1	CN 43016/CN45761	19	37
APS190010	F1	CN 45763/CN 43016	16	44
APS190011	F1	Rondo/CN 31237	16	48
APS190015	F2	CN 112351/WANDO	21	48
APS190017	F2	CN 25969/CN 29579	17	51
APS190020	F2	CN 25969/CN 43016	19	37
APS190021	F2	CN 25969/WANDO	17	50
APS190022	F2	CN 31237/G611764	19	44
APS190024	F2	Big pea/Green pea	25	53
APS190027	F2	G611764/DAKOTA	17	37
APS190028	F2	G611764/WANDO	16	37
APS190029	F2	RONDO/CN 112351	19	51
APS190030	F3	CN 25969/ CN 29579	14	42
APS190047	F3	DAKOTA/G611764	16	49
APS190048	F3	G611764/DAKOTA	14	50
APS190049	F3	G611769/Wando	14	46
APS190052	F3	ILCA/WANDO	19	39
APS190054	F4	G611764/WANDO	17	48
APS190055	F4	ILCA/DAKOTA	15	41
APS190056	F4	ILCA/WANDO	15	41
APS190057	F4	ILCA5074/DAKOTA	17	43

Table 3.2. Different wild varieties of field pea grown and assessed for physical parameters.

Accession number	Plant height (cm)	Number of branches	Number of pods/plant	Green pod length/width (cm)	Number of seed/green pod	100-seed mass dry (gm)
CN 43016	55	15	12	7/1.5	5	21
CN 112351	32	20	21	7/1.5	5	23
CN 29526	60	9	14	7/1.7	6	22
CN 29579	107	24	17	5.5/1.1	6	19
CN 29600	54	9	8	5.4/1.4	4	21
CN 42819	75	14	6	6.4/1.5	7	30
CN 45760	46	9	7	5.6/1.2	5	19
PI 280626	48	14	6	6.7/1.7	6	22
PI 285732	43	11	8	5.4/1.3	3	20
PI 285740	55	14	9	7.8/1.6	5	15
PI 288263	54	23	30	5/1.1	7	15
PI 341889	74	28	16	8.4/1.9	8	21
PI 343257	76	32	24	9/1.8	8	23
PI 347490	107	36	15	8/1.8	5	18
PI 347518	75	44	26	7/2.0	7	25
PI 358637	114	27	18	6/1.3	7	16
PI 365420	56	18	13	7/1.3	9	22
PI 404218	24	6	4	6/1.4	5	15
PI 411143	38	16	7	7/1.8	7	24
PI 413684	51	29	18	9/2.0	5	11
PI 471406	55	27	15	8/2.0	6	16

The plants were visually observed while advancing from F1 generation to F2 generation. Variations were observed in different traits in the same variety (pedigree), such as the plant's height, leaf density, and early flowering. In G611764/Dakota, the F1 generation seeds planted showed an observable difference in plant height as one plant is smaller in height than the other; the smaller plant has a higher leaf density (Figure 3.4). The CN 25969/CN 29579 F1 generation seeds planted (Figure 3.5) had size variation, with one is short and the other taller. Leaf density was higher on the smaller plant. As shown in Figure 3.6, the CN 112351/CN

43016 F1 generation seeds planted resulted in no observable difference between plants, with all plants having high leaf density and short height. Figure 3.7 shows Rondo/CN 112351 F1 generation seeds planted in different pots, and Figure 3.8 shows CN 31237/G611764 F1 generation seeds planted. There was no significant variation in plants, with all having high leaf density and short height.

The crossed generations shown in Table 3.1 can be accessed easily in the inventory for further advancement. The 100 seed mass was observed to range from 12 to 25 grams with an average of 17 grams. The days to flowering range from 37 to 53, with an average of 43 days. The 100 seed mass was measured and correlated to the lipid content (analysed using NMR). A positive correlation (with a correlation coefficient of 0.56) between 100 seed mass and lipid content was reported in Chapter 2. The F4 generations (hybrids) must be tested for total lipid content and compared to their parental generations to check for the relative change in the lipid content. The generations F1, F2, and F3 were advanced further and tested for lipid content at F4. The advancing of generation from F1 to F2 shows interesting results with variation in plant height as shown in Figures 3.6 and 3.7 and variation in leaf density in Figure 3.3. The plants have poor ability to stand upright, as observed in Figures 3.4 and 3.5, compared to plants in Figures 3.6, 3.7, and 3.8. Variation within the seeds obtained from the same parental generation confirms the presence of segregation between the different offspring (Figure 3.4 to Figure 3.8). It is necessary to stabilize the segregation to a stage where the traits associated with each offspring are consistent. The data on traits noted varieties grown in the field suggest the growing pattern and requirements. These varieties in Table 3.2 can be used to make new crosses and be incorporated into the breeding program. Plant height and number of branches can be associated with the plant's biomass; the number of pods and pod dimensions can be related to the yield. The trait that associates with the lipid content based on this limited data is the 100 seed mass.

In this study, the initial phase of the breeding program has been carried out. The inventory of the wild and domesticated varieties was set up. There were crosses made and propagated to further generations for segregation. This study can be used to take the breeding program further. Another trait that should be taken into account for further research is stem diameter, as a high correlation (0.85) has been seen to lipid content (Chapter 2). Wrinkled seeds, 100 seed mass, and stem diameter can be used as parameters for selecting wild varieties in further expansion of the breeding program.

(a) (b)

(c) (d)

Figure 3.4. G611764/Dakota; F1 generation seeds planted in different pots growing on a bench in the greenhouse. (a) low leaves density and relatively taller. (b) dense leaves, shorter height (c) medium density of leaves, tall in size (d) low leaf density, and taller.

Figure 3.5. CN 25969/CN 29579; F1 generation seeds planted in different pots growing in the greenhouse.

Figure 3.6. CN 112351/CN 43016; F1 generation seeds planted in different pots growing in the greenhouse.

Figure 3.7. Rondo/CN 112351; F1 generation seeds planted in different pots growing in the greenhouse.

Figure 3.8. CN 31237/G611764; F1 generation seeds planted in different pots growing in the greenhouse.

3.5 Conclusion

The breeding program is in its initial phase, and more work is required. The plants at F1, F2, and F3 generations need to be followed through to the F4 or F5 generation. Along with plants already at F4 and F5, there should be a check for the hybridity and analysis of lipid content to see if there is any improvement. Breeding should be continued to produce more crossed varieties and advanced further for segregation. The parameters such as plant height, number of branches, number of pod per plant, green pod length and width, number of seeds per pot, and 100 seed dry mass; represent the physical traits that play a significant role. These parameters can be used in establishing a commercial variety if lipid is found to be elevated in

crossed varieties. So far, three physical traits have been identified as indicators of lipid content wrinkled seeds, 100 seed mass, and stem diameter (Chapter 2). These positively correlate with the lipid content; they can be used in further research and selection of better parental lines for breeding, from a large pool of wild accessions.

Chapter 4: General conclusion and future research

4.1 General conclusion

The global market of vegetable oil is rising due to edible oil applications in the food and feed industry, medicine, cosmetic and oleochemical industry. Vegetable oil can act as an alternative for the petroleum-based product, including energy storage. A massive push towards sustainable usage of natural resources and strict climate laws has opened up the possibilities for organically produced oil. An alternative oilseed crop can supplement the increasing demand. Field pea is a valuable legume crop in western Canada, predominantly grown for starch and protein, but the seeds' lipid content has not been considered for vegetable oil production. The field pea can be developed as a novel oilseed crop by elevating lipid content using genetic modification and breeding techniques. This objective would require various tools, one of which would be lipid analysis. A novel, rapid spectroscopic technique HRMAS-NMR, was used to study lipid content in intact pea seeds with no pre-treatment. The methodology evaluated lipid content in wild accessions and genetically modified accessions. The lipid content in peas ranged from 2.37 % to 5.89 %. There was 100 % germination (post lipid analysis) of pea seeds observed after a tiny (7 mg) sample cut off from the seed was analysed by NMR. There were two parameters found that correlated with lipid content; 100 seed mass and stem diameter with a correlation coefficient of 0.57 and 0.85, respectively. The breeding program was initiated using the interbreeding technique. The crosses were made manually, and the subsequent filial generations were advanced further for segregation. The summer field experiment included observing the physical traits of the wild accessions of known lipid content (analysed with NMR) and increasing the sample size. There have been three parameters identified that correlate to lipid content: wrinkled seed, stem diameter, and 100 seed weight. This correlation can be used for selective breeding in the next phase of the breeding program and aid in developing pea as an oilseed crop.

4.2 Future research

1. The NMR methodology should be used for evaluating lipid content in genetically modified varieties and F4/ F5 seeds from the breeding program. The lipid content between parental and modified versions should be compared.

2. More crossing should be done in peas; the selection from the pool of wild accessions can be made using the data and parameters obtained related to lipid content. The plant growth parameters for the selected varieties should be observed in the Canadian climate.

References:

Aboobucker, S. I., & Suza, W. P. (2019). Why do plants convert sitosterol to stigmasterol? *Frontiers in Plant Science, 10*, 354.

Acquaah, G. (2009). *Principles of plant genetics and breeding*: John Wiley & Sons.

Akoh, C. C. (2017). *Food lipids: chemistry, nutrition, and biotechnology*: CRC press.

Almasi, S., Ghobadian, B., Najafi, G., & Soufi, M. D. (2021). A review on bio-lubricant production from non-edible oil-bearing biomass resources in Iran: Recent progress and perspectives. *Journal of Cleaner Production*, 125830.

Ansari, S. B. (2021). Mutation breeding for quality improvement: A case study for oilseed crops shazia bi ansari, aamir raina. *Mutagenesis, Cytotoxicity and Crop Improvement: Revolutionizing Food Science*, 171.

Arnoldi, A., Zanoni, C., Lammi, C., & Boschin, G. (2015). The role of grain legumes in the prevention of hypercholesterolemia and hypertension. *Critical Reviews in Plant Sciences, 34*(1-3), 144-168.

Baran, R., Chivot, M., Shalita, A., Lewis, A., & Wechsler, A. (2005). Acne. Baran R, Maibach HI editors. Textbook of Cosmetic Dermatology. In: London: Taylor & Francis.

Barison, A., Pereira da Silva, C. W., Campos, F. R., Simonelli, F., Lenz, C. A., & Ferreira, A. G. (2010). A simple methodology for the determination of fatty acid composition in edible oils through 1H NMR spectroscopy. *Magnetic resonance in chemistry, 48*(8), 642-650.

Bastianelli, D., Grosjean, F., Peyronnet, C., Duparque, M., & Regnier, J. (1998). Feeding value of pea (Pisum sativum, L.) 1. Chemical composition of different categories of pea. *Animal Science, 67*(3), 609-619.

Bastida, G. J. L., González-Ronquillo, M., Dominguez Vara, I. A., Romero-Bernal, J., & Castelan Ortega, O. (2011). Effect of field pea (Pisum sativum L.) level on intake, digestion, ruminal fermentation and in vitro gas production in sheep fed maintenance diets. *Animal science journal, 82*(5), 654-662.

Brescia, M. A., Di Martino, G., Fares, C., Di Fonzo, N., Platani, C., Ghelli, S., . . . Sacco, A. (2002). Characterization of Italian durum wheat semolina by means of chemical analytical and spectroscopic determinations. *Cereal chemistry, 79*(2), 238-242.

Brescia, M. A., Jambrenghi, A. C., Martino, V. d., Sacco, D., Giannico, F., Vonghia, G., & Sacco, A. (2002). High resolution nuclear magnetic resonance spectroscopy (NMR) studies on meat components: potentialities and prospects. *Italian Journal of Animal Science, 1*(2), 151-158.

Brescia, M. A., Sgaramella, A., Ghelli, S., & Sacco, A. (2003). 1H HR-MAS NMR and isotopic investigation of bread and flour samples produced in southern Italy. *Journal of the Science of Food and Agriculture, 83*(14), 1463-1468.

Brown, A. C. (2018). *Understanding food: principles and preparation*: Cengage learning.

Brummer, Y., Kaviani, M., & Tosh, S. M. (2015). Structural and functional characteristics of dietary fibre in beans, lentils, peas and chickpeas. *Food Research International, 67*, 117-125.

Burstin, J., Gallardo, K., Mir, R., Varshney, R., & Duc, G. (2011). 20 Improving Protein Content and Nutrition Quality. *Biology and breeding of food legumes*, 314.

Burstin, J., Salloignon, P., Chabert-Martinello, M., Magnin-Robert, J.-B., Siol, M., Jacquin, F., . . . Delaitre, C. (2015). Genetic diversity and trait genomic prediction in a pea diversity panel. *BMC genomics, 16*(1), 1-17.

Cahoon, E. B. (2003). Genetic enhancement of soybean oil for industrial uses: prospects and challenges.

Caprioli, G., Giusti, F., Ballini, R., Sagratini, G., Vila-Donat, P., Vittori, S., & Fiorini, D. (2016). Lipid nutritional value of legumes: Evaluation of different extraction methods and determination of fatty acid composition. *Food chemistry, 192*, 965-971.

Carranca, C., De Varennes, A., & Rolston, D. (1999). Biological nitrogen fixation by fababean, pea and chickpea, under field conditions, estimated by the 15N isotope dilution technique. *European journal of agronomy, 10*(1), 49-56.

Ceballos, H., Kawuki, R. S., Gracen, V. E., Yencho, G. C., & Hershey, C. H. (2015). Conventional breeding, marker-assisted selection, genomic selection and inbreeding in clonally propagated crops: a case study for cassava. *Theoretical and Applied Genetics, 128*(9), 1647-1667.

Chen, Y., Qi, X., & Liu, R. R. (2008). Prediction of solubility. *Water-insoluble drug formulation*, 27-63.

Control, C. f. D., & Prevention. (2012). Prevalence of cholesterol screening and high blood cholesterol among adults--United States, 2005, 2007, and 2009. *MMWR. Morbidity and mortality weekly report, 61*, 697-702.

Corsaro, C., Cicero, N., Mallamace, D., Vasi, S., Naccari, C., Salvo, A., . . . Dugo, G. (2016). HR-MAS and NMR towards foodomics. *Food Research International, 89*, 1085-1094.

Crivelli, S. M., Giovagnoni, C., Visseren, L., Scheithauer, A.-L., de Wit, N., den Hoedt, S., . . . de Vries, H. E. (2020). Sphingolipids in Alzheimer's disease, how can we target them? *Advanced drug delivery reviews, 159*, 214-231.

D'Imperio, M., Mannina, L., Capitani, D., Bidet, O., Rossi, E., Bucarelli, F. M., . . . Segre, A. (2007). NMR and statistical study of olive oils from Lazio: a geographical, ecological and agronomic characterization. *Food chemistry, 105*(3), 1256-1267.

Dahl, W. J., Foster, L. M., & Tyler, R. T. (2012). Review of the health benefits of peas (Pisum sativum L.). *British Journal of Nutrition, 108*(S1), S3-S10.

Damodaran, S., Parkin, K. L., & Fennema, O. R. (2007). *Fennema's food chemistry*: CRC press.

Daryanto, S., Wang, L., & Jacinthe, P.-A. (2015). Global synthesis of drought effects on food legume production. *PloS one, 10*(6), e0127401.

Daveby, Y. D., Abrahamsson, M., & Åman, P. (1993). Changes in chemical composition during development of three different types of peas. *Journal of the Science of Food and Agriculture, 63*(1), 21-28.

Davies, A. M., Coxon, D. T., Gavrel, G. M., & Wright, D. J. (1985). Determination of starch and lipid in pea flour by near infrared reflectance analysis. The effect of pea genotype on starch and lipid content. *Journal of the Science of Food and Agriculture, 36*(1), 49-54.

Demirbas, A. (2008). *Biodiesel*: Springer.

Deppe, C. (2000). Breed your own vegetable varieties. *Chelsea Green, White River Junction, VT*.

Duc, G., Blancard, S., Hénault, C., Petit, M., Bernicot, M.-H., Bizouard, F., . . . Bonnin, E. (2010). Potentiels et leviers pour développer la production et l'utilisation des protéagineux dans le cadre d'une agriculture durable en Bourgogne. *Innovations Agronomiques, 11*, 157-173.

Duprat-de-Paule, S., Guilbot, J., Roso, A., Cambos, S., & Pierre, A. (2018). Augmented bio-based lipids for cosmetics. *OCL, 25*(5), D503.

Erhan, S. Z., Sharma, B. K., Liu, Z., & Adhvaryu, A. (2008). Lubricant base stock potential of chemically modified vegetable oils. *Journal of Agricultural and Food Chemistry, 56*(19), 8919-8925.

Fahy, E., Subramaniam, S., Brown, H. A., Glass, C. K., Merrill Jr, A. H., Murphy, R. C., . . . Shaw, W. (2005). A comprehensive classification system for lipids. *European Journal of Lipid Science and Technology, 107*(5), 337-364.

FAOSTAT. (2019). Food and Agriculture Organization of the Untited Nations. Retrieved from http://www.fao.org/faostat/en/#data

Farm energy, E. (2019). Oilseed Crops for Biodiesel Production. Retrieved from https://farm-energy.extension.org/oilseed-crops-for-biodiesel-production/

Fick, G. N., & Miller, J. F. (1997). Sunflower breeding. *Sunflower technology and production, 35*, 395-439.

Food and Agricultural Organisation of United Nations, F. (2014). Oilcrops, Oil and Meals. Retrieved from http://www.fao.org/economic/est/est-commodities/oilcrops/en/

Frankel, E. N. (2014). *Lipid oxidation*: Elsevier.

Goodwin, J. (2009). *Colloids and interfaces with surfactants and polymers*: John Wiley & Sons.

Guillén, M. D., & Ruiz, A. (2003). 1H nuclear magnetic resonance as a fast tool for determining the composition of acyl chains in acylglycerol mixtures. *European Journal of Lipid Science and Technology, 105*(9), 502-507.

Guillén, M. D., & Ruiz, A. (2008). Monitoring of heat-induced degradation of edible oils by proton NMR. *European Journal of Lipid Science and Technology, 110*(1), 52-60.

Gülüm, M., Yesilyurt, M. K., & Bilgin, A. (2019). The performance assessment of cubic spline interpolation and response surface methodology in the mathematical modeling to optimize biodiesel production from waste cooking oil. *Fuel, 255*, 115778.

Gunstone, F. (2011). *Vegetable oils in food technology: composition, properties and uses*: John Wiley & Sons.

Gunstone, F. D., & Norris, F. A. (2013). *Lipids in foods: chemistry, biochemistry and technology*: Elsevier.

Haas, M. J. (2005). Animal fats. *Bailey's industrial oil and fat products*.

Hallauer, A. R. (2011). Evolution of plant breeding. *Crop breeding and applied biotechnology, 11*(3), 197-206.

Hanaki, K., & Portugal-Pereira, J. (2018). The effect of biofuel production on greenhouse gas emission reductions. In *Biofuels and sustainability* (pp. 53-71): Springer, Tokyo.

Hartung, F., & Schiemann, J. (2014). Precise plant breeding using new genome editing techniques: opportunities, safety and regulation in the EU. *The Plant Journal, 78*(5), 742-752.

Hayer, F., Bonnin, E., Carrouée, B., Gaillard, G., Nemecek, T., Schneider, A., & Vivier, C. (2010). *Designing sustainable crop rotations using Life Cycle Assessment of crop combinations.* Paper presented at the 9th European IFSA Symposium Vienna (Austria).

Hayes, D. G. (2021). Oils and Their Use Beyond the Food Industry. *Oil and Oilseed Processing: Opportunities and Challenges*, 119-148.

Herridge, D. F., Peoples, M. B., & Boddey, R. M. (2008). Global inputs of biological nitrogen fixation in agricultural systems. *Plant and soil, 311*(1), 1-18.

Hornberg, A. (2006). *Handbook of machine vision*: John Wiley & Sons.

Huang, M., Wang, Q., Zhu, Q., Qin, J., & Huang, G. (2015). Review of seed quality and safety tests using optical sensing technologies. *Seed Science and Technology, 43*(3), 337-366.

Huang, T.-H., Wang, P.-W., Yang, S.-C., Chou, W.-L., & Fang, J.-Y. (2018). Cosmetic and therapeutic applications of fish oil's fatty acids on the skin. *Marine drugs, 16*(8), 256.

Jensen, E. S., Peoples, M. B., Boddey, R. M., Gresshoff, P. M., Hauggaard-Nielsen, H., Alves, B. J., & Morrison, M. J. (2012). Legumes for mitigation of climate change and the provision of feedstock for biofuels and biorefineries. A review. *Agronomy for sustainable development, 32*(2), 329-364.

Jesch, E. D., & Carr, T. P. (2017). Food ingredients that inhibit cholesterol absorption. *Preventive nutrition and food science, 22*(2), 67.

Johnson, L. A., White, P. J., & Galloway, R. (2015). *Soybeans: chemistry, production, processing, and utilization*: Elsevier.

Kalia, R. (2016). Correlation of lipid content and phenotypic markers of Canadian field peas (Pisum sativum): a thesis submitted for the degree master of science. *Quebec: McGill Univ*.

Kausch, A. P., Hague, J., Oliver, M., Li, Y., Daniell, H., Mascia, P., . . . Stewart, C. N. (2010). Transgenic perennial biofuel feedstocks and strategies for bioconfinement. *Biofuels, 1*(1), 163-176.

Kaye, J. P., & Quemada, M. (2017). Using cover crops to mitigate and adapt to climate change. A review. *Agronomy for sustainable development, 37*(1), 4.

Keneni, Y. G., & Marchetti, J. M. (2017). Oil extraction from plant seeds for biodiesel production.

Khodapanahi, E., Lefsrud, M., Orsat, V., Singh, J., & Warkentin, T. D. (2012). Study of pea accessions for development of an oilseed pea. *Energies, 5*(10), 3788-3802.

Kjellin, M., & Johansson, I. (2010). *Surfactants from renewable resources*: John Wiley & Sons.

Knothe, G., Krahl, J., & Van Gerpen, J. (2015). *The biodiesel handbook*: Elsevier.

Kosson, R., Czuchajowska, Z., & Pomeranz, Y. (1994). Smooth and wrinkled peas. 2. Distribution of protein, lipid, and fatty acids in seed and milling fractions. *Journal of Agricultural and Food Chemistry, 42*(1), 96-99.

Kruk, J., Doskocz, M., Jodłowska, E., Zacharzewska, A., Łakomiec, J., Czaja, K., & Kujawski, J. (2017). NMR techniques in metabolomic studies: A quick overview on examples of utilization. *Applied magnetic resonance, 48*(1), 1-21.

Kusumi, A., Fujiwara, T. K., Chadda, R., Xie, M., Tsunoyama, T. A., Kalay, Z., . . . Suzuki, K. G. (2012). Dynamic organizing principles of the plasma membrane that regulate signal transduction: commemorating the fortieth anniversary of Singer and Nicolson's fluid-mosaic model. *Annual review of cell and developmental biology, 28*, 215-250.

Laghi, L., Picone, G., & Capozzi, F. (2014). Nuclear magnetic resonance for foodomics beyond food analysis. *TrAC Trends in Analytical Chemistry, 59*, 93-102.

Letzelter, N. S., Wilson, R. H., Jones, A. D., & Sinnaeve, G. (1995). Quantitative determination of the composition of individual pea seeds by Fourier transform infrared photoacoustic spectroscopy. *Journal of the Science of Food and Agriculture, 67*(2), 239-245.

Lewis, G. P., Schrire, B., Mackinder, B., & Lock, M. (2005). *Legumes of the World*: Royal Botanic Gardens Kew.

Li, J., Vosegaard, T., & Guo, Z. (2017). Applications of nuclear magnetic resonance in lipid analyses: An emerging powerful tool for lipidomics studies. *Progress in lipid research, 68*, 37-56.

Li, W. (2006). Multidimensional HRMAS NMR: a platform for in vivo studies using intact bacterial cells. *Analyst, 131*(7), 777-781.

Liao, A.-h. (2009). *Frictional Contact Analysis of Turbocharger Compressor-Shaft Sleeve-Shaft.* Paper presented at the 2009 International Conference on Measuring Technology and Mechatronics Automation.

Lindon, J. C., Beckonert, O. P., Holmes, E., & Nicholson, J. K. (2009). High-resolution magic angle spinning NMR spectroscopy: Application to biomedical studies. *Progress in Nuclear Magnetic Resonance Spectroscopy, 2*(55), 79-100.

Litchfield, C. (2012). *Analysis of triglycerides*: Elsevier.

Lohumi, S., Lee, S., Lee, H., & Cho, B.-K. (2015). A review of vibrational spectroscopic techniques for the detection of food authenticity and adulteration. *Trends in Food Science & Technology, 46*(1), 85-98.

MacWilliam, S., Wismer, M., & Kulshreshtha, S. (2014). Life cycle and economic assessment of Western Canadian pulse systems: the inclusion of pulses in crop rotations. *Agricultural Systems, 123*, 43-53.

Maheshwari, P., & Kovalchuk, I. (2014). Genetic engineering of oilseed crops. *Biocatalysis and Agricultural Biotechnology, 3*(1), 31-37.

Mandua, C., Barrera-Arellano, D., Santana, M., & Fernandes, G. (2020). Waxes used as structuring agents for food organogels: A Review. *Grasas Y aceites*.

Maphosa, Y., & Jideani, V. A. (2017). The role of legumes in human nutrition. *Functional food-improve health through adequate food, 1*, 13.

Marles, M. S., Warkentin, T. D., & Bett, K. E. (2013). Genotypic abundance of carotenoids and polyphenolics in the hull of field pea (Pisum sativum L.). *Journal of the Science of Food and Agriculture, 93*(3), 463-470.

Mazurek, B., Chmiel, M., & Górecka, B. (2017). Fatty Acids Analysis Using Gas Chromatography-Mass Spectrometer Detector (GC/MSD)-Method Validation Based on Berry Seed Extract Samples. *Food Analytical Methods, 10*(8), 2868-2880.

Miller, P. R., Bekkerman, A., Jones, C. A., Burgess, M. H., Holmes, J. A., & Engel, R. E. (2015). Pea in rotation with wheat reduced uncertainty of economic returns in southwest Montana. *Agronomy Journal, 107*(2), 541-550.

Mitchell, D. C., Lawrence, F. R., Hartman, T. J., & Curran, J. M. (2009). Consumption of dry beans, peas, and lentils could improve diet quality in the US population. *Journal of the American dietetic association, 109*(5), 909-913.

Miyake, Y., Yokomizo, K., & Matsuzaki, N. (1998). Determination of unsaturated fatty acid composition by high-resolution nuclear magnetic resonance spectroscopy. *Journal of the American Oil Chemists' Society, 75*(12), 1091-1094.

Msagati, T. A. (2013). *The chemistry of food additives and preservatives*: Wiley Online Library.

Muanruksa, P., & Kaewkannetra, P. (2020). Combination of fatty acids extraction and enzymatic esterification for biodiesel production using sludge palm oil as a low-cost substrate. *Renewable Energy, 146*, 901-906.

Müllertz, A., Ogbonna, A., Ren, S., & Rades, T. (2010). New perspectives on lipid and surfactant based drug delivery systems for oral delivery of poorly soluble drugs. *Journal of pharmacy and pharmacology, 62*(11), 1622-1636.

Munro, D. B., & Small, E. (1997). *Vegetables of Canada*: NRC Research Press.

Murcia, M., & Rincón, F. (1992). Size as source of variance in lipid composition of pea. *Food chemistry, 44*(1), 29-35.

Murphy, D. J. (2014). Using modern plant breeding to improve the nutritional and technological qualities of oil crops. *OCL, 21*(6), D607.

Nemecek, T., Kägi, T., & Blaser, S. (2007). Life cycle inventories of agricultural production systems. *Final report ecoinvent v2. 0 No, 15*.

Nemecek, T., von Richthofen, J.-S., Dubois, G., Casta, P., Charles, R., & Pahl, H. (2008). Environmental impacts of introducing grain legumes into European crop rotations. *European journal of agronomy, 28*(3), 380-393.

Ohyama, T. (2010). Nitrogen as a major essential element of plants. *Nitrogen assimilation in plants, 37*(2), 2-17.

Oram, R. N., Salisbury, P. A., Kirk, J. T., & Burton, W. A. (1999). *Development of early flowering, canola-grade Brassica juncea germplasm.* Paper presented at the Proceedings of the 10th International Rapeseed Congress, Canberra.

Osagie, A. U. (1987). Total lipids of sorghum grain. *Journal of Agricultural and Food Chemistry, 35*(4), 601-604.

Patel, J., Borgohain, S., Kumar, M., Rangarajan, V., Somasundaran, P., & Sen, R. (2015). Recent developments in microbial enhanced oil recovery. *Renewable and sustainable energy reviews, 52*, 1539-1558.

Petropoulos, S. A., Fernandes, Â., Dias, M. I., Vasilakoglou, I. B., Petrotos, K., Barros, L., & Ferreira, I. C. (2019). Nutritional value, chemical composition and cytotoxic properties of common purslane (Portulaca oleracea L.) in relation to harvesting stage and plant part. *Antioxidants, 8*(8), 293.

Pruett, S. T., Bushnev, A., Hagedorn, K., Adiga, M., Haynes, C. A., Sullards, M. C., . . . Merrill Jr, A. H. (2008). Thematic Review Series: Sphingolipids. Biodiversity of sphingoid bases ("sphingosines") and related amino alcohols. *Journal of lipid research, 49*(8), 1621-1639.

Rahman, A., & Cho, B.-K. (2016). Assessment of seed quality using non-destructive measurement techniques: a review. *Seed Science Research, 26*(4), 285-305.

Rawat, I., Kumar, R. R., Mutanda, T., & Bux, F. (2013). Biodiesel from microalgae: a critical evaluation from laboratory to large scale production. *Applied energy, 103*, 444-467.

Ritota, M., Marini, F., Sequi, P., & Valentini, M. (2010). Metabolomic characterization of Italian sweet pepper (Capsicum annum L.) by means of HRMAS-NMR spectroscopy and multivariate analysis. *Journal of Agricultural and Food Chemistry, 58*(17), 9675-9684.

Rosdahl, C. B., & Kowalski, M. T. (2008). *Textbook of basic nursing*: Lippincott Williams & Wilkins.

Ryan, E., Galvin, K., O'Connor, T. P., Maguire, A. R., & O'Brien, N. M. (2007). Phytosterol, squalene, tocopherol content and fatty acid profile of selected seeds, grains, and legumes. *Plant Foods for Human Nutrition, 62*(3), 85-91.

Sacchi, R., Addeo, F., & Paolillo, L. (1997). 1H and 13C NMR of virgin olive oil. An overview. *Magnetic resonance in chemistry, 35*(13), S133-S145.

Sainju, U. M., Lenssen, A. W., Allen, B. L., Jabro, J. D., & Stevens, W. B. (2019). Pea Growth, Yield, and Quality in Different Crop Rotations and Cultural Practices. *Agrosystems, Geosciences & Environment, 2*(1), 1-9.

Salinero, C., Feás, X., Mansilla, J. P., Seijas, J. A., Vázquez-Tato, M. P., Vela, P., & Sainz, M. J. (2012). 1H-nuclear magnetic resonance analysis of the triacylglyceride composition of cold-pressed oil from Camellia japonica. *Molecules, 17*(6), 6716-6727.

Sarwar, M., Ahmad, N., Siddiqui, Q., Rajput, A., & Tofique, M. (2003). Efficiency of different chemicals on Canola strain Rainbow (Brassica napus L.) for aphids control. *Asian J. Plant Sci, 2*(11), 831-833.

Sarwar, M. F., Sarwar, M. H., Sarwar, M., Qadri, N. A., & Moghal, S. (2013). The role of oilseeds nutrition in human health: A critical review. *Journal of Cereals and oilseeds, 4*(8), 97-100.

Schick, F., Eismann, B., Jung, W. I., Bongers, H., Bunse, M., & Lutz, O. (1993). Comparison of localized proton NMR signals of skeletal muscle and fat tissue in vivo: two lipid compartments in muscle tissue. *Magnetic resonance in medicine, 29*(2), 158-167.

Schlegel, R. H. (2017). *History of plant breeding*: CRC Press.

Schmidt, M. A., Dietrich, C. R., & Cahoon, E. B. (2005). Biotechnological enhancement of soybean oil for lubricant applications. *Synthetics, mineral oils, and bio-based lubricants: chemistry and technology*, 389-398.

Seefeldt, H. F., Larsen, F. H., Viereck, N., Petersen, M. A., & Engelsen, S. B. (2011). Lipid composition and deposition during grain filling in intact barley (Hordeum vulgare) mutant grains as studied by 1H HR MAS NMR. *Journal of Cereal Science, 54*(3), 442-449.

Seo, Y.-W., Ahn, C. K., Lee, H., Park, E., Mo, C., & Cho, B.-K. (2016). Non-destructive sorting techniques for viable pepper (Capsicum annuum L.) seeds using Fourier transform near-infrared and raman spectroscopy. *J. Biosyst. Eng, 41*(1), 51-59.

Seyis, F., Friedt, W., & Luhs, W. (2005). Development of resynthesized rapeseed (Brassica napus L.) forms with low erucic acid content through in ovulum culture. *Asian Journal of Plant Sciences*.

Shahzad, N., Khan, W., Shadab, M., Ali, A., Saluja, S. S., Sharma, S., . . . Abdel-Wahab, A. F. (2017). Phytosterols as a natural anticancer agent: Current status and future perspective. *Biomedicine & Pharmacotherapy, 88*, 786-794.

Shand, P., Ya, H., Pietrasik, Z., & Wanasundara, P. (2007). Physicochemical and textural properties of heat-induced pea protein isolate gels. *Food chemistry, 102*(4), 1119-1130.

Sharma, M., Gupta, S., & Mondal, A. (2012). Production and trade of major world oil crops. In *Technological Innovations in Major World Oil Crops, Volume 1* (pp. 1-15): Springer.

Shyu, P., Wong, X. F. A., Crasta, K., & Thibault, G. (2018). Dropping in on lipid droplets: insights into cellular stress and cancer. *Bioscience reports, 38*(5).

Sipeniece, E., Mišina, I., Qian, Y., Grygier, A., Sobieszczańska, N., Sahu, P. K., . . . Górnaś, P. (2021). Fatty Acid Profile and Squalene, Tocopherol, Carotenoid, Sterol Content of Seven Selected Consumed Legumes. *Plant Foods for Human Nutrition*, 1-7.

Smith, J., & Charter, E. (2011). *Functional food product development*: John Wiley & Sons.

Smýkal, P., Coyne, C. J., Ambrose, M. J., Maxted, N., Schaefer, H., Blair, M. W., . . . Besharat, N. (2015). Legume crops phylogeny and genetic diversity for science and breeding. *Critical Reviews in Plant Sciences, 34*(1-3), 43-104.

Sobolev, A. P., Testone, G., Santoro, F., Nicolodi, C., Iannelli, M. A., Amato, M. E., . . . Mannina, L. (2010). Quality traits of conventional and transgenic lettuce (Lactuca sativa L.) at harvesting by NMR metabolic profiling. *Journal of Agricultural and Food Chemistry, 58*(11), 6928-6936.

Solis, M. I. V., Patel, A., Orsat, V., Singh, J., & Lefsrud, M. (2013). Fatty acid profiling of the seed oils of some varieties of field peas (Pisum sativum) by RP-LC/ESI-MS/MS: Towards the development of an oilseed pea. *Food chemistry, 139*(1-4), 986-993.

Sosulski, F., Hoover, R., Tyler, R., Murray, E., & Arntfield, S. (1985). Differential scanning calorimetry of air-classified starch and protein fractions from eight legume species. *Starch-Stärke, 37*(8), 257-262.

Sosulski, F., & McCurdy, A. (1987). Functionality of flours, protein fractions and isolates from field peas and faba bean. *Journal of Food Science, 52*(4), 1010-1014.

Srivastava, A., & Prasad, R. (2000). Triglycerides-based diesel fuels. *Renewable and sustainable energy reviews, 4*(2), 111-133.

Tarranum, A., Chauhan, R., Kumar, R., & Chauhan, M. (2015). Comparison of Physicochemical Properties of Some Edible Oils. *Advertisement Tariff*, 28.

The Business Research Company, B. (2020). Oilseed Farming Global Market Report 2020-30: Covid 19 Impact And Recovery. Retrieved from https://www.thebusinessresearchcompany.com/report/oilseed-farming-global-market-report-2020-30-covid-19-impact-and-recovery

Tinto, W., Elufioye, T., & Roach, J. (2017). Waxes. In *Pharmacognosy* (pp. 443-455): Elsevier.

United States Department of Agriculture, U. (2021). U.S. National Plant Germplasm System. Retrieved from https://npgsweb.ars-grin.gov/gringlobal/site?id=26

Valdés, A., Beltrán, A., Mellinas, C., Jiménez, A., & Garrigós, M. C. (2018). Analytical methods combined with multivariate analysis for authentication of animal and vegetable food products with high fat content. *Trends in Food Science & Technology, 77*, 120-130.

van der Voet, H. (1994). Comparing the predictive accuracy of models using a simple randomization test. *Chemometrics and intelligent laboratory systems, 25*(2), 313-323.

Vance, J. E., & Vance, D. E. (2008). *Biochemistry of lipids, lipoproteins and membranes*: Elsevier.

Verheye, W. H. (2010). *Soils, Plant Growth and Crop Production-Volume III*: EOLSS Publications.

Vollmann, J., & Rajcan, I. (2009). Handbook of plant breeding. *Oil Crops*.

Walker, P., & Wood, E. (2010). *Physical science experiments*: Infobase Publishing.

Wang, T. L., Domoney, C., Hedley, C. L., Casey, R., & Grusak, M. A. (2003). Can we improve the nutritional quality of legume seeds? *Plant Physiology, 131*(3), 886-891.

Warkentin, T. D., Smýkal, P., Coyne, C. J., Weeden, N., Domoney, C., Bing, D.-J., . . . Boros, L. (2015). Pea. In *Grain legumes* (pp. 37-83): Springer.

Weiss, E., & Zohary, D. (2011). The Neolithic Southwest Asian founder crops: their biology and archaeobotany. *Current Anthropology, 52*(S4), S237-S254.

Welch, R. W., & Wynne Griffiths, D. (1984). Variation in the oil content and fatty acid composition of field beans (Vicia faba) and peas (Pisum spp.). *Journal of the Science of Food and Agriculture, 35*(12), 1282-1289.

Willcox, G., Buxo, R., & Herveux, L. (2009). Late Pleistocene and early Holocene climate and the beginnings of cultivation in northern Syria. *The Holocene, 19*(1), 151-158.

Wong, A., & Lucas-Torres, C. (2018). High-resolution Magic-angle Spinning (HR-MAS) NMR Spectroscopy. In.

Wu, D., & Sun, D.-W. (2013). Advanced applications of hyperspectral imaging technology for food quality and safety analysis and assessment: A review—Part I: Fundamentals. *Innovative Food Science & Emerging Technologies, 19*, 1-14.

Yadava, D., Vasudev, S., Singh, N., Mohapatra, T., & Prabhu, K. (2012). Breeding major oil crops: Present status and future research needs. *Technological Innovations in Major World Oil Crops, Volume 1*, 17-51.

Yi, Q., Zhao, Y., Huang, Y., Wei, G., Hao, Y., Feng, J., . . . Li, W. (2018). Life cycle energy-economic-CO2 emissions evaluation of biomass/coal, with and without CO2 capture and storage, in a pulverized fuel combustion power plant in the United Kingdom. *Applied energy, 225*, 258-272.

Yoshida, H., Tomiyama, Y., Tanaka, M., & Mizushina, Y. (2007). Characteristic profiles of lipid classes, fatty acids and triacylglycerol molecular species of peas (Pisum sativum L.). *European Journal of Lipid Science and Technology, 109*(6), 600-607.

Yunus, R., Rasheed, H. S., & Zulkifli, N. W. M. (2020). Wear and friction behavior of semi synthetic engine oil blended with palm oil/TMP ester and nano glass powder additive. *Jurnal Tribologi, 26*, 16-36.

Zohary, D., Hopf, M., & Weiss, E. (2012). *Domestication of Plants in the Old World: The origin and spread of domesticated plants in Southwest Asia, Europe, and the Mediterranean Basin*: Oxford University Press on Demand.