

Developing Macrolipidomics Profiling Methods for Dietary Food Samples

by

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AUTHOR'S DECLARATION

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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STATEMENT OF CONTRIBUTIONS

This thesis uses a lipidomic approach to characterize numerous lipid species in different food items (vegetable oils, a breakfast sandwich and meal homogenates). This research also involved food sample preparation and examined sample preparation and lipid extraction techniques. The sole author of this thesis, Tianchunlu Yu, designed the experiments with feedback from Ken Stark and prepared all the food items, processed the samples, isolated the lipids and performed the fatty acid analyses and processed all the data. Dani Chalil supervised and assisted with pulverization of frozen food homogenate sample preparation. Juan Jose Aristizabal Henao supervised and assisted with the addition of internal standards for mass spectrometry, ran samples on the liquid chromatograph tandem mass spectrometer and provided feedback interpretation of the mass spectrometry data.

Abstract

Diet has a major impact on health and disease and poor nutrition increases the risk of chronic disease. The amount and type of dietary fat has been a key target for dietary guidelines and health policy. Dietary fat in the food databases and on food labels is usually analyzed and described by the fatty acid composition of the fat, but dietary fat is a mix of fatty acyl specific species of triacylglycerols (TG) with minor contributions from other complex lipids such as phospholipids. The actual native lipid species information can be captured with a “macrolipidomic” approach that characterizes the individual fatty acyl species of the highly abundant lipids of a biological sample and provide additional information and insight about the dietary form and source of important nutritional fatty acids such as omega-3 polyunsaturates. The overall purpose of this thesis was to assess the ability of and identify challenges with the use of a macrolipidomic analytical approach for the characterization of dietary lipids by analysing food items and meals. Tandem mass spectrometry coupled to ultra-high-performance liquid chromatography was used to characterize the dietary lipid species in individual food items and meal homogenates and compared with traditional fatty acid profiles generated by gas chromatography-flame ionization detection and food profiles from the Canadian Nutrient File database. Analyses were performed on two highly consumed commercial plant oils (soybean and canola oils), a breakfast sandwich, and homogenates of traditional Canadian versions of breakfast, lunch, snack and dinner. The fatty acid profiles of the plant oils differed with 18:2n-6 (54 wt%) being the main fatty acid in soybean oil and 18:1n-9 (62 wt%) in canola oil but there were no fatty acids detected that were unique to an oil. Lipidomic analyses revealed the two oils shared only 56 identified acyl lipid species with soybean oil having 105 and canola oil having

105 unique lipids. For the sandwich and meal analyses, that the fatty acid content by biochemical determination was lower than the Canadian Nutrient File estimates. Blending and homogenizing large volumes of mixed food items was a challenge and requires additional processing strategies in the future that need to be evaluated. The macrolipidomic analysis of the breakfast sandwich and the meals confirmed phospholipid content in the diet with egg likely being the main source, but there were challenges with quantitation. Macrolipidomics estimated that 2.7% breakfast sandwich lipid was phospholipid while gas chromatography determinations estimated the 17% of the fatty acids were associated with phospholipids. The extent of the lipidomic profiling was also limited by challenges in making correct fatty acyl lipid species identifications as the processing software was unable to import MS/MS data acquired by data dependent acquisition. Despite this, it was estimated that in meals that mimic a typical Canadian diet, the most abundant lipid consumed was TG 18:1_18:1_18:1 at 12.6 g/d. These macrolipidomic studies provided a better understanding of the challenges in adapting lipidomic analyses to nutrient profiling. Advances in lipidomic methodology and data processing are still required and experiments assessing of food homogenization techniques and the impact of this sample preparation at the lipidomic level are necessary before large scale lipidomic profiling of the food supply is attempted.

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Table of Contents

AUTHOR'S DECLARATION	ii
STATEMENT OF CONTRIBUTIONS	iii
Abstract	iv
Acknowledgements	vi
List of Figures	xi
List of Tables	xii
List of Abbreviations	xiii
Chapter 1 Introduction	1
Chapter 2 Background and Databases	5
2.1 Lipids in Food Samples.....	5
2.2 Assessing Fat Intake in the Diet.....	10
2.3 Macrolipidomics versus Microlipidomics.....	13
Chapter 3 Foundations of Fatty Acid and Lipid Analyses.....	16
3.1 Lipid Extraction Techniques	16
3.2 Principles of Chromatography	17
3.3 Lipid Fractions Thin Layer Chromatography (TLC)	18
3.4 Derivatization of Fatty Acid Methyl Ester (FAME) for GC.....	18
3.5 Gas Chromatography – Flame Ionization Detection (GC-FID).....	19
3.6 Principles of High-Performance Liquid Chromatography	20
3.7 Principles of Mass Spectrometry.....	22
Chapter 4 General Methods and Materials	24

4.1 Chemicals and Materials	24
4.2 Instrumentation.....	24
4.3 Lipid Extraction.....	25
4.4 Thin Layer Chromatography.....	25
4.5 Fatty Acid Analysis.....	26
4.6 Lipidomic Analyses.....	26
4.7 Data Handling, Automated Lipid Identifications and Statistical Analyses.....	27
Chapter 5 Macrolipidomic Analysis of Soybean and Canola Oil.....	29
5.1 Rationale and Objectives.....	29
5.2 Hypotheses	31
5.3 Methods, Materials and Study Design	31
5.3.1 Sample Collection and Lipid Extraction	31
5.3.2 Fatty Acid Analysis	31
5.3.3 Macrolipidomic Profiling	32
5.4 Results	32
5.4.1 Fatty Acid Composition.....	32
5.4.2 Lipidomic Profile.....	33
5.5 Discussion	39
5.6 Conclusion.....	42
Chapter 6 Macrolipidomic Analysis of a Homemade Breakfast Sandwich	43
6.1 Rationales and Objectives	43
6.2 Hypotheses	44

6.3 Methods, Materials and Study Design	44
6.3.1 Sample Collection and Lipid Extraction	44
6.3.2 Nutrient Composition	44
6.3.3 Lipid Extraction	45
6.3.4 Fatty Acid Analysis	45
6.3.5 Macrolipidomic Profiling	46
6.3.6 Statistics	46
6.4 Results	49
6.4.1 Fatty Acid Composition of Total Lipids.....	49
6.4.2 Lipidomic Profile.....	50
6.4.3 Fatty Acid Composition of Triacylglycerols and Phospholipids.....	51
6.5 Discussion	56
6.6 Conclusion.....	60
Chapter 7 Macrolipidomic Analysis of Traditional Canadian Meals	62
7.1 Rationale and Objectives.....	62
7.2 Hypotheses	63
7.3 Methods, Materials and Study Design	64
7.3.1 Sample Preparation and Fatty Acid Analyses	64
7.3.2 Macrolipidomic Profiling	65
7.3.3 Statistics.....	65
7.4 Results	69
7.4.1 Fatty Acid Composition of the Meals.....	69

7.4.2 Lipidomic Profiles of the Meals	70
7.4.3 Comparing Lipidomics with Fatty Acid Analyses	72
7.5 Discussion	78
7.6 Conclusion.....	83
Chapter 8 General Discussion.....	85
8.1 Overview	85
8.2 Addressing the Challenges and Limitations.....	86
8.3 Conclusion.....	91
References.....	93

List of Figures

Figure 1.	Venn diagram of lipid species in soybean and canola oil	38
Figure 2.	Illustration of the workflow for the fatty acid and lipidomic analyses of a breakfast sandwich.	48
Figure 3.	Thin layer chromatography of a breakfast sandwich	54
Figure 4	Illustration of the workflow for the fatty acid and lipidomic analyses of traditional Canadian meals	68
Figure 5	Amount of total fatty acids in each meal and daily amount after blending versus pulverizing preparation as compared with estimates calculated from the Canadian Nutrient File (CNF) Database.	73

List of Tables

Table 1.	Fatty acid composition of soybean and canola oils	35
Table 2.	Percentage of lipid species with acyl identification in Soybean oil	36
Table 3.	Percentage of lipid species with acyl identification in Canola oil	37
Table 4.	The fatty acid composition of breakfast sandwich by biochemical analyses and by estimates from the Canadian Nutrient File	52
Table 5.	High abundant lipid species with acyl identification in breakfast sandwich	53
Table 6.	Fatty acid composition of the breakfast sandwich TG and phospholipids	55
Table 7.	Composition of the meals	67
Table 8.	Fatty acid composition of the meal homogenates as compared with estimates calculated from the Canadian Nutrient File Database	74
Table 9.	Abundances of different lipids classes in a commercial standard	75
Table 10.	Most abundant fatty acyl lipid species in the breakfast, lunch, dinner and snack meals	76
Table 11.	Fatty acyl lipid species identified in a traditional Canadian diet by abundance	77

List of Abbreviations

16:0	Palmitic Acid
18:0	Stearic Acid
18:1n-9	Oleic Acid
18:2n-6	Linoleic Acid
18:3n-3	Linolenic acid
22:3n-3	Docosatrienoic Acid
AOAC	Association of Official Analytical Chemists
DHA	Docosahexaenoic Acid (C22:6n-3)
EPA	Eicosapentaenoic Acid (C20:5n-3)
ESI	Electrospray Ionization
FAME	Fatty Acid Methyl Ester
GC-FID	Gas Chromatography-Flame Ionization Detection
HDL	High-density lipoprotein
HPLC	High-Performance Liquid Chromatography
HPLC-MS/MS	High-Performance Liquid Chromatography-Tandem Mass Spectroscopy
ISO	International Organization for Standardization
LDL	Low-density lipoprotein
m/z	Mass-to-charge ratio
MS/MS	Tandem Mass Spectrometry
PCA	Principal Component Analysis
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PS	Phosphatidylserine
PUFA	Poly Unsaturated Fatty Acid
QTOF	Quadrupole-Time Of Flight
RT	Retention Time
TG	Triacylglycerol

TLC	Thin Layer Chromatography
TLE	Total Lipid Extract
UHPLC	Ultra-high-Performance Liquid Chromatography

Chapter 1

Introduction

Lipids are a group of organic compounds that are insoluble in water but soluble in non-polar organic solvents, and together with carbohydrates and proteins constitute the main structural material for living cells. There are eight categories of lipids based on their chemically functional backbone and historical categorizations and include fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides [1]. Lipids are found in all plants and animals and are basic constituents of our diet. Although lipids are mainly thought of as a source of energy, they have important functional roles in biological processes as components of cell membranes, signaling molecules, cellular mediators [2]. As such, they can be considered to be either beneficial for health or contribute to various pathologies depending on the amount and type consumed [3]. For instance, high saturated fatty acid intakes are related to the development of obesity and chronic diseases such as coronary heart disease [4], whereas very low intakes may cause hemorrhagic stroke [5]. In contrast, increased omega-3 polyunsaturated fatty acid (PUFA) intakes are associated with numerous health benefits, such as decreasing the risk of cardiovascular diseases, supporting neural development and reducing the risk of inflammatory based diseases [2]. The amount and type of dietary fat is a key target of major dietary guidelines and health policy. Therefore, it is important to examine the intake of dietary fat at a detailed level for insights on how it impacts human health [3].

Traditionally, lipid nutrition has focused on the fatty acid content and composition of food, but fatty acids are found largely as components of complex glycerol and glycerophospholipids.

Analytical advancements within the field of lipidomics can allow these complex lipids in the diet to be measured. Initially lipidomic endeavors set out to identify and quantify the lipidome of cell systems to provide better understanding of function and interactions of very specific lipids in cell signaling and biochemical pathways [6, 7]. These analytical techniques can be applied to characterizing the food supply, so adapting lipidomic approaches to measuring lipids in food will be examined in this thesis.

Advances in mass spectrometry have played a central role in the emergence lipidomics, and developments in mass spectrometry continue to impact the field of lipidomics [8, 9]. Traditionally, targeted and untargeted lipidomic strategies have been employed using MS-based platforms. The targeted approach is used for determining the amount of a known set of specific lipids based on prior knowledge of the sample. Since only the targeted lipids are measured, information about other “unknown” lipids that could potentially be important is not collected. Targeted analyses are, however, easier to complete and true quantitation is possible when the appropriate internal standards are available [10, 11]. In contrast, untargeted analyses usually attempt to measure all the detectable lipids in a sample. This unbiased approach is preferred for the discovery of novel lipids. However, the effective detection of lipid species in low abundance and accurate quantification of all lipids by untargeted methods remains challenging [12]. Recently, due to the diversity in the types and roles of lipids in biology and analytical challenges, lipid analytical strategies based on the amount or abundance of lipids in a biological system/sample have been proposed with “macrolipidomics” defining approaches focused on highly abundant lipids and “microlipidomics” defining approaches focused on low abundant, but often very bioactive lipids [12].

Dietary fat is mainly in the form of triacylglycerols (TG) with minor contributions from other lipids. As TG are anhydrous, they can be stored in biological systems without water and therefore serve as a compact source of energy. It has been estimated that 95% of dietary fat is TG, with phospholipids contributing 4.5% and sterols and other lipids accounting for the remaining 0.5% [13], but empirical data is limited and the variation in the type of lipid classes is largely unknown. The nutritional quality of dietary fat is largely defined by fatty acid analyses [2] that tend to be optimized to extract nonpolar TG from food samples [14] and not necessarily the recovery of polar lipids such as phospholipids [15]. This is particularly important as fatty acids with potent bioactive properties in signaling pathways such as arachidonic acid and docosahexaenoic acid (DHA) tend to be found mainly in the phospholipids of cell membranes rather than TG [16]. For example, DHA intake has been associated more strongly with protein intake (rich in cell membranes containing phospholipids), than with actual fat intake as fat intake is mainly TG from seed oils, the adipose of meats, and dairy fats [17]. A macrolipidomic approach to food analysis would provide information about the type of lipids consumed in their natural molecular form and potentially link specific fatty acids to specific lipid classes. Understanding the molecular format of the parent lipids of the food supply, could lead to improved monitoring of the food supply, novel developments in enriching foods with lipids, and establishing unique lipidomic profiles of foods could enhance food traceability efforts [18].

This thesis aimed to assess the ability to use lipidomic analyses for the assessment of nutritional fat. The overall hypothesis is that “food lipidomics” can replace traditional fatty acid analyses to define and characterize nutritional fat, but there will be challenges to define. As such, the goal of this thesis was to use a defined lipidomic approach and apply it to the analyses

of a food component, a food item, and meals that mimic the daily intake of Canadians to generate preliminary data and define the analytical challenges that need to be overcome. Macrolipidomic and fatty acid analyses were performed on canola and soybean oils (simple food components), a homemade breakfast sandwich (common food item) and breakfast, lunch, snack and dinner meal homogenates (based on traditional Canadian daily dietary intakes). These traditional Canadian meals reflect the daily diet of a typical Canadian male between 20–64 years of age, as outlined by Health Canada's Overview of Canadians' Eating Habits [19]. Lipids were extracted and analyzed by tandem mass spectrometry coupled to ultra-high performance liquid chromatography [20] and the fatty acid compositions were determined by gas chromatography-flame ionization detection [21]. The fatty acyl lipid species were identified preliminarily using automated software followed by confirmation with manual inspection of the mass spectra. These lipidomic approaches can enhance our knowledge on dietary fat intake at the level of lipid classes and identify the most common fatty acyl species in human diet and have the potential to be adapted to other types of food items and samples. The lipidomic profiles of the plant oils could serve as references for nutrient databases in the future.

Chapter 2

Background and Databases

2.1 Lipids in Food Samples

Lipids are generally defined as those components which are soluble in organic solvents but are insoluble in water. This group of substances includes free fatty acids, triacylglycerols (TG), diacylglycerols, monoacylglycerols, phospholipids, sterols, and fat-soluble vitamins (such as vitamins A, D, E, and K) [22]. Lipids are one of the main constituents in foods and are essential in our diet for diverse reasons. They are a major source of energy but also serve as essential nutrients. Lipids can act as structural components of the cell membranes and serve as substrates for signaling molecules. Dietary fat intake also aids the absorption of the lipid soluble vitamins A, D, E and K. In many foods, lipid components also play an important role in determining overall physical characteristics such as flavor, texture, mouthfeel and appearance. Lipids also influence food stability and shelf-life as fats can be susceptible to lipid oxidation, resulting potentially harmful products and the formation of odors and off tastes [23]. Because each type of lipid can have a different fatty acid profile which determines the exact nature of its nutritional and physiochemical properties, it is important to fully characterize all highly abundant lipid species in food samples.

The lipid portion of food contains a complex mixture of different types of molecules. Even so, TG are the main component of most foods and main carriers of energy and fatty acids [24], typically accounting for more than 95% of total lipids, which is about 42-73g intake per day depending on age and sex [25]. TG are esters of three fatty acids with a glycerol molecule and

are therefore nonpolar lipids that can be stored without water [22]. The fatty acyls within TG vary in chain length, degree of unsaturation and position on the glycerol molecule resulting in different “acyl” species of TG. However, while there are a large number of possible TG species, fatty acids of 16-18 carbon chain length (ex. 16:0, 18:0, 18:1n-9, 18:2n-6) tend to be highly abundant in TG [26]. As a result, the distribution of the amounts of specific acyl species of TG is skewed with a subset of species being found in high concentrations and numerous other species found in low concentrations in food products. Related glycerolipids, monoacylglycerols (MG) and diacylglycerols (DG), have esters of one and two fatty acids on the glycerol molecule, respectively. They are present in small amounts in lipids of both animal and plant origin, but account for about 75% of the world production of emulsifiers for food purposes [27].

Most fatty acids in food are found as fatty acyls of complex lipids. While, free fatty acids in food can be found, they are mainly produced by the hydrolysis or oxidation of TG, DG and phospholipids during long-term storage or processing at high temperatures during heating or frying that releases the fatty acyls from their glycerol backbones [28]. There are basically 3 different groups of fatty acids found in food: saturated, unsaturated and unsaturated trans fatty acids. The amount saturation versus unsaturation in the fatty acyls directly influence the physical properties of food fat such as solid versus liquid states, and stability/shelf life, but can also have different impacts on human health [23]. Saturated fats are defined as fatty acids that contain no double bonds. They are found in high amounts in dairy products, including butter, cheese and whole milk and animal-based foods, including beef, chicken, lamb, pork. They are also found in palm oil, coconut oil, lard and shortening. The consumption of saturates as compared with unsaturates are believed to increase the risk of developing coronary heart disease by increasing

triacylglycerol levels, low density lipoprotein (LDL) concentrations, and the ratio of LDL to HDL (high density lipoproteins) in blood, and promoting clotting and vascular smooth muscle cell proliferation [29]. Unsaturated fatty acids with cis (*Z*) configuration are generally categorized as polyunsaturated and monounsaturated fat, with polyunsaturated fats further defined as omega-3 and omega-6 polyunsaturated fatty acids based on the location of the carbon-carbon double bonds closest to the methyl end of the fatty acid. Monounsaturated fatty acids are found in high amounts in avocados, nuts, seeds, and vegetable oils including olive, canola, peanut, sesame, safflower, and sunflower [30]. The polyunsaturates are also found largely in nuts and seeds oils with omega-6 polyunsaturates found in high amounts in corn, safflower, sunflower and soybean oils [30]. Soybean oil contains some omega-3 polyunsaturates but considerable amounts of n-6 polyunsaturates, while canola and flaxseed oils contain considerable omega-3 polyunsaturates [30]. Fatty fish such as herring, mackerel, salmon and trout are good sources of the very long chain omega-3 polyunsaturates, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [31]. Both polyunsaturated and monounsaturated fatty acids decrease circulating LDL-cholesterol when substituted for saturated fats in the diet and potentially reduce the risk of coronary heart disease [32]. When unsaturated fatty acids contain one or more non-conjugated double bonds in a trans (*E*) configuration, they are considered as a distinct group commonly referred to as “trans” fatty acids. Trans fatty acids have chemical/physical properties that tend to resemble saturated fatty acids, but their metabolism differs from the other fatty acids. They can be found naturally in some animal-based foods such as beef, lamb and dairy products as a result of bond rearrangement in ruminant stomachs, but the main source in the food supply was through the hydrogenation of vegetable oils to improve the shelf life and texture of food such as hard

margarines and baked foods [33]. However, the consumption of trans fats was found to increase LDL-cholesterol and reduce HDL-cholesterol levels in blood and increase the risk of cardiovascular disease [34]. Reducing the amount of trans fat in the global food supply is currently an ongoing effort [35].

As the major component of cellular membranes, phospholipids are a component of food, but due to their different physiological role, they have a different structure than TG. Specifically, phospholipids are polar lipids with a hydrophilic head group and hydrophobic “tails” which are fatty acids. The most abundant phospholipids are the glycerophospholipids that contain a glycerol backbone, two fatty acids and a phosphate group, which can be linked to compounds with a hydroxy group such as choline, ethanolamine, serine or inositol [22]. As such, there are “classes” of phospholipids that vary by their headgroup, and then within each phospholipid class, the type of fatty acyls and their arrangement can differ [36]. While 16-18 carbon chain fatty acids are also abundant in glycerophospholipids, there can be a high content of very long chain polyunsaturated fatty acids (LC-PUFA) or “highly unsaturated fatty acids” (≥ 20 carbons, ≥ 3 carbon-carbon double bonds, HUFA), particularly in the sn-2 position on the molecule [36].

The dietary intake of phospholipids in westernized populations has been estimated at 2-8g/d [37] depending on the type of food consumed. All foods originating from living plants and animals contain phospholipids since they are integral components of cell membrane. However, the phospholipid content of different food products can vary dramatically in amount, class type and fatty acid composition [37]. In general, foods rich in phospholipids include red meat, fish, organ meats such as liver and kidney, eggs and certain dairy products [37]. In addition, phospholipids or “lecithins” commercially prepared from soy and corn oil are used as emulsifiers

by the food industry in manufactured food products such as margarines, baked goods, icing, non-dairy creamers, confectionery products, and ice cream [38].

Other dietary lipids include cholesterol and the fat-soluble vitamins. Cholesterol is an important component of animal cell membranes and a precursor to a variety of biomolecules, such as steroid hormones, bile acids and vitamin D₃ [39]. The characteristic structure of cholesterol is a fused four hydrocarbon ring (called a steroid core) with a hydrocarbon tail. A fatty acid can be esterified to the polar hydroxy group on free cholesterol to form nonpolar cholesteryl esters largely by lecithin-cholesterol acyltransferase as part of reverse cholesterol transport mediated by HDL [40]. As such, free cholesterol and cholesteryl esters are transported through blood as components of chylomicrons, very low-density lipoproteins (VLDL), LDL, and HDL. Serum cholesterol levels below 200 mg / dL are associated with significantly increased overall life expectancy [40]. The food sources of cholesterol are mainly from animal tissues such as meat, dairy products, poultry, shellfish and eggs [40], but serum cholesterol levels are also greatly influenced by the endogenous synthesis of cholesterol [40]. Finally, the fat-soluble vitamins include vitamins A, D, E, and K. They are only needed in small amounts and they are more efficiently absorbed through the intestinal tract in the presence of other dietary fat. They can be stored in the body for long periods of time and pose a greater risk for toxicity than water-soluble vitamins [41].

2.2 Assessing Fat Intake in the Diet

Dietary intake assessment evaluates the food intake and dietary pattern of an individual or a population. In general, the main goal of measuring dietary intake is to obtain quantitative information about the amount of energy and nutrients that an individual consumes and is available for metabolism. Dietary intake habits can also be linked to disease risk and/or biochemical measures of nutrient status (biomarkers). Dietary fat intake has been identified as a main contributor to noncommunicable diseases such as cardiovascular disease [42].

Dietary assessment relies on the availability of precise techniques to accurately estimate nutritional intake [4]. Assessing diet intakes can be completed prospectively or retrospectively. Prospective methods assess food intake as the food is consumed and consists mainly of different forms of food records, although duplicate diet collections are used on rare occasions [43]. Retrospective methods assess food intake through past recollection and include 24-hour recalls, food frequency questionnaires (FFQ), and dietary histories [44]. Retrospective methods do not affect respondents' food selections and dietary patterns and there is less burden on both respondents and researchers as compared with prospective methods. However, retrospective methods rely heavily on the respondent's memory, and recall bias may exist since respondents can choose to report the food they would like to present during the recall. These methods also rely on the participant's ability to describe the food and to estimate its portion size [43]. In contrast, prospective methods can provide precise information on food amounts with less risk of omitted food items, but there is a tendency for respondents to change their diet to reduce recording burden and/or improve the quality of their diet [4]. Prospective dietary assessments also rely heavily on respondents with good literacy and numeracy skills.

Assessing dietary fat or fatty acid intake is particularly difficult for numerous reasons. It is challenging to obtain information on the type and amount of fat used in food preparation for those who do not prepare their own food. In addition, there are inconsistencies in the food composition from season to season, such as the content of linoleic acid in milk, which may affect the calculation of fatty acid intake [45]. Moreover, oil manufacturers may change the component of oils based on production costs and there can be changes in the fatty acid composition of food during cooking and processing [18]. Given that there are numerous individual fatty acids in the diet with considerable differences in amounts, it can be challenging to maintain fatty acid information in nutrition databases [45].

Food labels provide a direct way to deliver product information from the producer to the consumer to allow the consumer to assess their own dietary intake. In Canada, almost all packaged food labels must have a list of ingredients, net quantity, calories and a nutrient fact table. In terms of fats, the nutrition facts table provides the % Daily Value (% DV), the category (i.e. saturated and trans-fat) and the amount of total fat. The % DV used in nutrition labeling is based on recommendations for a healthy diet including 30% of calories from fat, which translates to 65 g from a 2000 calorie reference diet. In general, the total fat on the food label is obtained by gravimetric methods after isolating the fat. The food is mechanically broken down and the lipids are extracted with solvents using procedures such as the ISO (International Organization for Standardization) conventional Soxhlet method with the fatty acid composition of the lipid extract being determined by gas chromatography (GC) analysis [46]. These methods often rely on the use of nonpolar solvents such as hexane and only provide information about the fatty acids in foods while the information about the complex lipids and the arrangement of the fatty

acids in these lipids is lost. Overall, food label and nutrient database fat determinations are biased for assessing the fatty acids associated with TG. TG do represent the most relevant lipid for calculating the energy in a food [47], but the assessment of fatty acids known to preferentially partition to polar lipids such as EPA and DHA may not be accurate.

The Canadian Nutrition File is a standard reference food composition database that reports the amount of nutrient content of foods commonly consumed in Canada [48]. As mentioned previously, many factors affect the variability of fat composition in the food supply and the high cost of laboratory analysis can make achieving accurate and up to date product-specific information challenging. Direct chemical analysis is the most accurate method for determining fat composition, and while this is usually done for individual food items with consideration of different cooking and preparation methods, the fats of complex dishes are usually calculated from the amount of fat in each subcomponent [49, 50]. In the Canadian Nutrient File, the average for a generic representation of food is described. These generic values are derived from a combination of similar brands. Individuals who are looking for brand-specific nutrient data need to look for the Nutrition Fact table found on all prepackaged foods sold in Canada [51]. In particular, micronutrients and individual fatty acids such as EPA and DHA, which are found only in a small number of foods and in small quantities, can be poorly represented in the Canadian Nutrient File and other nutrient databases [52].

Duplicate food collection is a dietary assessment approach that involves collection of a duplicate of all food and beverages consumed during a specific time period. The duplicated portions are set aside, weighed, and sent to the laboratory for chemical analysis to quantify actual nutrient content. It is extremely burdensome and costly, but it is a useful approach to estimate

intakes of targeted and specific nutrients where the quality of the data is inconsistent due to challenges in measuring the nutrient, or with rapid changes in the level of the nutrient in the food supply (ie. appearance of novel functional foods). For example, in 2004 the DHA intake of pregnant Canadian women was determined by food duplicate analysis as DHA intake was being promoted for fetal and infant brain development, but data in the food databases at the time was unreliable [53]. Compared with other methods, food duplication provides a more accurate representation of quantitated intake because it does not rely on respondents' memory, portion size estimates and nutritional information derived from the nutrition databases, but the burden of collecting the food may change dietary habits [54]. Previous studies examining micronutrients intake in the diet have shown that duplicate diet techniques accurately estimates low abundant nutrients that are hard to quantify [55]. A food duplicate approach would be a practical way to start initial assessments of the food supply at the lipidomic level.

2.3 Macrolipidomics versus Microlipidomics

Lipidomics is an emerging field that originally focused on measuring global molecular lipids in cells and tissues, as well as studies of their biological activity, subcellular localization and tissue distribution [56]. It can be applied to food research, as the analysis of lipid components from different food sources can be used for the evaluation of food safety and quality, and for assessing the biological function and nutritional level of lipids in living organisms [57]. As an emerging field, there is a lack of standardization in the field and different analytical approaches are not consistently defined. Due to the lack of defined terms to describe

different aspects of lipidomics across interdisciplinary researches, it has been proposed that lipidomics can be divided into macrolipidomic and microlipidomic strategies [12].

Macrolipidomics is defined as an analytical approach that aims to comprehensively characterize all the highly abundant lipid species of a biological sample and microlipidomics is an approach that examines low abundant lipids with potent bioactivity which often requires targeted analyses [12]. In addition to these categories, it was also proposed that simple terms be used to identify the level of information that is being reported about the lipid molecules. With lipidomics, different analytical techniques can provide different levels of information about the molecular structure of a molecule [58]. The most basic level of information of a lipid molecule is the total number of carbon atoms and the number of carbon-carbon double bonds across all fatty acyl chains can be deduced by using full-scan accurate mass data and has been described as the sum composition of a lipid molecule or “brutto” level of information [59]. It has been proposed that the terms “medio”, “genio” and “infinio” levels of information also be used when increasing levels of molecular information is known about the lipids [12]. At the medio level, MS/MS spectra can be used to discriminate between isomeric species and recognize the individual fatty acyl constituents. Genio level is used when different “regioisomers” or the precise position of the fatty acyls on the complex lipid backbone is defined. Finally, the infinio level is used when information of the positionality and configuration (cis/trans) carbon-carbon double bonds of each fatty acid constituent is known. For nutritional research that is based on fatty acid compositional data, techniques that provide specific fatty acyl information about the lipid molecules should be used. Therefore, the “medio” level of lipidomic information is required to allow new macrolipidomic information to be compared with existing fatty acid data about the

food supply and lipid nutrition. MS/MS techniques have been used for food analysis previously, but mainly with approaches that focused on the characterization of TG in seeds, nuts and their corresponding oils [57]. Using a macrolipidomic approach rather than a TG targeted approach can provide a more comprehensive examination of dietary lipids. While most dietary fat is TG, macrolipidomics can also assess other lipids such as phospholipids, which revealed important details about food fat and the source of individual fatty acids that are important for health.

Chapter 3

Foundations of Fatty Acid and Lipid Analyses

In recent years, lipid profiling has been used for research insights in a variety of disciplines including lipid biochemistry, clinical biomarker discovery and pathology [60]. Numerous analytical techniques are used to assess lipids in biological samples [61] and the advancement of the lipid research field is linked to the development of analytical technologies, particularly the recent advances in liquid chromatography mass spectrometry and chemometric methods [60]. This chapter aims to give an overview of the fatty acid and lipid analytical techniques used in this thesis. Specifically, sample extraction and derivatization techniques, the chromatographic separation techniques of gas-, liquid-, and thin layer chromatography, and the detection of compounds by mass spectrometry are examined.

3.1 Lipid Extraction Techniques

Extracting lipids from complex sample matrices is usually the first step for lipid analyses. The three widely used extraction methods are the Association of Official Analytical Chemists (AOAC) method used commonly in industry settings, the International Organization for Standardization (ISO) method used for nutritional labelling purposes, and the Folch method used mainly for nutritional and biochemical research [62]. As triacylglycerols are the major fatty acid-containing lipids in foods, both the AOAC and ISO methods are designed to mainly extract this non-polar lipid, and largely use non-polar solvents such as petroleum ether (AOAC) and hexane (ISO) [46]. In contrast, Folch based methods use a 2:1 ratio of chloroform and methanol to extract both polar and non-polar lipids [63]. Therefore, for comprehensive extraction of most

lipids, a Folch based method was used to isolate lipids from samples for analyses performed in this thesis.

3.2 Principles of Chromatography

Chromatography is based on the principle of applying a mixture of substances to a stationary phase that separates these molecules from each other as they move along with a mobile phase [64]. The stationary phase can be solid particles, or a layer of a liquid coated on a solid support, while the mobile phase is a liquid or a gaseous component that moves through or across the stationary phase. The analyte can move with the mobile phase with separation depending on molecular properties related to adsorption (liquid-solid separation), partition (gas-liquid separation), affinity or their different molecular weight. Because of these differences, some of the components in the mixture have a longer retention time in the stationary phase, while other components pass through with the mobile phase faster [64]. While chromatography separates the individual components of an analyte, measuring the amount or concentration of each component requires the use of a detector that is suitable for the chemical structure of the component (ex. UV-visible, fluorescent, flame ionization or mass spectrometry detectors) [64]. When chromatography is combined with mass spectrometry or nuclear magnetic resonance detection techniques that are capable of precise determinations of the chemical structures, numerous molecular compounds can be identified and characterized which is the basis of powerful analytical approaches such as lipidomics. Biological lipids are almost always complex mixtures; therefore, chromatography separates the lipids by class and allows the determination of complete profiles of lipid molecules. Thin layer chromatography (TLC), gas chromatography (GC) and high-pressure liquid chromatography (HPLC) can be used to separate lipids.

3.3 Lipid Fractions by Thin Layer Chromatography (TLC)

TLC is traditionally used for routine separation and identification of lipid classes, such as triacylglycerols, diacylglycerols, monoacylglycerols, cholesterol, and phospholipids. TLC consists of a coated plate placed vertically in a tank with a small amount of solvent touching the bottom of the plate. The TLC plate is coated with an appropriate adsorbent material such as silica gel that serves as the stationary phase, and organic solvents such as heptane and/or diethyl ether move up the plate by capillary action and act as the mobile phase [65]. Lipid extracts are spotted on the plate above the solvent line and separation takes place on the silica gel adsorbent layer on the glass plate as the solvent moves upward. Depending on the affinity of the lipids for the silica adsorbent and for the solvent mixture, the lipids move up at different rates with the mobile phase [66]. At the end of the separation, the plate is dried, and the lipid class spots can be visualized under ultraviolet light with dichlorofluorescein. The presence of lipids can be identified by comparing the distance traveled by the spots to reference standards of known composition. Spots containing the lipids can be scraped off the plate and the separated lipids can be further analyzed for quantitative determinations using techniques such as GC or mass spectrometry. TLC is relatively inexpensive, requires little infrastructure and modest technical skills for rapid separation of lipids in fatty food samples.

3.4 Derivatization of Fatty Acid Methyl Ester (FAME) for GC

Intact complex lipids such as triacylglycerols and phospholipids are not volatile and are not suitable for GC analysis directly (which requires lipids to be volatilized to a gas in the instrument). However, the fatty acyls of complex lipids are volatile enough for GC analysis if they are removed from the parent lipid. The polar carboxyl headgroup of free fatty acids tend to

be retained in GC capillary columns for long periods of time resulting in poor separations, but derivatizing fatty acids to non-polar FAME greatly improves GC analysis [67]. Traditionally, preparing FAME required a hydrolysis procedure to release fatty acids from the complex lipids, followed by an esterification step to add the methyl group to the carboxyl. To increase sample throughput, several "transesterification" protocols have been developed where hydrolysis and esterification occur in a single reaction [46]. In this thesis, boron trifluoride (BF₃), a rapid acting acid catalyst was used in the presence of methanol to transesterify fatty acids directly from the parent lipid and to esterify any free fatty acids into methyl ester derivatives [46]. After this derivatization, FAME were collected in a suitable organic solvent (hexane), stored and then injected into a GC for analyses, which will be discussed in detail in section 3.5.

3.5 Gas Chromatography – Flame Ionization Detection (GC-FID)

The gas chromatograph is essentially an oven in which a capillary column containing a liquid stationary phase adsorbed on an inert solid silica surface is installed between an inlet and a detector. An inert gas such as He, N₂, or H₂ flows through the column under high pressure and serves as the mobile phase. As such, gas chromatography is actually "gas-liquid" chromatography. The sample to be analyzed is introduced into the heated inlet where it is vaporized, and then the vapor enters the column. The components contained in the sample partition between the mobile gas phase and the liquid stationary phase of the column at different rates and thereby reach the detector at different "retention times". Gas chromatography is a robust and highly sensitive technique that can finely separate and quantitate small volatile molecules [64].

Gas chromatography coupled to flame ionization detection (GC-FID) is the most commonly used technique for fatty acid analysis [68]. The accuracy of the analysis depends on several factors, including sample preparation, injection procedures, column specifications, oven and FID temperatures, type of gas and flow rates, and the FID detector frequency. For fatty acid analysis, the FAME in a suitable organic solvent (heptane or hexane) are injected into the GC. Usually, a Carbowax type column is used where the fatty acids with longer and more unsaturated carbon chains are retained longer, although very high polarity columns are used on occasion when separating complex positional and geometric isomers is desired [68]. As combustible hydrocarbons, when the FAME reach the detector, they are readily ionized by a hydrogen flame and the ions are captured as an electrical signal in a linear response to their mass amount that is represented in the form of a chromatogram. Ideally, each of these peaks represent an individual fatty acid but co-elution can occur and should be monitored. An external standard of known mixed FAME is analyzed to establish retention times for and to check the resolution of each fatty acid. A known amount of internal standard that does not naturally occur in the sample can also be added to a sample before analysis to be used as a reference standard to determine the concentrations of the fatty acids within a sample.

3.6 Principles of High-Performance Liquid Chromatography

High performance liquid chromatography (HPLC), also known as high pressure liquid chromatography, is an analytical method in which a sample dissolved in a solvent is separated into its subcomponents for quantitative and/or qualitative purposes. HPLC and the more recent Ultra High Performance Liquid Chromatography (UHPLC) have the same principle, which rely on pumping liquid samples under high pressure (HPLC up to 6000 psi, UHPLC up to 15000 psi)

through a narrow column [69]. The basic difference is the column material particle size for UHPLC is smaller (less than 2- μm) than HPLC (3- μm – 5- μm) [69]. The column particles serve as the stationary phase and the solvent pumped through the column is the mobile phase. HPLC and UHPLC methods can be divided into normal phase or reverse phase. Normal phase HPLC consists of non-polar (non-aqueous) mobile phases with polar stationary phases in the column, and reverse phase HPLC uses an aqueous solvent system with a non-polar surface lining inside the column. Reverse phase has become the most common approach.

When a sample is introduced into one end of the column, the mobile phase system continuously pumps solvent at a specified flow rate to help direct the sample through the column. A single solvent system can be used (isocratic) but often mixtures of solvents are used with programming controlling the solvent gradients. As compounds are separated and emerge from the column, each component can be identified using detectors which can employ various techniques with the most common detector being a UV lamp in a Photodiode Array (PDA). The development and use of electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) to ionize the compounds enable HPLC to be coupled to mass spectrometers and is a technique that has rapidly increased.

In lipidomics, most HPLC- and UHPLC-based lipidomics methods employ a reverse phase C18 column because of their higher reproducibility and wider applicability compared to normal phase HPLC. As a result, polar molecules in the mobile phase pass through the column and are eluted first, and non-polar molecules in the polar mobile phase tend to adsorb to the hydrophobic stationary phase. These non-polar molecules can then be eluted from the column by decreasing the polarity of the mobile phase using an organic (non-polar) solvent, which reduces the

hydrophobic interactions [70]. Separation then occurs based on the polarity of different lipid species that is influenced by the number of fatty acyl components (i.e., monoacylglycerol versus diacylglycerol versus triacylglycerol), fatty acyl chain length, the degree of unsaturation, and the phospholipid head groups. In some cases, it has also been shown that specific reverse phase methods have the ability to resolve lipid species by sn-glycerol regioisomeric conformation [12, 71, 72]. More specialized methods also exist, and the use of columns with different C18 chemistry and bond phases can change the selectivity of the analysis.

3.7 Principles of Mass Spectrometry

Mass spectrometry (MS) is an analytical technique that provides both qualitative and quantitative information of molecules of interest by measuring the mass-to-charge ratio of ions. The results are typically presented as a mass spectrum, which is used to determine the masses of molecules and to elucidate the chemical identity or structure of molecules. Mass spectrometry is utilized in many different fields and is applied to pure samples as well as complex mixtures. Modern mass spectrometry relies upon three basic concepts: ion evaporation and generation, ion separation, and ion detection [73]. The target molecules for analysis are first introduced into the ionization source of the mass spectrometer where they are ionized to obtain positive or negative charges. Then the ions travel through the mass analyzer and reach different parts of the detector based on the mass/charge (m/z) ratio. The ions are then detected and converted to an electrical signal that can be recorded [73]. The signal graphically displays as a mass spectrum showing the relative abundance of the signal based on its m/z ratio.

There are four main types of mass spectrometers that process and separate ions: quadrupole mass filters, time of flight (TOF) mass analyzers, linear ion traps, and Orbitrap™ analyzers. In

addition, there are hybrid instruments that use more than one ion separation and can combine different techniques to achieve tandem mass spectrometry (MS/MS). Common examples are triple quadrupole (QQQ), quadrupole time-of-flight (QTOF), and quadrupole-orbitrap instruments. In MS/MS, the analyte ion of interest (commonly referred to as the precursor ion) is selected by the first quadrupole mass analyzer and is collides with a gas (usually argon or nitrogen) in the collision cell for further fragmentation. This process is called collision induced dissociation (CID) and the resulting product ions are related to the structure of the compound and can be monitored by the second mass analyzer to provide addition information about the molecule. In this thesis, a quadrupole time-of -flight instrument is employed (QToF; Waters Corporation, Milford, MA, USA). This MS/MS instrument has high resolution, high sensitivity and high mass accuracy which makes it a powerful technology for fragment ion scanning required to identify fatty acyl chains on complex lipids.

Chapter 4

General Methods and Materials

4.1 Chemicals and Materials

All solvents, including chloroform, methanol, isopropanol, n-butanol, acetonitrile and hexane (HPLC grade or higher) were purchased from Thermo Fisher Scientific (Mississauga, ON, Canada). Citric acid, disodium phosphate, ammonium formate, formic acid, and 14% BF_3 in methanol were purchased from Sigma-Aldrich (Oakville, ON, Canada). All lipid standards used for peak area normalization were purchased from Avanti Polar Lipids (Alabaster, AL, USA).

4.2 Instrumentation

Gas chromatography analyses were performed using a Varian 3900 gas chromatograph equipped with a DB-FFAP 15 m x 0.1 mm x 0.1 μm film thickness nitroterephthalic acid-modified polyethylene glycol capillary column (J&W Scientific from Agilent Technologies, Mississauga, ON, Canada).

Tandem mass spectrometry (MS/MS) coupled to ultra-high-performance liquid chromatography (UHPLC) analyses were performed using a Waters Acquity UHPLC system coupled to a Waters Synapt G2Si Quadrupole-Time-of-Flight mass spectrometer (QToF; Waters Corporation, Milford, MA, USA). The columns used included a Waters Acquity UHPLC Charged Surface Hybrid (CSH) 150mm x 2.1mm x 1.7 μm and a Waters Acquity UHPLC Ethylene Bridged Hybrid (BEH) 100mm x 2.1mm x 1.7 μm setup in series. This serial column coupling setup has been demonstrated to improve TG species separation [74].

4.3 Lipid Extraction

In general, total lipid extracts were obtained from samples (soybean and canola oils, breakfast sandwich and meals) using a modified Folch-based protocol [63]. In brief, samples were mixed with 3mL of 2:1 chloroform/methanol (v/v) containing the appropriate internal standard(s). This was followed by vortexing for 1min, the addition of 500 μ L of 0.2M NaH₂PO₄ buffer, inversion, and centrifugation at 1734rcf at room temperature for 5min. The organic layer containing the lipids was then collected and the aqueous layer was reextracted by adding 2 mL of chloroform, vortexing and then centrifuging again. The second organic layer was collected and combined with the initial collection. The combined lipid extracts were dried under N₂ gas, reconstituted in chloroform and stored at -80°C until analysis. The total lipid extracts were aliquoted for fatty acid or lipidomic analyses. The fatty acid analyses were completed on the total lipid extracts and/or on isolated lipid classes after separation using thin layer chromatography (TLC).

4.4 Thin Layer Chromatography

A portion of the total lipid extracts were dried down, reconstituted into 50 μ L of chloroform, and then loaded on to a TLC plate (Silica Gel G, 20 x 20 cm, 250 μ m, Analtech Inc., Newark, DE). Plates were resolved in a TLC tank containing a mixture of 60:40:2 heptane:diethyl ether:acetic acid (v/v/v) as the mobile phase. After separation, the plates were dried, and the lipids were visualized by treating with dichlorofluorescein and exposing to ultraviolet light. Lipid classes of interest were collected for fatty acid analysis as described below.

4.5 Fatty Acid Analysis

Fatty acid analyses were completed using GC-FID. The total lipid extracts, and/or isolated lipids classes after TLC were dried under N₂ gas. Transesterification to fatty acid methyl esters (FAME) was completed by adding 1.0 mL 14% BF₃ in methanol with 300 mL hexane, vortexing for 1 min and then block heating for 1 hour at 95 °C [74]. Samples were then allowed to cool to room temperature, after which 1mL of ddH₂O and 1mL of hexane were added, followed by vortexing for 1min and centrifugation for 5min. The hexane layer containing the FAME was collected and evaporated under N₂ gas. FAME were resuspended in 65µL of heptane and briefly stored in vials at 4 °C until analysis.

FAME in heptane were analyzed by GC-FID using a Varian 3900 gas chromatograph. Hydrogen was used as the carrier gas at a flow rate of 0.5 mL / min with a split ratio of 100:1. The inlet temperature was 250 °C, and the detector was 300 °C with a sampling frequency of 50 Hz. An air flow rate of 100 mL / min, hydrogen flow rate of 30 mL / min, and nitrogen purge gas flow rate of 25 mL / min was used. The initial oven temperature was 150 °C for 0.25 min, with a 35 °C / min ramp to 200 °C, an 8 °C/min ramp to 245 °C followed by a 15 min hold. Peaks were identified by comparing retention times with an external standard mixture (GLC-462, Nu-Chek Prep, Elysian, MN, USA).

4.6 Lipidomic Analyses

Lipid extracts were prepared for lipidomic analyses by drying under N₂ gas and reconstituting into appropriate volumes of 65:30:5 acetonitrile:isopropanol:water + 0.1% formic acid and adding the appropriate internal standards according to each study. The samples were then analyzed by UHPLC-MS/MS with the QToF platform described above. The mobile phase

consisted of (A) 60:40 acetonitrile/water (v/v) +10mM ammonium formate +0.1% formic acid, and (B) 90:10 isopropanol/acetonitrile (v/v) +10mM ammonium formate +0.1% formic acid. A multi-step gradient was used as follows: initially solvent A at 68% and solvent B at 32% from 0-1 min, then B linearly increasing to 55% from 1-12 min, to 70% from 12-24 min, to 80% from 24 - 35 min, to 95% from 35-60 min, followed by a decrease back to 32% B at 60.1 min and held until 65 min. The column temperature was 50 °C, the autosampler temperature was 4 °C, and the injection volume was 5 µL.

The mass spectrometer was operated in positive ESI mode with a spray voltage of 1.0kV, enhanced resolution mode (approximately 60,000 resolution), scan range m/z from 100 to 1200, scan time of 0.2s/scan, and cone voltage set at 40V. The cone gas flow rate was 100 L/hr, the desolvation gas flow rate was 600 L/hr, the nebulizer gas flow rate was 7.0 bar, the source temperature was 140 °C, and the desolvation temperature was 400 °C. Lock mass correction was performed using leucine enkephalin (m/z 554.2615). Data dependent acquisition (DDA) was used to identify compounds with top 5 ion selection with a scanning window interval of ±1.0 Da. This raw data was acquired using Masslynx Workstation UPLC-QTOF/MS Acquisition software (version 4.1; Waters Company, Milford, MA, USA). Scan frequency was 0.1 s/scan, the transfer cell collision energy ramp was 30 V to 50 V for low mass (m/z 100) and 40 V to 60 V for high mass (m/z 1200) compounds.

4.7 Data Handling, Automated Lipid Identifications and Statistical Analyses

After data acquisition, the QTOF/MS raw data was transferred and imported into Progenesis QI software (version 2.3; Nonlinear Dynamics, Newcastle upon Tyne, UK) for automated lipid identifications [19]. The workflow for data processing and analysis included

entering the experimental design set-up, followed by peak picking, the identification of compounds and statistical analysis. ChemSpider and LipidBlast databases were used to match the compounds with 25 ppm precursor and 5 ppm product mass tolerances used to filter the ions. Retention time and m/z datasets were also used to characterize the detected ions with the help of LIPID MAPS® Online Tools [75, 76]. Manual inspection of diagnostic fragments from single TG precursor ions in MS/MS scans were performed in Masslynx. Generally, tandem MS methods generate fragments which lose a fatty acyl chain. Therefore, scans were performed for the m/z of the precursor ion plus adduct and the m/z of neutral loss of individual fatty acyl chain plus adduct. Principal Component Analysis (PCA) were completed using Progenesis QI software to determine if the lipidomic profiles of similar samples clustered together. Statistical analyses of individual experiment were described in appropriate chapters.

Chapter 5

Macrolipidomic Analysis of Soybean and Canola Oil

5.1 Rationale and Objectives

Cooking oil is often used in the food industry during frying and baking but is also used as an edible oil in food to add desirable physical, tastes and/or nutritional properties. While determinations of the fatty acid composition of oils are common, comprehensive lipidomic assessments of oils are rare. Although the fatty acid composition data of an oil is informative, the ability to characterize and quantify the fatty acyl chains of complex lipids in their natural state in an oil can provide further understanding of their nutritional value, flavor, stability, influences on human health, and the unique lipidomic profile of an oil can help to detect adulterated oils [77, 78].

In this thesis, the macrolipidomic profile of soybean and canola oil were examined since they are the two most consumed vegetable oils in Canada [79, 80]. Conventional fatty acid analyses were completed by GC-FID for comparison. Traditionally, the fatty acid composition of soybean oil is mainly linoleic acid (18:2n-6), with considerable amounts of oleic (18:1n-9), and a smaller amount of linolenic acid (18:3n-3). The fatty acid composition of canola oil mainly consists of oleic (18:1n-9), with a considerable amount of linoleic acid (18:2n-6) and a slightly higher amount of linolenic acid (18:3n-3) than soybean oil [29].

Plant-based oils are largely triacylglycerols (TG) [29]. Measuring triacylglycerol species is difficult due to considerable fatty acyl diversity with different carbon chain lengths, degree of unsaturation, isomeric configuration and double bond position. The soybean oil TG profile has

been examined at the medio level previously [81-83]. The three most prevalent lipid species in soybean oil are TG 18:2_18:2_18:2, TG 18:2_18:2_18:3 and TG 18:1_18:2_18:2. The actual concentrations of each TG species is inconsistent across studies, as soybean oil composition can vary due to genetic strains, environmental conditions and agricultural management [84]. TG species data about canola oil at the medio level is not as readily available, with some reports that are not entirely consistent. At the brutto level, TG 54:6 has been identified as the most abundant lipid species in canola oil [85] and based on the fatty acid profile of canola oil, TG 54:6 is likely TG 18:1_18:2_18:3 or possibly TG 18:2_18:2_18:2. Alternatively, TG 18:1_18:2_18:2, TG 18:2_18:2_18:3, TG 18:2_18:3_18:3, TG 18:1_18:1_18:1 and 18:1_18:1_18:2 were identified as the 5 main TG species in almost equal amounts but these results were generated in 1997 with a single quadrupole MS, so fatty acyl chains were not confirmed by MS/MS [86]. There is a recent study analyzing different rapeseed strains [87]. Canola was created from rapeseed through cross-breeding to remove erucic acid (22:1n-9) which was shown to have toxic effects on the heart in rat experiments [87]. It is shown that the rapeseed oil was dominated mainly by TG with 52 and 54 acyl species carbon atoms. The relative content of TG having 54 acyl carbon atoms was greater than 80% with the main species being TG 18:1_18:1_18:1 (71.75%), TG 18:1_18:1_18:2 (7.56%), TG 18:1_18:1_18:3 (4.81%) and TG 18:0_18:1_18:1 (4.74%) [88].

Our macrolipidomic analyses aimed to confirm the previous information available on TG species for soybean oil and canola oil, but also to determine if there are lipids from other classes that may be present in the oil.

5.2 Hypotheses

- TG 18:2_18:2_18:2 will be the most abundant lipid at the medio level for soybean oil.
- TG 18:1_18:1_18:1 will be the most abundant lipid at the medio level for canola oil.
- Diacylglycerol and phospholipid species will be found and characterized at the medio level.
- There will be unique species of lipids in the oils that can be used to distinguish soybean and canola oils.

5.3 Methods, Materials and Study Design

5.3.1 Sample Collection and Lipid Extraction

Soybean oil and canola oil were purchased locally in a grocery store (Walmart, Waterloo, ON, Canada) in November of 2018. Technical replicates of lipid extracts were obtained from the soybean and canola oil in quadruplicate for fatty acid analyses and in triplicate for lipidomic analyses. Briefly, 30 μ mol of triheptadecanoin (TG 17:0/17:0/17:0) was added to 50 μ L of oil aliquots to serve as the internal standard in fatty acid and lipidomic analyses. Oil aliquots were then extracted using the modified Folch-based protocol described in Section 4.3. The lipid extracts in chloroform were then stored at -80°C until follow up analyses.

5.3.2 Fatty Acid Analysis

Technical replicates of total lipid extracts (n=4) were transesterified using 14% boron trifluoride in methanol to FAME and analyzed by gas chromatography according to procedures in section 4.5.

5.3.3 Macrolipidomic Profiling

Lipidomic analyses were completed in triplicate using UHPLC-MS/MS on a QToF platform as described in section 4.2 and 4.6. Two μL of the lipid extract was dried under N_2 gas and 2500 μL of the reconstitution solvent (65:35:5 acetonitrile/isopropanol/water (v/v/v) +0.1% formic acid) was added and stored in vials at 4°C until analysis. UHPLC was completed using an Ascentis Express C18 column and a Waters Acquity BEH C18 column in a dual column serial set-up with 2 solvent systems on a gradient program as described in Chapter 4. Spectra were acquired in positive electrospray ionization mode under top-5 DDA conditions. Compound identifications and manual confirmations were completed using Progenesis QI software as described in section 4.7.

5.3.4 Statistics

In this chapter, the fatty acid composition and the lipidomic profiles of soybean and canola oil are compared. The fatty acid composition was compared using an independent t-test with significance accepted at $P < 0.05$ (Excel, v1908, Microsoft). For the lipidomic profiles, principal component analysis was completed using Progenesis QI. Plots were visually inspected to determine to confirm independent clustering followed by sorting the highly abundant lipid species into a Venn diagram to highlight the unique and common lipids.

5.4 Results

5.4.1 Fatty Acid Composition

The most abundant fatty acid in soybean oil was 18:2n-6 which accounted for 54.2% of total fatty acids (Table 1). There was also considerable amount of 18:1n-9 (22.8%) followed by

18:3n-3 (6.0%) and 16:0 (5.2%). In canola oil, 18:1n-9 had the highest abundance, accounting for 62.2% of total fatty acids followed by 18:2n-6 (18.6%) and 18:3n-3 (7.5%). The higher content of n-6 PUFA in soybean oil and higher content of MUFA in canola oil resulted in a much higher n-6/n-3 ratio of 9:1 in soybean oil as compared with the canola oil n-6/n-3 ratio of 2.5:1 despite only slightly more n-3 PUFA in the canola oil.

5.4.2 Lipidomic Profile

The identified lipid species of soybean oil and canola oil were sorted based on their abundance, presented as a relative percent of total identified lipids and grouped by abundance categories of $\geq 1.0\%$, $\leq 1.0 > 0.5\%$, $\leq 0.5 > 0.2\%$, $\leq 0.2 > 0.1\%$, $\leq 0.1 > 0.01\%$ (Table 2 and Table 3, respectively). Over 90 individual TG species and 7-8 diacylglycerol species were identified in the soybean and canola oils, but no phospholipids or sterols species were identified.

The most three abundant lipid species in the soybean oil were TG 18:2_18:2_18:2 (7.28%), TG 18:1_18:2_18:2 (7.08%) and TG 18:2_18:2_18:3 (6.90%). There were 24 lipids (all TG) with abundances $> 1\%$ that accounted for 73.3% of the total lipids in the soybean oil. Of these 24 high abundant species in soybean oil, 18:2 was found in 15, 18:1 in 11, 18:3 in 7, 16:0 in 6, and 18:0 in 5. Trilinoleate, trioleate, and trilinolenate were identified as the first, sixth, and seventeenth most abundant lipids, respectively. There were also 6 dilinoleate, 3 dioleate and 3 dilinolenate TG species in the top 24 abundant lipids in soybean oil. Seven DG species were identified in soybean oil, with DG 18:2_18:3 being the most abundant DG (0.96%) followed by DG 18:2_18:2 (0.35%) and DAG 18:3_18:3 (0.24%).

In canola oil, TG 18:1_18:2_18:3 (7.86%), TG 18:1_18:1_18:1 (7.67%), and TG 18:1_18:2_18:2 (7.51%) were the three highest abundant lipids. In the present analysis, TG

18:1_18:1_18:2 was the eighth most abundant lipid in canola oil at 3.31% of the total lipids identified. There were 27 lipids (26 TG and one DG) with abundances >1% that accounted for 77.2% of the total lipids in the canola oil. Of these 27 high abundant species in canola oil, 18:1 was found in 23, 18:2 in 12, 18:3 in 9, 16:0 in 4, and 18:0 in 2. Trilinoleate was not identified in canola oil, while trioleate, and trilinolenate were identified as the second and twentieth most abundant lipids, respectively. The only dilinoleate species was a DG, while there were 7 dioleate and 2 dilinolenate TG species in the top 27 abundant lipids in canola oil. Seven DG species were also identified in canola oil, and again no phospholipids were identified. DG 18:2_18:2 was the most abundant DG (1.21%) followed by DG 18:2_18:3 (0.51%) and DG 18:3_18:3 (0.21%).

The PCA plot of the lipidomic profiles of soybean oil and canola oil clustered separately indicating the oils were unique (data not shown). To examine specific differences, lipids with greater than 1% of the total abundance were plotted in a Venn diagram (Figure 1). This included 105 lipids from soybean oil and 105 lipids from canola oil with 56 lipids species being found in both oils. TG 18:2_18:2_18:2 and TG 18:2_18:2_18:3 were unique and highly abundant (>5%) in soybean oil and TG 18:1_18:2_18:3 and TG 18:1_18:3_18:3 were unique and highly abundant (>5%) in canola oil.

Table 1. Fatty acid composition of soybean and canola oils

Fatty Acid	Soybean Oil	Canola Oil	T-test
14:0	0.053 ± 0.053	0.045 ± 0.045	0.13
16:0	5.19 ± 0.07	3.88 ± 0.02	<0.001
18:0	3.96 ± 0.16	1.66 ± 0.08	<0.001
20:0	0.10 ± 0.01	3.45 ± 0.05	<0.001
22:0	0.08 ± 0.01	0.21 ± 0.02	0.84
24:0	0.04 ± 0.01	0.07 ± 0.01	0.20
Total SFA	9.42 ± 0.23	6.37 ± 0.14	<0.001
14:1	0.002 ± 0.002	0.005 ± 0.003	0.09
16:1	0.08 ± 0.01	0.19 ± 0.02	<0.001
18:1n-7	1.54 ± 0.02	3.45 ± 0.05	<0.001
18:1n-9	22.83 ± 0.55	62.16 ± 0.86	<0.001
20:1n-9	0.13 ± 0.02	0.95 ± 0.05	<0.001
22:1n-9	0.014 ± 0.005	0.03 ± 0.01	0.001
24:1n-9	0.01 ± 0.01	0.04 ± 0.03	0.046
Total MUFA	24.62 ± 0.55	66.82 ± 0.85	<0.001
18:2n-6	54.19 ± 0.77	18.57 ± 0.45	<0.001
18:3n-6	0.01 ± 0.01	0.008 ± 0.003	0.67
20:2n-6	0.02 ± 0.01	0.036 ± 0.005	0.001
20:3n-6	0.010 ± 0.004	0.01 ± 0.01	0.30
20:4n-6	0.008 ± 0.003	0.01 ± 0.01	0.54
22:4n-6	0.01 ± 0.01	0.01 ± 0.01	0.50
22:5n-6	0.01 ± 0.01	0.01 ± 0.01	0.82
Total n-6 PUFA	54.28 ± 0.77	18.67 ± 0.46	<0.001
18:3n-3	6.00 ± 0.17	7.52 ± 0.39	<0.001
20:5n-3	0.01 ± 0.01	0.01 ± 0.01	0.72
22:5n-3	0.01 ± 0.01	0.01 ± 0.01	0.17
22:6n-3	0.01 ± 0.01	0.03 ± 0.03	0.14
Total n-3 PUFA	6.03 ± 0.18	7.57 ± 0.41	<0.001

Values are mean ± S.D. of n = 4 technical replicates. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids, PUFA, polyunsaturated fatty acids. The data is shown as relative percent of total fatty acids weight.

Table 2. Percentage of lipid species with acyl identification in soybean oil

#	Lipids >1.0%	%	#	Lipids ≤1.0 >0.5%	%	#	Lipids ≤0.5 >0.2%	%	#	Lipids ≤0.2 >0.1%	%	#	Lipids ≤0.1 >0.01%	%
1	TG 18:2_18:2_18:2	7.28	25	TG 18:1_18:1_23:0	0.99	45	TG 16:0_17:1_18:2	0.50	70	TG 14:0_18:2_18:2	0.19	87	TG 18:2_18:3_25:0	0.10
2	TG 18:1_18:2_18:2	7.08	26	TG 18:2_18:3_22:0	0.96	46	TG 18:2_18:2_23:0	0.49	71	TG 18:1_18:2_25:0	0.19	88	TG 16:0_18:1_21:0	0.09
3	TG 18:2_18:2_18:3	6.90	27	DG 18:2_18:3	0.96	47	TG 15:1_18:2_18:2	0.49	72	TG 14:0_18:2_18:3	0.18	89	TG 18:0_18:1_25:0	0.08
4	TG 16:0_18:2_18:2	6.39	28	TG 18:1_18:2_24:0	0.85	48	TG 17:0_18:2_18:3	0.49	73	TG 16:1_18:3_18:3	0.18	90	TG 18:2_18:3_26:0	0.08
5	TG 18:2_18:3_18:3	6.17	29	TG 17:2_18:2_18:2	0.84	49	TG 17:2_18:2_18:3	0.48	74	TG 18:0_18:2_25:0	0.17	91	TG 18:2_18:2_26:0	0.07
6	TG 18:1_18:1_18:1	5.46	30	TG 16:0_18:0_18:1	0.83	50	TG 18:2_18:3_24:0	0.48	75	TG 17:0_18:0_18:1	0.17	92	TG 18:2_18:3_20:1	0.07
7	TG 18:0_18:2_18:2	4.00	31	TG 18:0_18:0_18:1	0.81	51	TG 16:1_18:2_18:3	0.47	76	TG 18:0_18:1_18:2	0.16	93	TG 18:3_18:3_20:1	0.06
8	TG 18:0_18:1_18:1	3.78	32	TG 17:0_18:1_18:1	0.76	52	TG 16:0_16:0_18:2	0.45	77	DG 18:1_18:2	0.15	94	TG 16:0_18:0_20:0	0.06
9	TG 16:0_18:0_18:2	2.61	33	TG 17:0_18:2_22:0 TG 16:0_18:2_23:0 TG 18:0_18:2_21:0	0.75	53	TG 18:2_18:2_21:0	0.45	78	TG 18:3_18:3_20:0	0.15	95	DG 18:1_18:1	0.06
10	TG 16:0_18:2_18:3	2.57	34	TG 18:2_18:3_20:0	0.75	54	TG 18:1_18:2_19:1	0.43	79	TG 16:0_16:0_18:1	0.14	96	TG 18:2_18:3_19:2	0.05
11	TG 18:1_18:1_20:0	2.37	35	TG 18:1_18:2_23:0	0.74	55	TG 18:1_18:1_18:2	0.39	80	TG 18:1_18:2_26:0	0.14	97	DG 16:0_18:2	0.05
12	TG 18:1_18:1_22:0	2.37	36	TG 18:0_18:1_22:0	0.71	56	TG 16:0_16:0_18:3	0.37	81	TG 18:2_18:3_23:0	0.14	98	TG 17:2_18:3_18:3	0.05
13	TG 17:0_18:2_18:2	1.65	37	TG 17:1_18:2_18:2	0.71	57	DG 18:2_18:2	0.35	82	TG 18:0_18:2_26:0	0.14	99	TG 16:0_18:0_18:0	0.05
14	TG 18:1_18:2_20:0	1.59	38	TG 16:0_17:0_18:2	0.66	58	TG 18:2_18:2_19:1 TG 18:2_18:3_19:1	0.30	83	TG 18:3_18:3_22:0	0.13	100	TG 18:3_18:3_24:0	0.04
15	TG 18:1_18:2_22:0	1.56	39	TG 16:0_18:1_22:0	0.66	59	TG 18:0_18:1_23:0 TG 16:0_18:1_25:0	0.29	84	TG 15:1_16:0_18:2	0.13	101	TG 14:0_18:1_18:2	0.04
16	TG 18:1_20:1_22:0	1.53	40	TG 18:0_18:1_24:0	0.64	60	TG 16:0_18:2_21:0	0.29	85	DG 18:0_18:2	0.11	102	TG 18:0_18:3_20:0	0.04
17	TG 18:3_18:3_18:3	1.44	41	TG 16:1_18:2_18:2	0.62	61	TG 18:1_18:2_19:0	0.29	86	TG 15:1_16:0_18:1	0.10	103	TG 18:1_18:2_18:3	0.04
18	TG 18:0_18:3_18:3	1.39	42	TG 16:0_17:2_18:2	0.58	62	TG 18:1_18:1_26:0	0.26				104	TG 12:0_18:1_18:2	0.04
19	TG 16:0_18:1_18:2	1.26	43	TG 18:2_18:2_24:0	0.58	63	TG 18:0_18:1_21:0	0.26				105	DG 16:0_18:3	0.02
20	TG 16:0_18:0_18:3	1.25	44	TG 18:1_18:2_21:0	0.52	64	DG 18:3_18:3	0.24						
21	TG 18:2_18:2_22:0	1.23				65	TG 18:1_18:1_25:0	0.23						
22	TG 17:0_18:1_18:2	1.18				66	TG 15:1_18:2_18:3	0.23						
23	TG 16:0_18:3_18:3	1.16				67	TG 18:2_18:2_25:0	0.22						
24	TG 18:1_18:2_20:1	1.08				68	TG 56:8*	0.22						
						69	TG 16:0_17:0_18:1	0.21						
Total		73.30			14.92			8.86			2.55			1.10

Identifications were based on n = 3 technical replicates. *MS/MS spectrum was not available to identify acyl species. TG, triacylglycerol, DG, diacylglycerol. Identifications made automatically by Progenesis QI with manual confirmation of the top 10 lipids.

Table 3. Percentage of lipid species with acyl identification in canola oil

#	Lipids >1.0%	%	#	Lipids ≤1.0 >0.5%	%	#	Lipids ≤0.5 >0.20%	%	#	Lipids ≤0.2 >0.1%	%	#	Lipids ≤0.1 >0.01%	%
1	TG 18:1_18:2_18:3	7.86	28	TG 18:1_18:2_19:0	1.00	46	TG 18:2_18:3_19:0	0.49	67	TG 18:1_22:1_22:2	0.20	80	TG 18:1_18:3_21:0	0.10
2	TG 18:1_18:1_18:1	7.67	29	TG 17:1_18:1_18:2	0.90	47	TG 18:1_22:0_24:1	0.49	68	DG 18:1_18:2	0.19	81	TG 18:1_18:2_28:1	0.09
3	TG 18:1_18:2_18:2	7.51	30	TG 16:1_18:2_18:2	0.84	48	TG 15:1_18:1_18:2	0.48	69	TG 18:2_18:2_23:0	0.18	82	TG 16:0_17:0_18:1	0.08
4	TG 18:1_18:3_18:3	6.99	31	TG 17:0_18:1_18:1	0.78	49	TG 18:2_18:3_22:0	0.45	70	TG 14:0_18:2_18:3	0.17	83	TG 15:1_18:2_18:3	0.08
5	TG 18:1_18:1_20:1	4.08	32	TG 18:1_22:1_22:1	0.73	50	TG 18:1_18:1_21:0	0.43	71	DG 18:1_18:1	0.17	84	TG 17:1_18:3_18:3	0.08
6	TG 18:0_18:1_18:3	3.79	33	TG 17:1_18:1_20:2	0.71	51	TG 18:2_18:3_24:0	0.43	72	TG 16:0_16:1_18:2	0.17	85	TG 18:0_18:1_21:0 TG 17:0_18:1_22:0 TG 16:0_18:1_23:0	0.08
7	TG 18:2_18:3_18:3	3.46	34	TG 18:1_18:1_23:0	0.70	52	TG 18:1_18:1_28:1	0.42	73	TG 16:0_16:0_18:2	0.16	86	TG 18:2_18:3_24:1	0.07
8	TG 18:1_18:1_18:2	3.31	35	TG 18:0_18:1_22:0	0.69	53	TG 18:1_18:2_21:0	0.39	74	TG 18:3_18:3_22:0	0.15	87	TG 12:0_14:1_18:1	0.07
9	TG 18:0_18:1_18:1	2.75	36	TG 16:1_18:3_18:3	0.65	54	TG 18:3_18:3_20:1	0.39	75	TG 14:0_16:1_18:1	0.14	88	TG 18:3_18:3_24:0	0.07
10	TG 18:1_18:1_20:0	2.58	37	TG 18:1_18:2_23:0	0.65	55	TG 16:0_18:0_18:1	0.37	76	TG 14:1_16:1_18:1	0.13	89	TG 18:1_18:2_25:1	0.07
11	TG 18:1_20:1_22:1	2.50	38	TG 18:0_18:1_24:0	0.64	56	TG 16:0_17:1_18:1	0.37	77	TG 18:2_18:3_19:1	0.13	90	DG 18:2_20:1	0.07
12	TG 18:1_18:1_22:0	2.50	39	TG 18:0_18:1_20:0	0.61	57	TG 18:1_18:1_19:0	0.27	78	TG 16:0_16:0_18:1	0.12	91	TG 17:0_18:0_18:1	0.07
13	TG 18:1_20:1_22:0	2.34	40	TG 17:1_18:1_18:3	0.60	58	TG 18:1_22:0_22:0 TG 18:1_20:0_24:0 TG 18:0_18:1_26:0	0.27	79	TG 18:1_18:3_26:0	0.11	92	TG 18:1_18:3_25:1	0.06
14	TG 18:1_18:3_20:0	2.26	41	TG 18:0_18:0_18:1	0.59	59	TG 18:1_18:2_25:0	0.26				93	TG 18:2_22:1_22:2	0.06
15	TG 16:0_18:1_18:2	1.85	42	TG 15:0_18:1_18:2	0.59	60	TG 15:1_18:2_18:2	0.24				94	DG 18:0_18:2	0.05
16	TG 18:1_18:2_22:0	1.70	43	TG 18:1_18:1_25:0	0.53	61	TG 10:0_18:1_18:1	0.22				95	TG 16:0_18:1_18:1 TG 16:0_18:0_18:2	0.04
17	TG 18:1_18:3_20:1	1.69	44	TG 18:2_18:3_20:1	0.53	62	TG 17:1_18:2_18:3	0.22				96	DG 18:1_20:1	0.03
18	TG 18:1_18:2_22:1	1.66	45	DG 18:2_18:3	0.51	63	TG 10:0_18:1_18:2	0.22				97	TG 16:0_18:1_25:0 TG 18:0_18:1_23:0	0.03
19	TG 16:0_18:1_18:1	1.37				64	TG 18:1_18:2_23:1	0.22				98	TG 16:0_18:1_21:0	0.03
20	TG 18:3_18:3_18:3	1.31				65	TG 16:1_16:1_18:2	0.22				99	TG 18:2_18:3_26:0	0.03
21	TG 16:0_18:1_18:3	1.28				66	DG 18:3_18:3	0.21				100	TG 18:0_18:1_18:2	0.03
22	TG 18:1_18:1_26:0	1.23												
23	DG 18:2_18:2	1.21												
24	TG 17:0_18:1_18:2	1.14												
25	TG 18:1_18:2_24:1	1.05												
26	TG 16:0_18:2_18:3	1.05												
27	TG 16:1_18:1_18:2	1.01												
Total		77.18			12.24			7.25			2.13			1.19

Identifications were based on n = 3 technical replicates. TG, triacylglycerol, DG, diacylglycerol. Identifications made automatically by Progenesis QI with manual confirmation of the top 10 lipids.

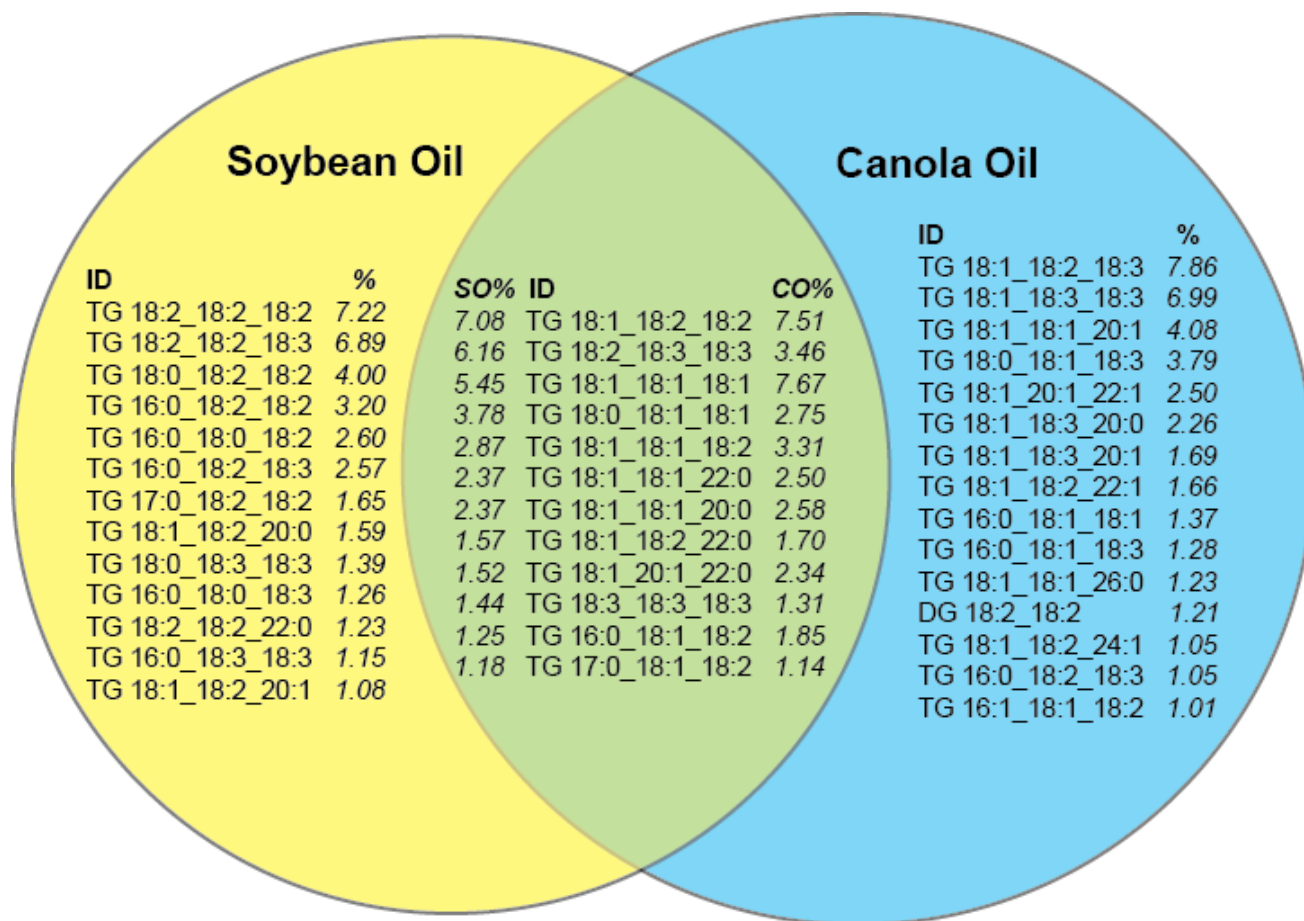


Figure 1. Venn diagram of lipid species in Soybean and Canola oil

Identifications were based on n = 4 technical replicates for each oil. The lipids were greater than 1 percent and shown by abundance. The intersection area were common species between soybean oil and canola oil. ID, identification; SO, soybean oil; CO, canola oil; TG, triacylglycerol, DG, diacylglycerol.

5.5 Discussion

Overall, the fatty acid composition for both oils showed consistency with the data in the literature, while lipidomic results were partially in agreement with the data available in the literature. The fatty acid composition of soybean oil was over 50% linoleic acid (18:2n-6) and just under 25% oleic (18:1n-9) with 6% linolenic acid (18:3n-3). The fatty acid composition of canola oil was over 60% oleic acid (18:1n-9), with less than 20% linoleic acid (18:2n-6) and 7.5% linolenic acid (18:3n-3), indicating that the oil analyzed represented typical commercial versions of these oils. At the lipidomic level, there were several similarities to the reported data but also some differences.

Based on the literature [79-81], it was hypothesized that TG 18:2_18:2_18:2 would be the most abundant lipid in soybean oil which was confirmed. In this analysis, TG 18:2_18:2_18:2 was 7.28% of the total identified lipid while the literature has reported it to be 8-25%. Earlier older studies [81, 82] tended to report much higher percentages of TG 18:2_18:2_18:2, but these percentages were determined using either single quadrupole or low-resolution instruments, resulting in low numbers of identified lipids based on nominal masses. Examinations of soybean oil by high resolution tandem MS instruments allows for determination using accurate mass with acyl species confirmations by MS/MS fragmentations and report TG 18:2_18:2_18:2 abundances relative to the current determinations. The 7.28% is also likely low due to the fact that 105 of lipid species were identified in the present analysis, where previous reports have identified 12-93 lipids [79-81]. We also confirmed that TG 18:2_18:2_18:3 and TG 18:1_18:2_18:2 were the other top 3 most abundant lipid species in soybean oil at abundancies that reflect more recent estimates [83].

For canola oil, it was hypothesized that TG 18:1_18:1_18:1 would be the most abundant oil based on a recent rapeseed oil study [87] but it was the second most abundant lipids in the present analysis at 7.7% of total lipids. The most abundant lipid was TG 18:1_18:2_18:3 (7.9% of total lipids) which agrees with an older single quadrupole determination that TG 54:6 is the most abundant lipid in canola oil [85]. Comparing the canola oil results to the literature is difficult as there are several varieties of canola with considerable variation in the fatty acid and triacylglycerol compositions [86]. The abundancies observed in the present analyses are within the ranges observed for different varieties, but they do not exactly match the previous reports of single varieties [86, 87]. It is possible that the commercial product examined herein was a blend of canola varieties and/or the lipid composition was influenced by seasonal and geographic growing conditions.

As hypothesized, diacylglycerols were detected in the both the oils. Previous reports examining the anti-obesity effects of DG consumption on human and rodent health have indicated that DG levels in commercially refined soybean and canola oils are approximately 1% of the lipid weight although DG enriched cooking oils (up to 55% DG by weight) are produced and consumed in Japan [89]. Unfortunately, our untargeted analysis we are not able to precisely quantitate the weights of the individual species and provided just the mass spectrometer detection abundance. The total abundance of the eight DG species in soybean oil was 1.89% and of the eight DG species in canola oil was 2.44%, suggesting confirmation of previous findings. Data on DG species in soybean oil and canola oil are very difficult to find in the literature as most lipid species characterizations of these oils focus on triacylglycerols. Wu et al. did report the detection of DG 36:4 and DG 36:5 in soybean oil and DG 36:2, DG 36:3, and DG 36:1 in

canola oil [85]. The current analysis at the medio level of identification partially agrees with these canola oil previous reports, but the present results identified more DG species and DG species with more polyunsaturates were found in a greater abundance.

Phospholipids were not detected in either oils with the present analysis contradicting our hypothesis. This hypothesis was based on the knowledge that soybeans and canola contain lecithin (mixed phospholipids) [38] and that phosphates were detected in a previous assessment using a Fourier-transform ion cyclotron resonance mass spectrometry although the identification of actual phospholipids were not attempted [85]. It is clear that we underestimated the efficiency of commercial oil processing in removing phospholipids. Specifically, degumming removes most of the seed oil lecithins as lecithins are an important commercial by-product, but the refining, bleaching and deodorization steps in oil processing can also remove residual polar lipids [90]

Finally, it was hypothesized that soybean oil and canola oil would have unique lipidomic profiles that could be used to distinguish them. These initial analyses indicate that this is definitely possible with the two oils compared as several highly abundant lipid species were identified to be unique in each oil. This work is limited to a simple comparison of two commercially purchased oils. Based on the number of unique species identified in each oil, lipidomic profiling of seed oils can be used for food traceability efforts. Additional work is needed to characterize very specific strains of each seed oil as well as examine the effects of harvesting location and seasonal conditions in order to better link seed phenotypes, genotypes and the resulting lipotypes.

5.6 Conclusion

This study indicated that adopting a macrolipidomic approach could enable the characterization of hundreds of molecular species of lipids with numerous targets. However, identifications of the specific lipids remain challenging. Due to the number of mass spectrometry features, it is time-consuming to ensure correctness, completeness and consistency, as automated identifications remain problematic in regard to mismatches. In addition, the present work was an untargeted approach to mainly discover and identify lipid species. Proper quantitation of each and every species will require more targeted approaches and additional labour to ensure accuracy. However, the present study should enable such future work, as the identified lipids can be used to generate inclusion lists for targeted analyses. If these analyses can be improved and completed cost effectively, there is considerable potential for macrolipidomic profiling to be applied to several applications such as confirming oil quality, detecting adulterated oils, improving food traceability, and ensuring safety of the food supply. Additionally, this work reports on specific acyl species of diacylglycerols present in soybean and canola oils that are not usually reported. The characterization of these DG species could provide further insights regarding the oxidative stability, palatability, and nutritional value of these oils and lead to the development of food products and evaluation of food functionality, bioactivity, and toxicity.

Chapter 6

Macrolipidomic Analysis of a Homemade Breakfast Sandwich

6.1 Rationales and Objectives

Most food lipidomics studies focus mainly on the identification of triacylglycerols (TG) [91] and the content of other biologically important lipid species that can be in food such as phospholipids have been largely neglected. It is known that LC-PUFA, especially n-3 LC-PUFA which are associated with various cardiovascular and neurological health benefits [92], are preferentially incorporated into phospholipids of animal tissues which appear to be the main dietary source of LC-PUFA by Canadians [17]. In addition, there is evidence that marine phospholipids rich in n-3 LC-PUFA are better absorbed than marine LC-PUFA TG [93].

In the present study, the objective was to characterize and quantitate the specific lipid species of a breakfast sandwich using an untargeted or “global” macrolipidomic profiling technique. This analysis allows for the determination of the molecular sources of fatty acids in lipids such as phospholipids in addition to TG. Fatty acid composition analyses of total lipids were completed by gas chromatography for traditional fatty acid profiling along with the estimated fatty acid composition of the sandwich using the Canadian Nutrient File. Finally, thin layer chromatography (TLC) was also used *ad hoc* to isolate the TG and phospholipids fractions for fatty acid analysis as an additional assessment of the lipidomic results.

6.2 Hypotheses

- Total fatty acid amounts measured in the sandwich by gas chromatography should agree with an estimate calculated from the Canadian Nutrient File.
- Phospholipid species will be present in the sandwich because of the egg and ham content and will contribute up to 5% of the total fatty acid content.
- The fatty acid data by gas chromatography analyses should be consistent with lipidomic data (the amount of different types of lipids and specific fatty acyl species)

6.3 Methods, Materials and Study Design

6.3.1 Sample Collection and Lipid Extraction

A flow chart of the procedures used to prepare and complete macrolipidomic and fatty acid analyses is available in Figure 2. All food products were purchased in December 2018 from a Walmart supermarket in Kitchener-Waterloo, Ontario. The sandwich was prepared from two slices of whole grain bread, two slices of processed ham, one large egg and 30 grams of spinach. During preparation, the mass of each food item was recorded. The entire sandwich was then blended to homogeneity with an equal weight of water in a 4-Liter Waring blender, aliquoted into 50mL Falcon tubes and stored at -80°C for fatty acid and lipidomic analyses.

6.3.2 Nutrient Composition

The nutrient composition of the breakfast sandwich was estimated using the Canadian Nutrient File (date modified: 2018-02-06). The nutrient content of each individual food

component in the sandwich was determined using the recorded weights and summed to produce a nutrient profile of the sandwich (Table 4).

6.3.3 Lipid Extraction

Lipids were extracted from the breakfast sample homogenate with 2:1 chloroform/methanol (v/v) as described in section 4.3. The homogenate was collected from a single Falcon tube on three different occasions as different internal standards were used. Each time the homogenate was allowed to thaw and then 2 mL, 100 μ L and 100 μ L of homogenate was collected for extraction separately. Technical replicates were generated from the homogenate with the extraction, chemical processing and analysis procedures be completed for each replicate. For the fatty acid composition of the total lipids, n = 4 technical replicates were performed while n = 3 technical replicates were completed for the lipidomic analyses due to the difficulty and cost of the latter analyses. A third extraction was added after the lipidomic determinations to complete the fatty acid composition of the triacylglycerol and phospholipid classes as an additional assessment (n = 4 technical replicates). For the fatty acid total lipid analyses, TG 17:0_17:0_17:0 was added as the internal standard and for the fatty acid composition of triacylglycerols and phospholipids, TG 17:0_17:0_17:0 and phosphatidylcholine 21:0_21:0 were added to the sandwich homogenate prior to lipid extraction. Due to the cost and requirement of adding multiple lipidomic internal standards, they were added after the lipid extraction (see details below).

6.3.4 Fatty Acid Analysis

Fatty acid methyl esters were prepared from the lipid samples by transesterification with BF_3 in methanol followed by determination of the fatty acid composition by GC-FID (section 4.5). Initially this was completed on the total lipid extracts. For the *ad hoc* analysis of the fatty acids of the TG and phospholipids fractions the lipid extract was separated into lipid classes using thin layer chromatography with 60:40:2 heptane:diethyl ether:acetic acid as described in section 4.4. The TG and phospholipids were then collected, and the fatty acid methyl esters were then prepared from the isolated lipid fractions as described above.

6.3.5 Macrolipidomic Profiling

Lipid extracts were prepared for lipidomic analysis by evaporating and reconstituting the samples in the liquid chromatography running buffer (section 4.7). The reconstituted samples were then spiked with TG 17:0_17:0_17:0, phosphatidylethanolamine (PE) 15:0_15:0, and phosphatidylcholines (PC) 11:0_11:0 and 21:0_21:0 as internal standards. The samples were then analyzed by UHPLC-MS/MS with the dual column setup (section 4.7). TG and phospholipids of high abundance were identified by comparison of accurate masses and by matching experimental spectra to libraries using Progenesis QI (Nonlinear Dynamics, Newcastle upon Tyne, UK).

6.3.6 Statistics

In this chapter, a breakfast sandwich homogenate was examined by three different analytical methods and the data was presented as the mean \pm the standard deviation. The data was compared with reference values from the Canadian Nutrient File and the results of the

different analyses were contrasted but formal statistical comparisons were not performed due to differences in the analytical output measures.

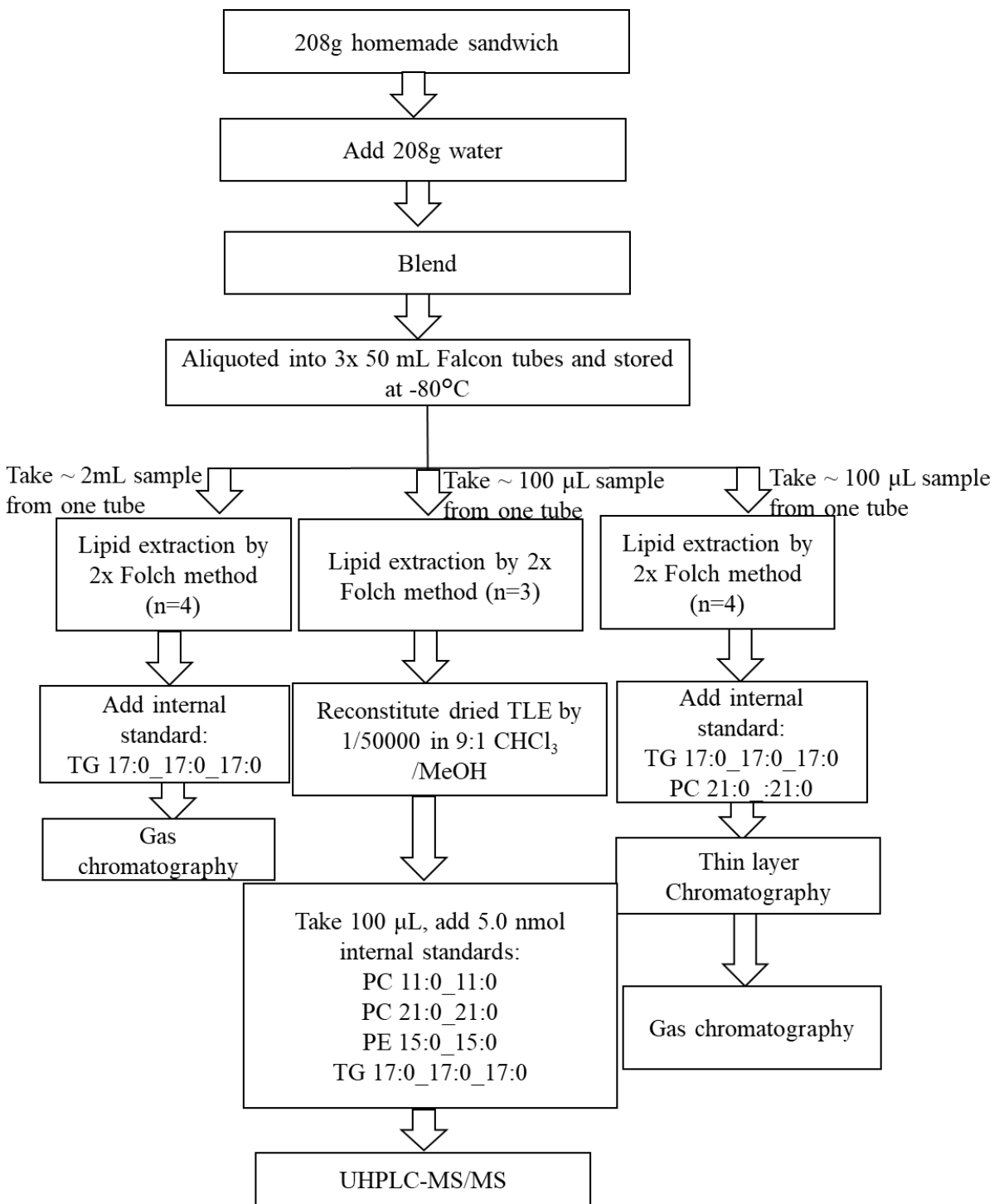


Figure 2. Illustration of the workflow for the fatty acid and lipidomic analyses of a breakfast sandwich. TG, triacylglycerol; TLE, total lipid extract; CHCl₃/MeOH, chloroform/methanol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; UHPLC-MS/MS, ultra-high pressure liquid chromatography tandem mass spectrometry.

6.4 Results

6.4.1 Fatty Acid Composition of Total Lipids

The theoretical total fat content of the breakfast sandwich was estimated to be 55.1 mg/g from the Canadian Nutrient file (Table 4) with 40.2% of the total fat being saturated, 34.8% being monounsaturated and 25.0 % being polyunsaturated. Theoretically, the highest amount of an individual fatty acid in the sandwich was 18:1n-9 (17.6 mg/g), followed by 16:0 (13.6 mg/g) and 18:2n-6 (10.9 mg/g). The fatty acid analysis of the total lipid extract indicated that the total fat content was much lower at 27.2 ± 0.9 mg/g sandwich. The proportions of the fatty acid classes also differed in the biochemical analysis with the monounsaturates being the highest at 41.8%, followed by saturates at 30.4% and then PUFA at 27.8%. The most abundant fatty acid of the total lipid extract was 18:1n-9 (10.3 ± 0.3 mg/g) followed by 16:0 (5.4 ± 0.2 mg/g) and 18:2n-6 (5.4 ± 0.2 mg/g). These three fatty acids accounted for 77.6% of the total fatty acids in the sandwich and adding 18:0 (7.1%) and 18:3n-3 (6.9%) accounted for 91.5% of the fatty acids in the sandwich. The biochemical analyses also indicated a lower n-3 polyunsaturated fatty acid status of the sandwich as determined by the sum of n-3 PUFA, EPA+DHA and N-6/N-3 as compared with the theoretical estimates. These differences can be attributed mainly to a higher estimate in the theoretical amount of DHA in the breakfast sandwich of 0.61 mg/g sandwich (1.12 wt%) versus the biochemical determination of 0.036 ± 0.003 mg/g sandwich (0.13 wt%).

6.4.2 Lipidomic Profile

The identified lipid species of breakfast sandwich were sorted by abundance and presented as mg lipid/g sandwich (Table 5). There were 17 TG, 7 PC and 1 PE lipid species with a concentration of over 0.01 mg lipid/g sandwich. At the level of individual lipid species, there were three TG species with concentrations over 1 mg/g sandwich including TG 16:0_18:1_18:1 at 2.72 ± 0.21 , TG 16:0_18:1_18:2 at 2.20 ± 0.27 and TG 18:1_18:2_18:3 at 1.02 ± 0.14 mg/g sandwich. The total identified TG content was 10.84 ± 1.16 mg/g sandwich which was $97.34 \pm 0.57\%$ of the identified lipid. The most abundant phospholipids were the phosphatidylcholine (PC) species, PC 16:0_18:1, and PC 16:0_18:2 at 0.10 ± 0.03 and 0.09 ± 0.02 mg/g sandwich. The total amount of the identified phospholipids was 0.29 ± 0.03 mg/g sandwich which was $2.66 \pm 0.57\%$ of the identified lipid.

The five most abundant fatty acids were found in all of the identified abundant lipids except one (PE 12:0_14:0) with 18:1 found in 15, 16:0 in 10, 18:2 in 7, 18:0 in 4 and 18:3 in 5 of the 25 lipids identified. Of these 25 lipids, the top five abundant fatty acids filled 49 of the 66 available positions for fatty acyls of all the identified lipids and 25 of 30 available acyl positions in the top 10 most abundant lipids. The amount of the abundant fatty acids was quantitated from the identified lipid species, but the amounts of 4.0 mg/g of 18:1, 2.2 mg/g of 16:0 and 1.5 mg/g 18:2 were lower than both the total lipid fatty acids and TG combined with phospholipids fatty acids estimates by gas chromatography.

6.4.3 Fatty Acid Composition of Triacylglycerols and Phospholipids

In order to confirm the lipidomic results of breakfast sandwich, thin layer chromatography was performed to isolate the triacylglycerol and phospholipid classes and then the fatty acid compositions of these classes were determined (Figure 3, Table 6). The total fatty acids in the TG fraction were 44.93 ± 2.25 mg/g and in the phospholipid fraction were 9.14 ± 0.19 mg/g, suggesting that 83% of the fatty acids in the sandwich came from TG and 17% of the fatty acids came from phospholipids. In regard to the pattern of fatty acids, the 5 most abundant fatty acids in the TG fraction were the similar as the 5 most abundant lipids in the total lipid fatty acid determinations with 18:1n-9 (41.5%) followed by 16:0 (17.8%), 18:2n-6 (19.1%), 18:0 (5.7%) and 18:3n-3 (8.2%). In phospholipids, 18:2n-6 was the most abundant fatty acid (26.1 wt%) followed by 16:0 (25.5 wt%), 18:1n-9 (19.8 wt%), 18:0 (13.1 wt%) and 20:4n-6 (5.99 wt%). Phospholipids were also the main source of DHA in the sandwich (0.17 ± 0.01 mg/g sandwich). Summing the individual fatty acids in TG and the phospholipid fractions, and then expressing the data as relative weight % resulted in very similar proportions of the individual fatty acids as compared with the proportions determined in the total fatty acid analysis with the exception of DHA. DHA when TG plus phospholipid were combined was 0.35 wt% and DHA in the total fatty acid analysis was 0.13 wt% (Table 6).

Table 4. The fatty acid composition of breakfast sandwich by biochemical analyses and by estimates from the Canadian Nutrient File

Fatty Acid	Concentration (mg/g)	Weight % of total fatty acids	Canadian Nutrient File (mg/g)	Weight % of total fatty acids
10:0	0.13 ± 0.01	0.49	0.41	0.74
12:0	0.16 ± 0.01	0.57	0.56	1.02
14:0	0.58 ± 0.02	2.13	1.78	3.23
16:0	5.41 ± 0.17	19.91	13.63	24.77
18:0	1.92 ± 0.07	7.08	5.72	10.38
20:0	0.042 ± 0.001	0.16	0.033	0.06
24:0	0.008 ± 0.004	0.03	0.010	0.02
SFA	8.27 ± 0.26	30.44	22.15	40.21
14:1	0.043 ± 0.005	0.16	0.16	0.29
16:1	0.33 ± 0.01	1.22	1.24	2.25
18:1n-9	10.28 ± 0.33	37.82	17.59	31.94
20:1n-9	0.090 ± 0.003	0.33	0.15	0.26
22:1n-9	0.004 ± 0.001	0.01	0.003	0.01
24:1n-9	0.004 ± 0.002	0.01	0.017	0.03
MUFA	11.36 ± 0.37	41.78	19.15	34.77
18:2n-6	5.39 ± 0.22	19.82	10.92	19.82
18:3n-6	0.009 ± 0.002	0.03	0.077	0.14
20:2n-6	0.017 ± 0.001	0.06	0.030	0.05
20:3n-6	0.012 ± 0.003	0.04	0.045	0.08
20:4n-6	0.16 ± 0.01	0.60	0.50	0.91
22:4n-6	0.011 ± 0.001	0.04	0.032	0.06
22:5n-6	0.021 ± 0.002	0.08	0.000	0.00
N-6 PUFA	5.62 ± 0.23	20.69	11.60	21.06
18:3n-3	1.88 ± 0.10	6.91	1.54	2.80
20:5n-3	0.004 ± 0.002	0.01	0.005	0.01
22:5n-3	0.009 ± 0.002	0.03	0.022	0.04
22:6n-3	0.036 ± 0.003	0.13	0.61	1.12
N-3 PUFA	1.93 ± 0.10	7.09	2.18	3.96
Total PUFA	7.55 ± 0.32	27.78	13.78	25.02
Total	27.2 ± 0.9	100	55.09	100.00

Values are mean ± S.D. of n = 12 technical replicates. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids, HUFA, highly unsaturated fatty acids (≥20 carbons, ≥3 carbon-carbon double bonds).

Table 5. High abundant lipid species with acyl identification in breakfast sandwich

m/z	Medio level ID	Concentration (mg lipid/g sandwich)
876.8008	TG 16:0_18:1_18:1	2.72 ± 0.21
874.7852	TG 16:0_18:1_18:2	2.20 ± 0.27
896.7689	TG 18:1_18:2_18:3	1.02 ± 0.14
901.7245	TG 18:3_18:3_20:3	0.83 ± 0.16
872.7694	TG 16:1_18:1_18:2	0.76 ± 0.07
894.7542	TG 18:1_18:3_18:3	0.55 ± 0.04
848.7692	TG 16:0_16:1_18:1	0.49 ± 0.01
904.8304	TG 18:0_18:1_18:1	0.43 ± 0.06
850.7842	TG 16:0_16:0_18:1	0.43 ± 0.04
899.7095	TG 18:2_18:3_20:5	0.41 ± 0.04
796.7376	TG 14:0_16:0_16:0	0.38 ± 0.08
909.7874	TG 18:0_18:1_20:4	0.22 ± 0.01
870.7527	TG 16:0_18:2_18:3	0.12 ± 0.02
766.6896	TG 10:0_16:0_18:1	0.11 ± 0.03
760.5835	PC 18:1_16:0	0.10 ± 0.03
758.5684	PC 18:2_16:0	0.09 ± 0.02
846.7528	TG 16:1_16:1_18:1	0.09 ± 0.01
684.6121	TG 8:0_14:0_16:0	0.06 ± 0.02
608.4288	PE 12:0_14:0	0.05 ± 0.03
738.6587	TG 12:0_12:0_18:1	0.04 ± 0.01
786.5998	PC 18:1_18:1	0.02 ± 0.01
788.6144	PC 18:0_18:1	0.01 ± 0.01
520.3395	lysoPC 18:2	0.01 ± 0.01
706.5352	PC 18:0_12:0	0.01 ± 0.01
810.5981	PC 18:0_20:4	0.01 ± 0.01
	Total TG	10.84 ± 1.16
	Total phospholipid	0.29 ± 0.03
	weight % TG	97.34 ± 0.57
	weight % phospholipid	2.66 ± 0.57

Values are mean ± S.D. of n = 3 technical replicates. TG, triacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine. Identifications made automatically by Progenesis QI followed by manual inspection.

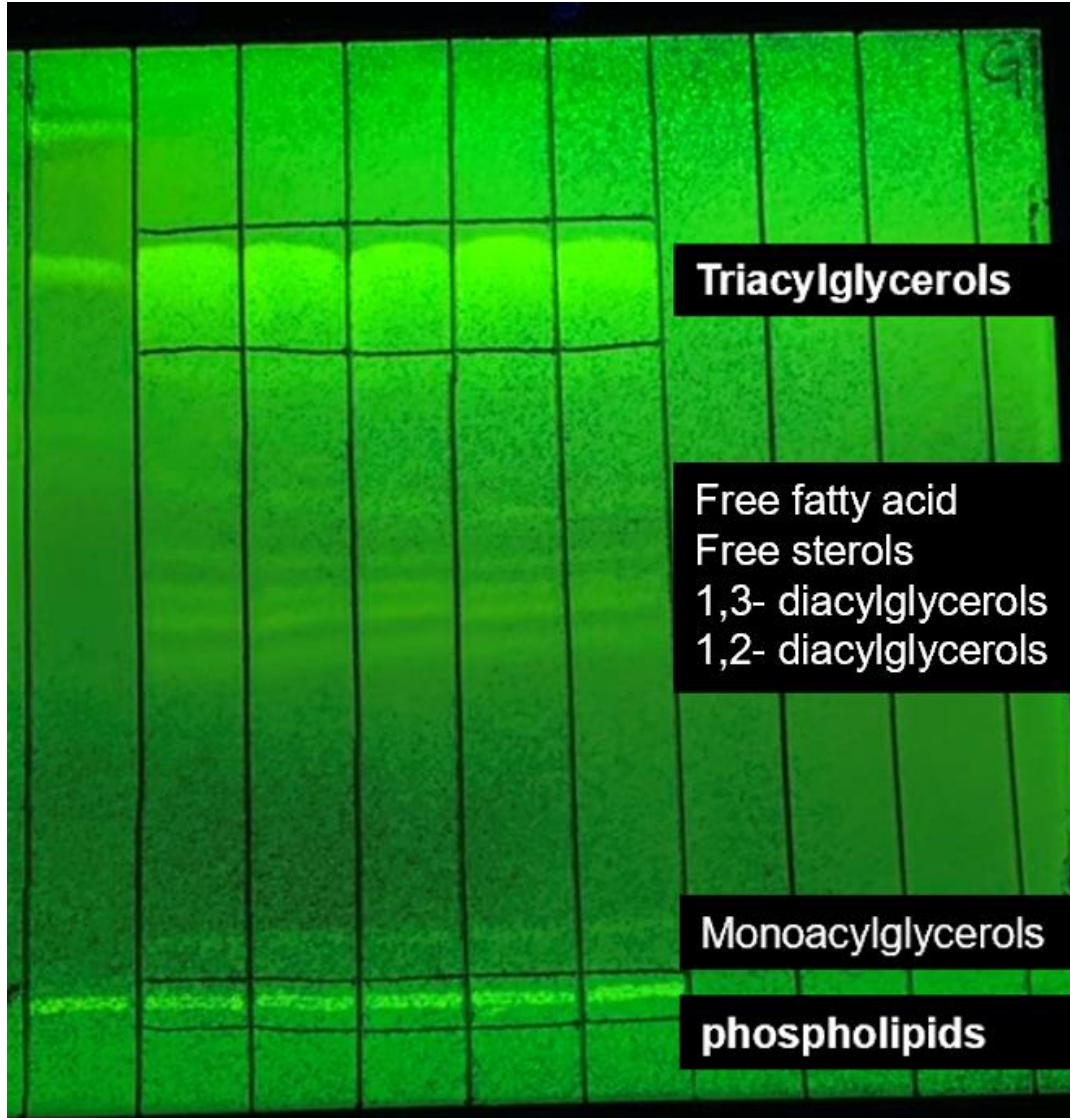


Figure 3. Thin layer chromatography of a breakfast sandwich. Lipids visualized with ultraviolet light after treating with dichlorofluorescein after separating on Silica Gel G plates with 60:40:2 heptane:diethyl ether:acetic acid (v/v/v).

Table 6. Fatty acid composition of the breakfast sandwich TG and phospholipids

Name	TG (mg/g)	Phospholipid (mg/g)	Sum of TG and Phospholipid	
			(mg/g)	(wt%)
12:0	0.22 ± 0.02	0.003 ± 0.001	0.22	0.40
14:0	0.83 ± 0.06	0.02 ± 0.01	0.86	1.58
16:0	7.99 ± 0.50	2.33 ± 0.07	10.32	19.08
18:0	2.55 ± 0.17	1.20 ± 0.02	3.74	6.92
20:0	0.10 ± 0.01	0.008 ± 0.001	0.11	0.20
22:0	0.05 ± 0.01	0.012 ± 0.001	0.06	0.11
24:0	0.02 ± 0.01	0.01 ± 0.00	0.03	0.06
SFA	11.88 ± 0.77	3.65 ± 0.06	15.53	28.72
16:1	0.61 ± 0.04	0.06 ± 0.01	0.67	1.24
18:1n-7	1.01 ± 0.06	0.11 ± 0.01	1.12	2.06
18:1n-9	18.64 ± 0.79	1.81 ± 0.02	20.44	37.81
20:1n-9	0.21 ± 0.01	0.01 ± 0.01	0.22	0.41
MUFA	20.58 ± 0.89	2.01 ± 0.01	22.59	41.78
18:2n-6	8.56 ± 0.60	2.39 ± 0.09	10.95	20.25
20:4n-6	0.07 ± 0.00	0.55 ± 0.02	0.61	1.14
N-6	8.73 ± 0.61	3.13 ± 0.12	11.86	21.93
18:3n-3	3.70 ± 0.19	0.16 ± 0.01	3.86	7.14
20:5n-3	0.005 ± 0.002	0.005 ± 0.001	0.01	0.02
22:5n-3	0.011 ± 0.002	0.022 ± 0.001	0.03	0.06
22:6n-3	0.02 ± 0.00	0.17 ± 0.01	0.19	0.34
N-3	3.74 ± 0.18	0.36 ± 0.01	4.10	7.58
PUFA	12.14 ± 1.08	3.48 ± 0.13	15.62	28.89
Total	44.93 ± 2.25	9.14 ± 0.19	54.07	100.00

Values are mean ± S.D. of n = 4 technical replicates. TG, triacylglycerol; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; n.d., not detected.

6.5 Discussion

Overall, there was agreement and disagreement between the fatty acid and lipidomic analyses of the breakfast sandwich. Given that direct biochemical quantitation by very different approaches were used and compared with data from a national database, complete agreement was not expected. Qualitatively, the analyses were relatively similar, but the lack of agreement on the concentration of lipid in the sandwich and the lower-than-expected levels of phospholipids was surprising. Determining the fatty acid content of the TG and phospholipids fractions were completed *ad hoc* in an attempt to resolve the differences. After reviewing the data in detail, it appears that precise determination of these discrepancies may require additional analyses and possibly repeating the experiment. Unfortunately, due to challenges with access to functional laboratory equipment as a result of the coronavirus pandemic, this was not possible. However, possible sources of error can be identified and discussed. There are known challenges in the quantitation of lipids when using untargeted lipidomics [74] but it appears that there was also error in the analysis of fatty acids by gas chromatography which is usually considered to be robust [68].

The first analysis of total fatty acids measured by gas chromatography indicated that the breakfast sandwich contained approximately half the amount of fat as compared with estimates generated from the Canadian Nutrient File database. In addition, there were decreased proportions of 16:0 and DHA and increased proportions of 18:1n-9 and 18:3n-3 by biochemical quantitation. The challenge of determining the precise nutrient composition of a single food item from a food database is well documented [53], therefore it felt reasonable to reject the first hypothesis that the biochemical determination and the Canadian Nutrient file data would be in

agreement. However, after the *ad hoc* analyses of the fatty acid composition of the isolated TG and phospholipid fractions were completed, the initial fatty acid analyses of the total lipid fraction may have underestimated the amount of lipid present in the breakfast sandwich. The sum of the total fatty acids from the TG and phospholipids fractions of 54.07 mg/g sandwich was twice the amount determined in the total fatty acid analysis (27.2 mg/g) and similar to the estimate based on the Canadian Nutrient file of 55.09 mg/g sandwich. This is somewhat surprising as the additional sample processing involved with preparing TG and phospholipid fractions by thin layer chromatography is often associated with some lipid loss based on anecdotal observations within our laboratory. Interestingly, the qualitative assessment of the fatty acids as determined by weight % was very similar by these two separate analyses with the exception of differences in fatty acids of low abundance such as DHA. This suggests that there were problems in quantitation.

Errors in quantitation may be related to failing to prepare a consistent representative homogenate across the multiple analyses. For each individual analysis, the variation of the technical replicates was low, suggesting that the blended sandwich preparation was homogeneous. However, due to the size and heterogenous nature of the original breakfast sandwich, it is possible that the additional water used to aid the blending process could have led to partitioning of the lipids if the sampling for extraction was not completed immediately after mixing. It has been suggested previously that food samples may require freezing and additional pulverization and mixing to achieve a true homogenate [94]. This can ideally be achieved by large scale freeze dryers available in the food industry, or they can be achieved in a research laboratory setting by using liquid nitrogen to freeze and then pulverize the sample [95]. The

other possible source of error between three analyses could be the different internal standards used. The concentration of the TG 17:0_17:0_17:0 standard used in the original fatty acid analysis of total lipids may have been higher than the 539 nmol recorded on storage source resulting in underestimation of the fatty acids in the sample. The internal standards are stored in solvents that can evaporate over time during storage and handling, resulting in a concentrating effect and this was not assessed.

Based on the lipidomic analysis, TG species were estimated at 97.3% of the lipid weight with 2.7% lipid weight being phospholipids in the most abundant lipids. The *ad hoc* analyses of the fatty acid composition of the isolated TG and phospholipid fractions using thin layer chromatography and gas chromatography suggested that 83% of the fatty acids came from TG and 17% from phospholipids. These fatty acid determinations of lipid classes also indicated that phospholipids may be a main dietary source of LC-PUFA. LC-PUFA have important bioactive roles such as eicosanoid synthesis [96]. While it was expected that TG would make up most of the lipids, based on the literature it was hypothesized that the phospholipid fraction would be approximately 5% of the total lipid [13]. Resolving the differences in the estimates of phospholipid content by lipidomics and by gas chromatography after phospholipid isolation is difficult. Again, if the lipids of the sample partitioned during the sampling process, this heterogeneity would impact the estimates of TG and phospholipid as the polar and nonpolar lipids may not have been distributed equally during sampling. However, limitations in the lipidomic method should be examined. Determining the total lipid amount is extremely difficult with lipidomics as there are thousands of possible compounds to quantitate, and untargeted lipidomic approaches are noncomprehensive. The present method used a top 5 data dependent

acquisition mode that only identifies 5 lipids at a specific time as the instrument is incapable of producing MS/MS fragments for all precursor ions and tracking them at a single time point. This form of data acquisition can lead to under sampling, especially of precursors of low abundance. A greater mass of phospholipids would have been measured if we identified and quantitated more lipids beyond the 0.01mg/g sandwich threshold, but it is not clear if this would have any effect on the proportion of phospholipids as other lipids from other lipid classes would also have been identified. Additionally, because it was known that the lipids in the samples were mainly TG, a dual column chromatography set up and positive mode ionization for MS was used in this experiment which favours TG species separations and detections.

The fatty acid concentrations determined by the gas chromatography and estimated from the lipidomic analyses did not agree quantitatively, however qualitatively, there was considerable agreement as the patterns and percentages of fatty acids within the total fatty acids measured were relatively similar. Quantitation is a well-documented challenge in lipidomics due to the diversity in the types of lipids and the diversity of fatty acyl species within a species type of lipid [12]. It may be necessary to re-examine sample preparation and chromatography and sample introduction to improve quantitation of the different lipid classes in food. Incomplete extraction can lead to errors in the total lipids and result in distortions among the relative amounts of component lipid classes [97]. In these analyses, a solvent system of mixed polarity (2:1 chloroform: methanol) was used for all the analyses rather than the nonpolar solvent systems traditionally used for food analysis in an attempt to extract the polar and nonpolar lipids. However, different internal standards were added at different stages of sample preparation. For the lipidomic analysis, the internal standards were added after the lipids were extracted due to

their financial cost, while the internal standards for fatty acid analysis were added before extraction. The standard for the fatty acid analysis of phospholipids would have acted as a “surrogate” standard and accounted for lipid losses during extraction while the lipidomic standards would only indicate what was left after extraction.

Method targeting may also improve lipidomic quantitation. Chromatography can be tailored for better separations of different types of lipids and running the samples using both positive and negative mode ionization can improve the determinations of the various types of lipids [98]. While 2:1 chloroform: methanol is commonly used to extract cellular lipids, some lipids are too polar and partition with the aqueous fraction [99] while some lipids remain tightly bound to aqueous cellular components such as proteins and require additional treatment for extraction [46]. Precise and accurate quantitation of food lipids may require a variety of thorough and comprehensive procedures. In addition, these procedures will need to be validated by using analytical chemistry procedures such as spike and recovery experiments to determine the efficiency of a procedure for quantitating specific types of lipids within a food matrix by an analytical system.

6.6 Conclusion

In this chapter, it was confirmed that TG are the main type of food lipids and that phospholipids are a component of dietary lipids in a breakfast sandwich. Unfortunately, despite considerable agreement on the qualitative fatty acid composition of the breakfast sandwich, the quantitative determinations of the fatty acids and lipids of breakfast sandwich was inconsistent. The breakfast sandwich is a heterogeneous mixed food item that requires extensive sample preparation to achieve a representative homogenate. The present experiment attempted to

achieve this by extensive blending, but the addition of water to the sample may have resulted in differential partitioning of the sample after blending. Different practices for the use of internal standards for the different methods may have also contributed inconsistencies. However, qualitative and semi-quantitative analyses such as those performed on the breakfast sandwich help to identify specific lipid species that are abundant in a food item and can inform the development of targeted analyses that are more quantitatively robust in the future.

Chapter 7

Macrolipidomic Analysis of Traditional Canadian Meals

7.1 Rationale and Objectives

Dietary assessment and advice on dietary fat is based mainly on the classes of fatty acids and dietary cholesterol. In food, most fatty acids are found as fatty acyls within a TG molecule. TG and cholesterol are both nonpolar molecules, so the analytical methods used to routinely measure dietary fat for food labels and nutrient databases use solvents optimized for nonpolar lipid extraction [62]. In Chapter 6, we have identified that a food item (a breakfast sandwich) can contain phospholipids, a polar lipid and that these polar lipids may be an important dietary source of bioactive LC-PUFA. Information about the intake of phospholipids at daily levels in populations is limited and there is no data on the intake of specific individual phospholipid acyl species. Global or “discovery” lipidomic analysis using untargeted techniques is a considerable analytical burden with several limitations especially regarding the determination of precise concentrations of the numerous lipid species in food.

In this Chapter, the primary objective was to perform a preliminary macrolipidomic characterization of Canadian dietary lipid intakes. This was completed using a daily set of meals representing the traditional Canadian diet as designed previously for confirming intakes of individual fatty acids (particularly EPA and DHA) using direct biochemical quantitation [100]. Due to the challenges in quantitation with lipidomic analyses observed in Chapter 6, the main goal was to identify abundant lipid species, although internal standards were still added to allow crude estimates of concentrations of the lipids to be determined. In addition to the

macrolipidomic profiling by UHPLC-MS/MS, fatty acid analyses by gas chromatography and estimates of nutrient compositions using the Canadian Nutrient File database were also completed for comparison. Given the quantitative inconsistencies observed in Chapter 6, sample preparation was examined in more detail. Cryomills are reported to be used by the U.S. Food and Drug Administration to homogenize challenging food items [101]. Therefore, in addition to analyzing a sample of the blended homogenate, the homogenate was also analyzed after additional pulverization after freezing, but this was completed only for fatty acid analyses due to restricted laboratory access due to the coronavirus pandemic. The macrolipidomic profiling, however, can be used to generate a list of lipids that exist in Canadian diets to improve the ability of future lipidomic analytical approaches to quantitate the lipid species of food items and the intakes of individuals with an eventual goal of using lipidomics to assess dietary intake at a population level.

7.2 Hypotheses

- According to the Canadian Nutrient File database the total amount of fatty acids should be 26.9g in the breakfast meal, 20.7g in the lunch meal, 12.2g in the afternoon snack and 36.7g in the dinner meal.
- The analysis of food samples using an untargeted macrolipidomic approach will detect and allow for semi-quantitation of individual fatty acyl species of lipid molecules in the meals.

- A profile of individual phospholipid acyl species consumed, and crude estimates of the amount consumed by the average Canadian will be created for future confirmation and validation.
- The fatty acid data determined by gas chromatography will be consistent with the fatty acid acyl species profiles of the lipidomic data.

7.3 Methods, Materials and Study Design

7.3.1 Sample Preparation and Fatty Acid Analyses

The diet examined in this study was initially designed with Food Processor SQL Edition dietary analysis software (version 10.1, ESHA Research, Salem, OR) to reflect the daily diet of a typical Canadian male between 20–64 years of age [100]. Briefly, this daily diet included an egg and toast breakfast, a deli sandwich lunch, an afternoon snack and a salad and pasta dinner meal (see details in Table 7). The food was purchased in December 2018 from a Walmart supermarket in Kitchener-Waterloo, Ontario. Individual meals were prepared as they would be at home with the masses of each food item recorded. The items of each meal were combined and blended to homogeneity in a 4-Liter blender. The total meal homogenates and pulverized food were weighed, aliquoted into 3x50mL Falcon tubes and stored at -80°C until analysis. At a later date the blended homogenates in the Falcon tubes of each blended meal were then pulverized with a large mortar and pestle that was prechilled to -80°C and liquid nitrogen was added during pulverization for additional homogenization [102].

7.3.2 Macrolipidomic Profiling

The macrolipidomic profiles and the fatty acid compositions of the total lipid extracts of each meal were determined in triplicate according to the workflow presented in Figure 4 and using methods as described previously in Chapter 4. For the fatty acid composition analyses of the blended and pulverized homogenates, an internal standard (21:0 ethyl ester) was added to the meal samples (0.25g) prior to extraction with chloroform/methanol. The total lipid extracts were then transesterified using 14% BF₃ in methanol to produce FAME for analysis by GC-FID. The Canadian Nutrient File (version 2015) was used as described in section 6.3.2 to determine nutrient composition of each food item, each meal and for daily intake. For the macrolipidomic analyses, the homogenate samples (0.25g) were extracted with chloroform/methanol in quadruplicate, dried and then reconstituted in the UHPLC running buffer. The reconstituted samples were then spiked with a mixture of 13 deuterated lipid internal standards at a concentration of 100µg/mL each (EquiSPLASH™ LIPIDOMIX® Quantitative Mass Spec Internal Standard, Avanti Polar Lipids, Inc. Alabaster AL, USA) and then analyzed by UHPLC-MS/MS. Lipid identifications were achieved by manual confirmations of automated identifications of lipids by Progenesis software QI (Nonlinear Dynamics, Newcastle upon Tyne, UK).

7.3.3 Statistics

In this chapter, the total fatty acid concentrations of mixed meals after preparing homogenates by blending or by blending then pulverizing were compared using an independent t-test with significance accepted at a $P < 0.05$ (Excel, v1908, Microsoft). The individual fatty acids of each meal after the pulverization preparation are compared by one-way ANOVA with

Tukey's post hoc testing after a significant F-value with significance accepted at a $P < 0.05$ (SPSS Statistics, v26, IBM). The fatty data is compared with the estimates from the Canadian Nutrient File database without formal statistical procedures. For the lipidomic analyses, the analytical responses of the internal standards of the EquiSPLASH, and the sums of the identified TG, phospholipid and total lipid species of each meal were compared using the one-way ANOVA procedures described above.

Table 7. Composition of the meals

Food Item	Weight in grams
Breakfast	
Large eggs (2 ea)	103
Margarine (1 tsp)	6
White Bread (1 piece)	35
Peanut Butter (1 tbsp)	21
Orange Juice (1 cup)	259
Lunch	
Lunchmeat, Chicken Breast	48
White Bread (2 pieces)	71
Margarine (2 tsp)	8
Cheddar Cheese (1" cube)	38
Peeled Apple (1 ea)	114
Vanilla Yogurt	100
Soda, Cola (1, 355 mL can)	369
Snack	
Peanut Butter (1 tbsp)	14
Saltine Crackers (4 ea)	12
Rice Krispies Square (2 squares)	44
Soda, Cola (1, 355 mL can)	401
Dinner	
Iceberg Lettuce (1 cup)	58
Tomato (0.25 cup)	48
Balsamic Vinegar (1 tbsp)	19
Olive Oil (1 tbsp)	15
Tomato Sauce (0.5 cup)	123
Lean Ground Beef (fried)	100
Pasta (2 cups, cooked)	237
Milk, 2% (1 cup)	260

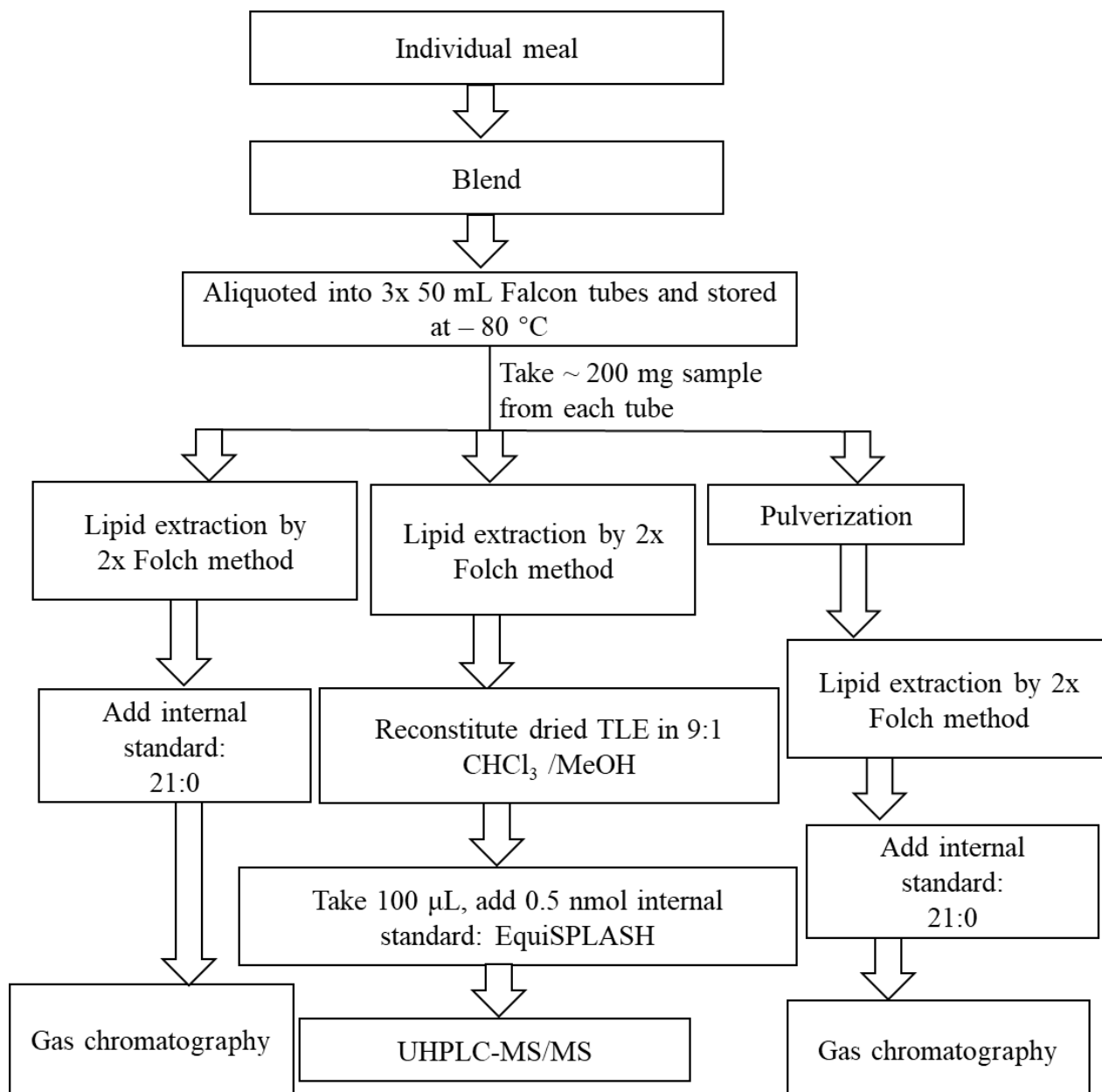


Figure 4. Illustration of the workflow for the fatty acid and lipidomic analyses of traditional Canadian meals. TLE, total lipid extract; CHCl₃/MeOH, chloroform/methanol; UHPLC-MS/MS, ultra-high pressure liquid chromatography tandem mass spectrometry.

7.4 Results

7.4.1 Fatty Acid Composition of The Meals

The total fatty acid content as estimated by the Canadian Nutrient File for breakfast (22.7g), lunch (19.7g), dinner (33.0g), snack (11.6g) and total (87.1g) were all higher than the biochemical determinations of the blended homogenates which were $17.3 \pm 1.9\text{g}$, $10.9 \pm 1.7\text{g}$, $11.9 \pm 0.2\text{g}$, $9.2 \pm 1.0\text{g}$ and $49.4 \pm 0.6\text{g}$, respectively (Figure 5). The experimental values were approximately 60-80% of the theoretical values except for the dinner meal which was only 37% of anticipated value. After pulverization, the measurements of fatty acid content of breakfast ($19.4 \pm 1.0\text{g}$), lunch ($14.3 \pm 0.7\text{g}$), dinner ($18.8 \pm 0.4\text{g}$) and total (61.7 ± 0.8) were significantly higher than the determinations made from the blended samples while snack ($9.2\text{g} \pm 0.6$) was similar.

The total mass of the breakfast meal was the smallest (Table 7), but it had the highest concentration of total fatty acids ($45.2 \pm 2.2\text{mg}$ fatty acids /g meal homogenate) as compared with the other meals which were similar (Table 8). Most of the fatty acid concentrations were the highest in the breakfast meal, but the much higher total fatty acid concentration was mainly due to a very high concentration of 18:1n-9 ($25.6 \pm 1.3 \text{ mg/g}$) that accounted for $56.6 \pm 0.1 \text{ wt\%}$ of the total fatty acids in the breakfast meal while the percentage of 18:1n-9 was 35-50% in the other meals (data not shown). As a result, the breakfast meal had the highest percentage of monounsaturates ($60.9 \pm 0.1 \text{ wt\%}$). In contrast, the dinner meal had the highest percentage of saturates ($45.3 \pm 1.8 \text{ wt\%}$), and the snack meal had the highest percentage of n-6 ($22.4 \pm 2.7 \text{ wt\%}$) and total polyunsaturates ($25.2 \pm 3.4 \text{ wt\%}$). The breakfast meal had the highest

concentration of n-3 polyunsaturates (1.09 ± 0.05 mg/g) but the lunch meal had the highest percentage (3.38 ± 0.32 wt%). However, 22:6n-3 was the highest in concentration (0.12 ± 0.01 mg/g) and percentage (0.26 ± 0.01 wt%) in the breakfast meal. The breakfast meal also had the highest concentration of 20:4n-6 (0.35 ± 0.02 mg/g). In general, the pattern of the fatty acid concentrations was similar but slightly lower than those estimated from the Canadian Nutrient File with the exception of the dinner meal. Biochemical determinations (21.6 ± 0.4 mg total fatty acids /g meal homogenate) were considerably lower than the estimated 37.8 mg/g. In the daily intake, the most abundant fatty acids in the meals were 18:1n-9 followed by 16:0, 18:2n-6 and 18:0 by both the biochemical determinations and the Canadian Nutrient File estimates. This abundance pattern was similar across the individual meals except for dinner where the abundance of 18:0 and 18:2n-6 were relatively similar.

7.4.2 Lipidomic Profiles of the Meals

Different lipid species have different chemical and physical properties which influence their analytical response especially with UHPLC-MS/MS technologies. To assess the present lipidomic analysis, the response factors of EquiSPLASH internal standards were examined (Table 9). EquiSPLASH contains equal concentrations of 13 deuterated lipids for quantitation of the appropriate lipid classes. The average abundances of 15:0-18:1(d7) PC, 18:1(d7) Lyso PC, 15:0-18:1(d7)-15:0 TG were all similar, but ranged from 28-61 times higher than the abundances of 15:0-18:1(d7) PI, 15:0-18:1(d7) PS and 18:1(d7) MG. Therefore, the present method was better suited for identification of PC and TG of the food macrolipidome, as compared with other lipid classes found in the food microlipidome.

Using the untargeted top 5 DDA approach, the top 42 most abundant lipids for the breakfast, lunch, dinner and snack meals were identified (top 20 for each meal shown in Table 10). Given each meal had different lipidomic profiles, statistical analyses were not completed on the individual lipid species, but they were completed for the sum of identified triacylglycerols, phospholipids and total lipids but not the individual species (Table 10). The dinner meal had the highest amount of TG (23.44 ± 0.83 mg/meal) followed by lunch (20.29 ± 1.96 mg/meal), breakfast (15.85 ± 0.98 mg/meal) and then snack (7.79 ± 5.79 mg/meal). The breakfast meal was the highest source of dietary phospholipid (1.26 ± 0.11 mg/meal) while the other meals provided less than 0.5 mg phospholipid/meal. As a result, the phospholipid was 7.9% of the identified breakfast lipids, while it was only 1.8% for lunch, 2.1% for dinner and 3.4% for the snack.

The daily intakes of individual lipid species were calculated by summing the results of the individual meals (Table 10). The estimated intake of total identified TG was 67.37 ± 4.26 g/d for 96.58% of the identified lipids. The most five abundant lipid species were all TG and consisted of TG 18:1_18:1_18:1 (18.28%), TG 16:0_18:1_18:1 (12.80%), TG 18:1_18:1_18:2 (7.02%), TG 18:1_18:1_18:3 (6.73%) and TG 18:0_18:1_18:1 (6.05%) which accounted for 50.88% of the total identified lipids. For phospholipids, the six phospholipid species observed were PC 16:0_18:1, PC 16:0_18:2, PC 18:1_18:1, lysoPC 18:2, PC 18:0_18:1 and lysoPC 18:3 with a total mass of 1.44 ± 0.07 g/d and 2.09% of the identified lipids. TG 60:6 was found in the top 20 of all the meals except dinner (28th most abundant) and was the 17th most abundant lipid in the total daily intake. This lipid was automatically identified as TG 16:1_22:1_22:4 but this fatty acyl pattern could not be confirmed after manual inspection of the MS/MS spectra.

7.4.3 Comparing Lipidomics with Fatty Acid Analyses

The amount of specific fatty acids within the lipid acyl species of the lipidomic data was estimated for comparison with the fatty acid data determined by gas chromatography. The mass of the fatty acids in the top 12 identified lipids were determined and summed. These 12 lipids accounted for 75.97% of the identified lipids with each of these lipids contributing at least 2% of the total identified lipid weight (data not shown). The 12 lipids contained seven individual fatty acids in different arrangements. The most abundant lipid was 18:1 estimated at 30.40 g/day which was relatively similar to the amount determined from the pulverized samples by GC-FID (28.03 g/d, data not shown). The amount of 18:2 was also similar (8.80 vs. 7.96 g/d), but lipidomics estimates were lower for 16:0 (7.80 vs. 11.84 g/d) and 18:0 (1.34 vs 4.46 g/d). The lipidomic estimates for 16:1 appeared to be higher (2.30 vs. 0.97 g/d) while 14:0 (0.70 vs. 1.73 g/d) and 20:1 (0.63 vs 0.36 g/d) were within the same range, considering that there would be additional lipid species containing these fatty acyls outside the top 12 most abundant lipids.

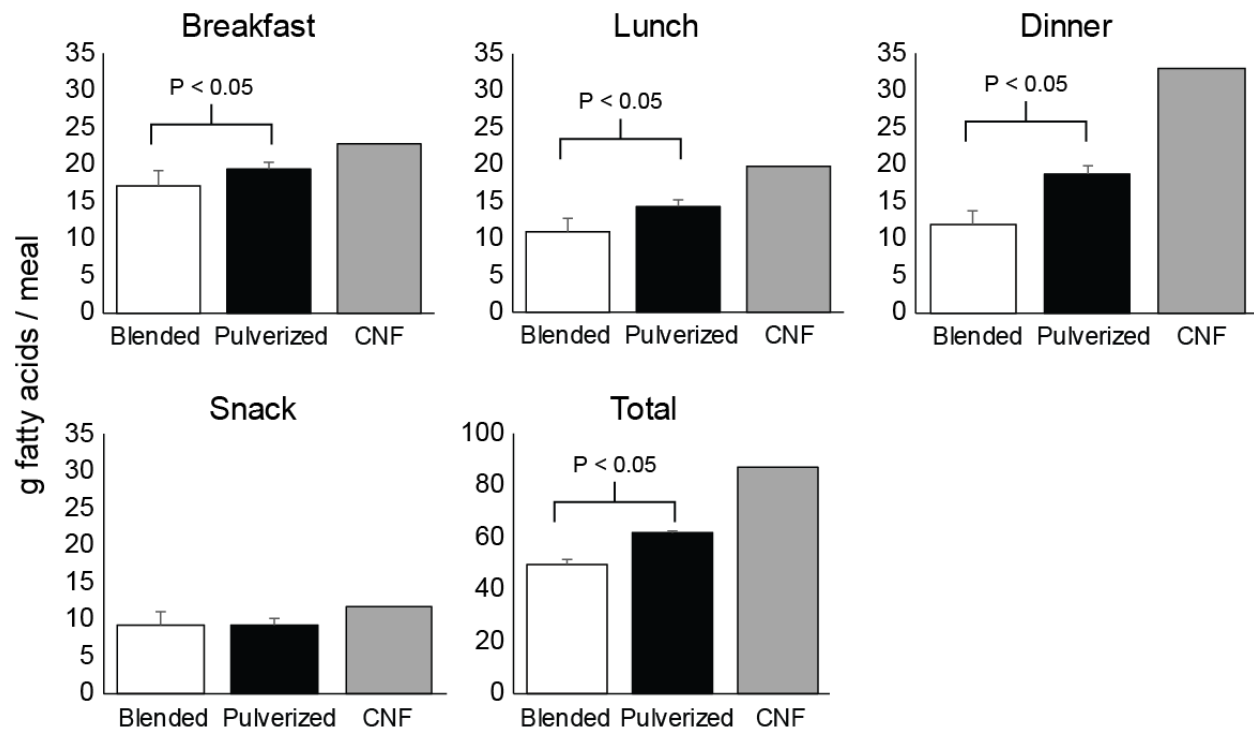


Figure 5. Amount of total fatty acids in each meal and daily amount after blending versus pulverizing preparation as compared with estimates calculated from the Canadian Nutrient File (CNF) database. Significant differences ($P < 0.05$) determined by independent t-test of $n = 3$ technical replicates.

Table 8. Fatty acid composition of the meal homogenates as compared with estimates calculated from the Canadian Nutrient File database

Name	Meal Homogenates (Pulverized)* mg fatty acid/g meal					Canadian Nutrient File Estimates mg fatty acid/g meal				Daily intake
	Breakfast	Lunch	Dinner	Snack	Daily intake	Breakfast	Lunch	Dinner	Snack	
12:0	0.167 ± 0.006 ^b	0.362 ± 0.048 ^c	0.171 ± 0.001 ^b	0.002 ± 0.001 ^a	0.196 ± 0.015	0.14	0.55	0.05	0.00	0.20
14:0	0.209 ± 0.012 ^b	1.077 ± 0.117 ^c	0.941 ± 0.020 ^c	0.005 ± 0.002 ^a	0.687 ± 0.037	0.17	1.69	0.96	0.01	0.87
16:0	7.06 ± 0.37 ^d	4.35 ± 0.44 ^b	5.59 ± 0.24 ^c	2.13 ± 0.09 ^a	4.83 ± 0.18	8.93	7.07	7.53	2.50	6.69
18:0	2.36 ± 0.12 ^c	1.40 ± 0.12 ^b	2.70 ± 0.32 ^c	0.59 ± 0.02 ^a	1.86 ± 0.08	3.33	2.70	3.17	1.08	2.67
20:0	0.176 ± 0.009 ^c	0.051 ± 0.005 ^a	0.042 ± 0.004 ^a	0.112 ± 0.006 ^b	0.081 ± 0.002	0.42	0.05	0.10	0.22	0.16
22:0	0.310 ± 0.014 ^c	0.030 ± 0.002 ^a	0.019 ± 0.001 ^a	0.244 ± 0.019 ^b	0.114 ± 0.003	0.87	0.02	0.03	0.51	0.26
24:0	0.156 ± 0.012 ^b	0.013 ± 0.001 ^a	0.011 ± 0.001 ^a	0.041 ± 0.054 ^a	0.042 ± 0.012	0.39	0.01	0.00	0.22	0.11
SFA	10.55 ± 0.54 ^b	7.52 ± 0.77 ^{ab}	9.77 ± 0.56 ^b	4.62 ± 2.47 ^a	8.28 ± 0.74	14.34	12.66	12.23	4.56	11.28
16:1	0.736 ± 0.038 ^d	0.235 ± 0.038 ^b	0.564 ± 0.025 ^c	0.018 ± 0.002 ^a	0.394 ± 0.018	0.80	0.63	1.08	0.03	0.70
18:1n-7	0.76 ± 0.02 ^c	0.33 ± 0.03 ^b	0.36 ± 0.11 ^b	0.17 ± 0.01 ^a	0.38 ± 0.04	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
18:1n-9	25.57 ± 1.28 ^c	6.66 ± 0.71 ^a	9.13 ± 0.45 ^b	9.65 ± 0.44 ^b	11.29 ± 0.38	22.94	8.23	20.07	9.68	15.10
20:1n-9	0.372 ± 0.021 ^c	0.080 ± 0.010 ^a	0.048 ± 0.005 ^a	0.205 ± 0.012 ^b	0.142 ± 0.004	0.35	0.02	0.11	0.20	0.14
MUFA	27.51 ± 1.36 ^c	7.41 ± 0.79 ^a	10.25 ± 0.39 ^b	10.08 ± 0.47 ^b	12.31 ± 0.41	24.14	9.28	21.65	9.93	16.21
18:2n-6	5.43 ± 0.24 ^d	2.73 ± 0.21 ^b	1.88 ± 0.09 ^a	4.22 ± 0.16 ^c	3.17 ± 0.08	11.63	3.32	3.21	8.79	5.72
20:4n-6	0.350 ± 0.017 ^b	0.002 ± 0.001 ^a	0.002 ± 0.001 ^a	0.005 ± 0.003 ^a	0.062 ± 0.003	0.38	0.04	0.05	0.01	0.09
N-6	5.99 ± 0.28 ^d	2.79 ± 0.21 ^b	1.94 ± 0.10 ^a	4.35 ± 0.18 ^c	3.33 ± 0.09	13.24	3.90	3.59	9.49	6.43
18:3n-3	0.96 ± 0.03 ^d	0.63 ± 0.06 ^c	0.22 ± 0.01 ^a	0.43 ± 0.02 ^b	0.50 ± 0.02	1.07	0.45	0.32	0.68	0.55
20:5n-3	0.004 ± 0.001 ^a	0.005 ± 0.001 ^{ab}	0.009 ± 0.001 ^c	0.008 ± 0.002 ^{bc}	0.007 ± 0.001	0.00	0.00	0.00	0.00	0.00
22:5n-3	0.012 ± 0.002 ^b	0.009 ± 0.001 ^{ab}	0.021 ± 0.001 ^c	0.006 ± 0.001 ^a	0.013 ± 0.001	0.02	0.02	0.00	0.00	0.01
22:6n-3	0.117 ± 0.011 ^b	0.002 ± 0.001 ^a	0.002 ± 0.001 ^a	0.014 ± 0.009 ^a	0.035 ± 0.010	0.17	0.00	0.00	0.00	0.03
N-3	1.09 ± 0.05 ^c	0.64 ± 0.06 ^b	0.25 ± 0.01 ^a	0.53 ± 0.08 ^b	0.57 ± 0.01	1.27	0.49	0.32	0.68	0.60
PUFA	7.09 ± 0.33 ^d	3.43 ± 0.28 ^b	2.20 ± 0.10 ^a	4.89 ± 0.25 ^c	3.90 ± 0.10	14.51	4.39	3.91	10.17	7.02
Total	45.15 ± 2.22 ^a	19.03 ± 0.88 ^b	21.56 ± 0.42 ^b	19.59 ± 2.12 ^b	24.45 ± 0.93	52.99	26.33	37.79	24.66	34.52

*Values are mean ± S.D. of n = 3 technical replicates. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; n.a., not available. Fatty acids of meals with different alphabetical superscripts were significantly different by Tukey's post hoc testing after a significant F-value by one-way ANOVA (P < 0.05).

Table 9. Abundances of different lipids classes in a commercial standard

Internal Standards*	Abundance
15:0-18:1(d7)-15:0 TG	9908 ± 2275 ^a
18:1(d7) Lyso PC	9068 ± 6380 ^{ab}
15:0-18:1(d7) PC	8445 ± 1871 ^{abc}
d18:1-18:1(d9) SM	3749 ± 987 ^{bcd}
15:0-18:1(d7) PG	2953 ± 4581 ^{cd}
15:0-18:1(d7) PE	2059 ± 469 ^d
18:1(d7) Cholesteryl Ester	1807 ± 482 ^d
18:1(d7) Lyso PE	1685 ± 366 ^d
15:0-18:1(d7) DG	1611 ± 330 ^d
C15 Ceramide-d7(d18:1-d7/15:0)	1145 ± 126 ^d
15:0-18:1(d7) PS	299 ± 199 ^d
15:0-18:1(d7) PI	166 ± 30 ^d
18:1(d7) MG	163 ± 31 ^d

*EquiSPLASH™ LIPIDOMIX® Quantitative Mass Spec Internal Standard, Avanti Polar Lipids, Inc. Alabaster AL, USA). Values are mean ± S.D. of n = 4 technical replicates. Abundances with different alphabetical superscripts were significantly different by Tukey's post hoc testing after a significant F- value by one-way ANOVA (P < 0.05). TG, triacylglycerol; PC, phosphatidylcholine, SM, sphingomyelin; PG, phosphatidyl glycerol; PE, phosphatidylethanolamine; DG, diacylglycerol; PS, phosphatidylserine; PI, phosphatidylinositol; monoacylglycerol.

Table 10. Most abundant fatty acyl lipid species in the breakfast, lunch, dinner and snack meals*

Breakfast		Lunch		Dinner		Snack	
Medio Level ID	Mass (g/meal)	Medio Level ID	Mass (g/meal)	Medio Level ID	Mass (g/meal)	Medio Level ID	Mass (g/meal)
TG 18:1_18:1_18:1	3.71 ± 0.38	TG 18:1_18:1_18:1	3.34 ± 0.30	TG 16:0_18:1_18:1	4.18 ± 0.28	TG 18:1_18:1_18:1	1.79 ± 1.54
TG 16:0_18:1_18:1	1.96 ± 0.19	TG 18:1_18:2_18:2	2.38 ± 0.39	TG 18:1_18:1_18:1	3.73 ± 0.22	TG 16:0_18:1_18:1	0.71 ± 0.73
TG 18:1_18:1_18:2	1.34 ± 0.08	TG 16:0_18:1_18:1	1.95 ± 0.13	TG 18:0_18:1_18:1	2.22 ± 0.11	TG 18:1_18:2_18:2	0.46 ± 0.09
TG 18:1_18:2_18:2	1.11 ± 0.09	TG 18:1_18:1_18:2	1.95 ± 0.22	TG 16:0_16:1_18:1	1.71 ± 0.06	TG 18:1_18:1_18:2	0.41 ± 0.12
TG 16:0_18:1_18:2	1.09 ± 0.07	TG 18:2_18:2_18:2	1.07 ± 0.21	TG 16:0_16:0_18:1	1.51 ± 0.08	TG 18:2_18:2_18:2	0.40 ± 0.05
TG 18:0_18:1_18:1	0.92 ± 0.05	TG 16:0_18:1_18:2	0.93 ± 0.10	TG 16:0_18:1_18:2	1.45 ± 0.04	TG 18:1_18:1_20:1	0.32 ± 0.16
TG 18:1_18:1_20:1	0.70 ± 0.04	TG 16:0_16:1_18:1	0.86 ± 0.07	TG 14:0_16:0_18:1	1.26 ± 0.06	TG 18:0_18:1_18:1	0.29 ± 0.12
PC 16:0_18:1	0.55 ± 0.04	TG 16:0_16:0_16:1	0.80 ± 0.06	TG 18:1_18:1_18:2	1.13 ± 0.03	TG 16:0_18:1_18:2	0.28 ± 0.10
TG 16:0_16:1_18:1	0.55 ± 0.03	TG 18:0_18:1_18:1	0.75 ± 0.10	TG 18:1_18:2_18:2	0.68 ± 0.05	TG 16:0_16:1_18:1	0.27 ± 0.32
TG 18:1_18:1_22:0	0.51 ± 0.04	TG 16:0_16:0_18:1	0.73 ± 0.05	TG 18:0_18:1_22:0	0.48 ± 0.05	TG 18:1_18:1_22:0	0.26 ± 0.04
TG 18:1_18:1_24:0	0.39 ± 0.04	TG 16:0_18:2_18:2	0.64 ± 0.09	TG 14:1_16:0_18:1	0.48 ± 0.01	TG 16:0_16:0_18:1	0.25 ± 0.28
TG 18:2_18:2_18:2	0.37 ± 0.05	TG 18:1_18:1_20:1	0.41 ± 0.04	TG 16:0_18:2_18:2	0.45 ± 0.03	TG 16:0_18:2_18:2	0.24 ± 0.05
TG 16:0_18:2_18:2	0.33 ± 0.03	TG 18:2_18:2_18:3	0.39 ± 0.08	TG 18:1_18:1_20:1	0.41 ± 0.03	TG 18:1_18:1_24:0	0.19 ± 0.02
TG 16:0_16:0_18:1	0.32 ± 0.04	TG 60:6**	0.38 ± 0.03	TG 20:0_20:1_20:1	0.38 ± 0.06	TG 14:0_16:0_18:1	0.19 ± 0.30
TG 18:1_18:1_20:0	0.30 ± 0.02	TG 12:0_14:0_16:0	0.35 ± 0.05	TG 16:0_16:0_16:0	0.32 ± 0.11	TG 16:0_18:0_20:0	0.17 ± 0.30
PC 16:0_18:2	0.26 ± 0.02	TG 12:0_12:0_16:0	0.34 ± 0.05	TG 16:0_16:0_18:0	0.31 ± 0.27	TG 18:1_18:1_20:0	0.16 ± 0.08
TG 20:0_20:1_20:1	0.21 ± 0.03	TG 12:0_16:0_16:0	0.32 ± 0.02	TG 16:0_16:0_18:3	0.28 ± 0.02	TG 16:0_16:0_16:0	0.16 ± 0.25
TG 14:0_16:0_18:1	0.20 ± 0.02	TG 14:1_16:0_18:1	0.28 ± 0.02	TG 18:2_18:2_18:2	0.26 ± 0.03	TG 18:2_18:2_18:3	0.16 ± 0.02
TG 60:6**	0.19 ± 0.01	TG 18:0_18:1_22:0	0.25 ± 0.01	TG 12:0_16:0_16:0	0.25 ± 0.01	TG 60:6**	0.15 ± 0.10
TG 16:0_16:0_18:0	0.16 ± 0.12	TG 16:0_16:0_16:0	0.23 ± 0.01	TG 12:0_14:0_16:0	0.24 ± 0.01	TG 18:0_18:0_20:1	0.13 ± 0.16
Total TG	15.85 ± 0.98 ^b		20.29 ± 1.96 ^{bc}		23.44 ± 0.83 ^c		7.79 ± 5.79 ^a
Total phospholipids	1.07 ± 0.09 ^d		0.12 ± 0.02 ^b		0.25 ± 0.02 ^c		0.01 ± 0.001 ^a
Total lipid	16.91 ± 1.04 ^b		20.41 ± 1.97 ^{bc}		23.68 ± 0.83 ^c		7.80 ± 5.80 ^a

*Top 20 individual lipid species shown with total TG and total phospholipids calculated from all identified lipid species (n=41).

**Compound could only be identified at the brutto level. Values are mean ± S.D. of n = 4 technical replicates. TG, triacylglycerol; PC, phosphatidylcholine. Identifications made automatically by Progenesis QI followed by manual inspection.

Table 11. Fatty acyl lipid species identified in a traditional Canadian diet by abundance

Medio Level Identification	g / day	% of Total Identified Lipids	Cumulative Abundance %
TG 18:1_18:1_18:1	12.57 ± 1.27	18.28	18.28
TG 16:0_18:1_18:1	8.81 ± 0.71	12.80	31.08
TG 18:1_18:1_18:2	4.83 ± 0.21	7.02	38.10
TG 18:1_18:2_18:2	4.63 ± 0.23	6.73	44.83
TG 18:0_18:1_18:1	4.17 ± 0.16	6.05	50.88
TG 16:0_18:1_18:2	3.74 ± 0.09	5.43	56.31
TG 16:0_16:1_18:1	3.38 ± 0.26	4.92	61.23
TG 16:0_16:0_18:1	2.82 ± 0.26	4.09	65.32
TG 14:0_16:0_18:1	2.45 ± 0.28	3.56	68.89
TG 18:2_18:2_18:2	2.10 ± 0.14	3.05	71.93
TG 18:1_18:1_20:1	1.85 ± 0.14	2.69	74.62
TG 16:0_18:2_18:2	1.65 ± 0.03	2.40	77.02
TG 14:1_16:0_18:1	0.98 ± 0.15	1.42	78.44
TG 18:0_18:1_22:0	0.94 ± 0.09	1.37	79.81
TG 20:0_20:1_20:1	0.92 ± 0.12	1.33	81.14
TG 18:1_18:1_22:0	0.88 ± 0.07	1.27	82.42
TG 60:6*	0.85 ± 0.08	1.24	83.65
TG 18:1_18:1_20:0	0.78 ± 0.09	1.14	84.79
TG 18:2_18:2_18:3	0.76 ± 0.06	1.10	85.89
TG 16:0_16:0_18:0	0.75 ± 0.26	1.08	86.98
TG 16:0_16:0_16:0	0.74 ± 0.10	1.08	88.05
TG 12:0_14:0_16:0	0.66 ± 0.02	0.95	89.01
PC 16:0_18:1	0.63 ± 0.04	0.91	89.92
TG 18:1_18:1_24:0	0.63 ± 0.05	0.91	90.83
TG 12:0_16:0_16:0	0.61 ± 0.03	0.88	91.72
TG 16:0_16:0_18:3	0.61 ± 0.04	0.88	92.60
TG 16:0_16:0_16:0	0.60 ± 0.25	0.88	93.48
TG 12:0_12:0_16:0	0.60 ± 0.03	0.88	94.36
TG 16:0_20:2_22:2	0.58 ± 0.08	0.84	95.20
TG 16:0_18:0_20:0	0.55 ± 0.30	0.80	96.00
TG 18:0_18:0_20:1	0.47 ± 0.16	0.68	96.68
TG 16:0_16:0_18:0	0.44 ± 0.12	0.64	97.32
PC 16:0_18:2	0.36 ± 0.02	0.53	97.85
TG 20:2_20:2_20:3	0.36 ± 0.01	0.52	98.37
TG 14:0_16:0_16:1	0.35 ± 0.02	0.51	98.88
TG 20:0_22:0_22:2	0.28 ± 0.05	0.41	99.29
PC 18:1_18:1	0.18 ± 0.01	0.26	99.55
lysoPC 18:2	0.15 ± 0.01	0.22	99.77
PC 18:0_18:1	0.11 ± 0.01	0.15	99.93
TG 18:1_16:1_18:1	0.040 ± 0.001	0.06	99.99
lysoPC 18:3	0.009 ± 0.001	0.01	100
Total TG	67.37 ± 4.26	97.91	
Total phospholipids	1.44 ± 0.07	2.09	
Total lipid	68.81 ± 4.30		

**Compound could only be identified at the brutto level. Identifications made automatically by Progenesis QI with manual confirmation of the top 20 lipids. Top 41 individual lipid species shown with values are mean ± S.D. of the sums of the n = 4 technical replicate analyses of the meals. TG, triacylglycerol; PC, phosphatidylcholine.

7.5 Discussion

In this Chapter, the fatty acid and macrolipidomic profiles of traditional Canadian meals [100] for a single day were examined. Single meals were prepared and cooked from food items purchased locally, and the blended to generate four meal homogenates. The total lipids of these samples were extracted using a mixed polarity solvent system and the fatty acids were determined by GC-FID and untargeted macrolipidomic profiling was completed using UHPLC-MS/MS. Internal standards were added to these samples in an attempt to quantitate the fatty acid and lipidomic results. The fatty acid analyses were then repeated after the frozen meal blends were pulverized under freezing conditions in an attempt to duplicate cryomill type conditions [101]. The fatty acid analytical results were also compared with fatty acid content estimates of the meals generated from the Canadian Nutrient File.

The fatty acid content of the blended meals was lower than the estimates from the Canadian Nutrient File. Pulverization of the blended meals prior to fatty acid analysis increased the measured fatty acid content, but the analytical results for each meal remained greater than 2 standard deviations away from the Canadian Nutrient File estimates. As such, the first hypothesis that the analytical and database estimates would agree, was rejected. Meals are large mixtures of different food items with different distributions of fat and can be considered liquid solid mixtures. Most food sample analysis is designed and tailored towards the composition of a specific food item and not meal composites [103]. However, appropriate sample preparation is required to obtain a representative sample homogenate and blending does not appear to be sufficient. Additional grinding on liquid nitrogen after homogenizing in the blender improved lipid recovery yields of the meals. Additional research examining the use of food analysis

protocols on whole meals appears to be warranted as most of the research is focused on single food items. In addition to the physical sample preparation, additional research evaluating chemical preparation of samples for lipid analysis is required. This would include examining different extraction procedures and different sample volumes for a variety of food matrices [62].

While the additional research in meal sample preparation is required, closer examination of the results that were generated did provide additional insights. The dinner meal analytical result (18.8 ± 0.4 g fatty acid) was much lower than the database value (33.0 g fatty acid) relative to the other meals. The dinner meal was pasta with meat sauce, a garden salad and a glass of 2% milk. The main sources of fat in the dinner meal were olive oil (1 tbsp – 15g), lean ground beef (100g) and the milk. It is most likely that the source of the discrepancy is the lean ground beef as it is the food product with the greatest possible amount of variation in the fat content prior to cooking. Cooking the ground beef also can add variation as the fat can render out of the beef and not be retained in the final meal product. As such, this suggests the database may have overestimated the fat content of the present meal as prepared for this study. In the future, additional analyses of each food item or at least food items with high fat content before and after preparation and cooking could provide further insights into the challenges of determining the fat content of mixed food item and/or meal homogenates.

Comparing the individual fatty acids of the meals provided information on the pattern of fat intake and the food sources of specific fatty acids in the diet. The breakfast meal of 2 large eggs, toast with peanut butter and a glass of orange juice was relatively small in mass but had a relatively high fat content. This high concentration of fat was mainly due to a much higher content of 18:1n-9. While the breakfast meal tended to have a higher content of most fatty acids,

the proportions of saturated/monounsaturated/polyunsaturated fatty acids was 23/61/16 for breakfast, while lunch was 40/39/18, dinner was 45/48/10 and snack was 24/51/25. The breakfast meal was also the highest in LC-PUFA, specifically 20:4n-6 and 22:6n-3. The peanut butter most likely contributed to the high amounts of 18:1n-9 in the breakfast meal [104], while the egg likely provided the LC-PUFA [105]. While confirmational analyses of individual food items is required, it would appear that eggs were the main dietary source of docosahexaenoic acid (DHA, 22:6n-3), an important n-3 LC-PUFA in this typical Canadian daily intake that did not contain fish or seafood. Considering the type breakfast meal, the potential for considerable individual and population variation in breakfast fat intake is high as it has been estimated that 10% of Canadians do not eat breakfast, and for those that do consume breakfast, the proportion of the meals that contain meat and meat alternatives such as eggs is approximately 37% [106].

The present macrolipidomic method was able to detect and identify 41 acyl species of TG and phosphatidylcholines. The ability to detect and quantify lipidomic profiles of the meals required a method to control for the different analytical responses of the distinct types of lipids in the mass spectrometer. Typically, this is done by the addition of internal standards to samples allows compensation for these sources of variability and critical for accurate quantification. In this experiment, EquiSPLASH which is a mixture of 13 deuterated lipid internal standards were used to measure several potential lipid species as these standards are recognized as having the closest chemical and physical properties as the lipids to be quantified but they are still distinguishable from the endogenous lipids in a mass spectrometer [98]. TG and phosphatidylcholine standards had the highest analytical response of all the standards in the present analytical workflow with the lowest abundant lipid in the meal contributing less than

0.03% of the total lipid identified suggesting the present method may be appropriate for macrolipidomic profiling of the highly abundant lipids in foods. This method, particularly the liquid chromatography, was optimized for TG detection [74] and it is well documented that phosphatidylcholines are easily ionized within the electrospray source [98]. A bias for detecting TG and phosphatidylcholines is problematic for “discovery” lipidomics as it is unclear if this analytical bias had a role in the lack of detection of the other species of lipids in this untargeted analysis, but given the abundance of the lipids identified, the mass contribution of unidentified lipids will be minor. Therefore, the method was capable of identifying and semi-quantitating the macrolipidome of the meals. However, targeted lipidomic analysis using diagnostic ions of low abundant lipid classes, and mass spectrometry and UHPLC parameters that improve the analytical response are required in the future to generate food content estimates of these low abundant lipids.

Forty-one high abundant lipids were identified in each meal. The most abundant lipid in these meals mimicking a traditional Canadian diet was TG 18:1_18:1_18:1 at 12.57 ± 1.27 g/d and this lipid was the most abundant in each of the meals except for dinner where it was the second most abundant after TG 16:0_18:1_18:1. TG 16:0_18:1_18:1 was also common across the other meals as the second most abundant lipid in breakfast and snack and third most abundant in lunch. TG 18:1_18:1_18:2 and TG 18:1_18:2_18:2 were also present in the top 4 abundant lipids of the breakfast, lunch and snack meals but not in the dinner meal. The dinner meal had TG 18:0_18:1_18:1 and TG 16:0_16:1_18:1 as the third and fourth most abundant lipids, respectively, which was unique amongst the meals. The overall pattern and abundance of lipids in the dinner meal was distinctive and it was the only meal that contains olive oil and beef fat

and no margarine and/or peanut butter. TG 18:0_18:1_18:1 and TG 16:0_16:1_18:1 are present in beef fat at moderately high abundances of 2-5% of TG lipids [107, 108]. Based on these observations and the results in Tables 10 and 11, it was determined that untargeted macrolipidomic analyses is capable to detect and semi-quantitate the individual fatty acyl species of lipid molecules of meals. However, there were challenges in identifying the lipid acyl species in the meals and manual inspection of the MS/MS spectra was required to correct several misidentifications made by the automated software. These errors appear to be related to issues with the Progenesis software relying on identifications based on accurate mass only (MS level) due to issues with importing the MS/MS fragmentation spectra which will be discussed in detail in Chapter 8.

Individual dietary phospholipid acyl species were identified, and intake masses were determined as hypothesized, but a phospholipid profile was not generated as only six phosphatidylcholines were identified in the top 41 lipids of the total daily intake. Other phospholipids such as phosphatidylethanolamines, phosphatidylserines, phosphatidylinositols and sphingomyelins that have been estimated previously were not identified [97, 109]. It was estimated that the daily phospholipid intake was 1.44 ± 0.07 g/d (2.09% of the total identified lipid). This is slightly lower than previous estimates for a Western diet of 2-8 g dietary phospholipid per day /d [109]. The breakfast meal (1.07 ± 0.09 g/meal) provided 74% of the phospholipid intake in the daily diet in this study. The two large eggs in the breakfast meal likely contributed the phospholipid content as it has been estimated that 28% of egg lipids are phospholipids [110] and that eggs contribute 10-40% of the daily intake of phospholipids in the United States [111]. The most abundant phospholipid in the breakfast meal was PC 16:0_18:1

(0.55 ± 0.04 g/meal) which is consistent with data on the fatty acid composition of egg yolk phosphatidylcholine with 62.8% of the *sn-1* position being 16:0 and 56% of the *sn-2* position being 18:1 [112]. Phospholipids containing LC-PUFA were not identified in the breakfast meal or any of the other meals, most likely because they were not within the top 41 abundant lipids. In contrast to the breakfast meal, none of the other meals contained a phospholipid in the top 20 dietary lipids and the most abundant phospholipid in these meals tended to be PC 16:0_18:2.

After summing the mass of the fatty acyls of the top 12 identified lipids, the estimated fatty acyl composition by macrolipidomics was relatively similar to the determinations by GC-FID and the estimated concentrations were in the same range. Therefore, the hypothesis that the fatty acid data from the two analytical systems would be consistent is acceptable. However, there was some evidence that the amount of 16:0 and 18:0 as lipid acyl species was being underestimated, while 16:1 and 20:1 was being overestimated. The use of accurate mass only (MS level) by the software rather than the MS/MS fragmentation patterns that was mentioned previously and will be discussed in more detail in the next chapter, is a likely cause of this problem.

7.6 Conclusion

In this chapter, the fatty acid and lipid species of traditional meals were examined. Quantitatively, the fatty acid content of the meals determined by GC-FID were lower than those estimated from the Canadian Nutrient File even after additional pulverizing of the frozen meal homogenates. The dinner meal estimates differed the most which may be due the fat content of ground beef used in the meal. The macrolipidomic analyses were successful in identifying and semi-quantitating the top 42 lipid acyl species and the fatty acids patterns in these lipids reflected

determinations made by GC-FID. However, manual inspection of the raw chromatograms and spectra were required to correct several misidentifications made by the automatic software that limited the number of lipids that could be identified. This challenge of manual integration limited the number of lower abundant lipids that were identified which compromised the attempt to profile dietary phospholipids. Detailed dietary phospholipid intake estimates will likely require lipidomic approaches targeted towards polar lipids in the future. Knowledge of the fatty acid content of the meals in this study assisted in determining which lipid species were misidentified but performing both fatty acid and lipidomic analyses of food items is not feasible for comprehensive food lipidomic profiling. Existing food nutrient databases such as the Canadian Nutrient File may be an alternative approach to identify lipidomic misidentifications despite differences in quantitation of fatty acids as the qualitative pattern of fatty acids (relative weight %) in the foods tended to be similar by the direct fatty acid analysis and the database. Improving automated identifications and developing systematic confirmations will be critical for future efforts of lipidomic profiling of the food supply.

Chapter 8

General Discussion

8.1 Overview

In summary, an untargeted macrolipidomic approach with an UHPLC-MS/MS method was used to analyse common edible oils, a breakfast sandwich and meals typical to Canadian daily intake. The overall goal of this thesis was to generate preliminary macrolipidomic data on abundant fatty acyl lipid species in the Canadian food supply and compare to traditional fatty acid analyses by GC-FID and fatty acid data in the Canadian Nutrient File database. While “true” quantitation is not possible with untargeted lipidomics, internal standards were used to allow semi-quantitation of the results to enable these comparisons, rather than generating only qualitative lipidomic data. Comparing lipid intake quantitatively (the mass concentration) and qualitatively (relative to other lipids) is common in nutrition and fatty acid research and therefore doing both enables a better assessment of a lipidomic approach for nutrient profiling.

The results of this thesis indicate that food macrolipidomic measurements are possible, especially for highly abundant lipid species, but this approach will not be capable of replacing traditional fatty acid analyses to define and characterize nutritional fat in the immediate future. The stated overall hypothesis that food lipidomics can replace traditional fatty acid analyses to define and characterize nutritional fat remains a possibility, but the challenges are greater than anticipated. Even with the traditional fatty acid analyses by GC-FID, we observed variation in analyses that suggested sample preparation and homogenization procedures need to be re-

examined. Lipidomics also has the ability to reveal changes in the structure of food lipids that fatty acid analysis of total lipids does not.

The untargeted macrolipidomic UHPLC-MS/MS procedure was able to separate and generate MS/MS spectra for most of the highly abundant lipids indicating that food lipidomic profiling is possible. However, correctly identifying the lipids species in an efficient manner is a major challenge. Manual inspection of the raw chromatograms was consistently required as the automated software identified the lipid correctly at the brutto level, but was not able to identify the correct acyl chain fragments for medio level identifications. As a result, the amount of manual inspection limited the number of lipids that could be identified, resulting in no data being generated for lipid classes other than TG and phosphatidylcholines in the meals and fatty acyl species were limited to the most common fatty acids. For example, we did not identify any phosphatidylethanolamines or any n-3 LC-PUFA containing lipids in the breakfast sandwich or meal homogenates. While the abundance of these lipids may be low relative to other lipids, their biological impact may be significant.

8.2 Addressing the Challenges and Limitations

Fatty acid determinations by GC-FID are known to be quantitatively robust [113], which was confirmed by the relatively small variation of the technical replicates in the analyses of this thesis. It appears, however that considerable variation in food analysis can be introduced in the preparation of food samples and lipid extracts prior to GC-FID. In both the sandwich and meal analyses, the measured fatty acid content of the food was below the estimates from the Canadian Nutrient File. The initial protocol attempted to use a 4-Liter commercial grade blender to homogenize the sample, but additional water was required. Additional pulverization of the

blended meals resulted in higher determinations of lipid content, but these remained lower than the database estimates. It is unclear if the databases are overestimating the lipid content, or if the blending and pulverizing procedures are limited in producing representative homogenates. Analysis of single food items as listed in the database is a possible strategy to better address this challenge in the future. The chloroform/methanol extraction used in this thesis is capable of properly quantitating lipids from a representative, small volume sample, but this was done using ground flaxseed [62]. Unlike ground flaxseed, the food items and meals examined in this thesis were heterogeneous in composition, and the procedures used may have failed to produce a representative homogenate. Freeze drying and cryomills may be required to process large volume, heterogeneous samples properly. It may also be necessary to extract the lipids from a larger mass of sample for each replicate in future studies. Although not examined in this thesis, the ability of the lipidomics to detect thousands of potential lipids in various structural and chemical states could also provide insights into the hydrolysis and oxidation of lipids over time and could be a useful tool in the study of food stability and storage in the future.

The untargeted macrolipidomic approach was tailored for the analysis of triacylglycerols, the most abundant type of lipid in the food supply [2]. As such, the liquid chromatography column set up and ramping were designed to separate the numerous possibilities of TG species. While these separations were not designed for other lipid classes, it is an approach that produces a high amount of resolution for analytical compounds and requires considerably more sample run time than most lipidomic analyses [74]. In theory, it should have been possible to resolve phospholipids chromatographically, but this could be optimized further. For the mass spectrometry settings, the samples were analysed using positive mode to identify the neutral loss

fragments of triacylglycerols as they hold a positive charge. Therefore, the triacylglycerols fatty acyls are identified by comparing the molecular ion to neutral loss fragments that have lost the mass of a specific fatty acid [82]. The fatty acyl fragments hold a negative charge and as such are undetectable in positive mode mass spectrometry. With phospholipids, the neutral loss of fatty acyl fragments can be very low in abundance and difficult to detect. Therefore, determining fatty acyl species of phospholipids is easier using negative mode mass spectrometry [114]. Future research in food lipidomics should examine using both positive and negative modes for food lipidomic analyses. In particular, food items known to contain phospholipids such as eggs and liver [109, 115] should be examined to assess the ability to detect a full range of phospholipids in a food matrix. Improving the ability to identify and quantitate food phospholipids may be critical to identify the intake of key bioactive nutrients as it appears that phospholipids may be the main source of n-3 and n-6 LC-PUFA for most Canadians [17]. Individuals that do eat fish and seafood, which is a small proportion of the Canadian population, likely consume appreciable amounts of TG containing n-3 LC-PUFA [17, 53].

The most frustrating limitation of the macrolipidomic analyses was processing of mass spectrometry data. For mass spectrometry analysis by the QToF, the instrument was controlled, and the data collected using the MassLynx software. The data was then transferred to the Progenesis QI software for processing. Progenesis is designed for systems biology research and discovery applications. The software is able to align analytical runs, compound results and identify compounds by matching spectra with known online databases such as Chempider, and LipidBlast. The identifications are assigned using a score based on mass, isotope intensities, and fragmentation patterns. Progenesis ranks multiple possible identifications and usually the

MS/MS spectra can be manually inspected within Progenesis for additional confirmation. After the mass spectrometry analysis of the samples and during the processing of the data, it was discovered that the Progenesis software was consistently unable to import the MS/MS data from the QToF instrument which limited the automatic identification process.

The Waters Synapt G2Si Quadrupole-Time-of-Flight mass spectrometer was designed primarily for proteomic applications. The speed of the Time-of-Flight detector is well suited for data independent acquisition (DIA) which was developed in 2003 [116]. Waters developed the Synapt G2Si to use DIA specifically, which they marketed as MS^E. With DIA, MS/MS is performed for every compound detected by MS and therefore has great potential for various discovery analytical approaches. In contrast, the older data dependent acquisition (DDA) mode, completes MS/MS for a set number of compounds based on their detected MS abundance. The Synapt G2Si QToF instrument can collect data using DDA but the software support is limited as compared with the support for DIA. With DIA in the Synapt G2Si QToF, the precursor ions from MS and the product ions from MS/MS are defined by time and not directly linked as precursor-product pairs. In lipidomic applications, this results in “noisy” and convoluted MS/MS spectra as product ions from multiple precursors are indistinguishable, resulting in it being very difficult to determine specific acyl species of lipids [74]. Due to these limitations the top 5 DDA technique used for the mass spectrometry analyses in this thesis was developed in our laboratory as a novel application of the Synapt G2Si QToF instrument [74]. With DDA, the MS precursor ions and the MS/MS products ions are precisely linked resulting in cleaner spectra with little analytical noise and increased confidence in lipid species identifications.

During the processing of the mass spectrometry data of this thesis, it became apparent that the Progenesis software was making identifications using scores based on MS data only (brutto level) and that the MS/MS data required for medio level identifications was not being imported into the program. Progenesis has the ability import MS/MS data from various instruments from different manufacturers using different acquisition modes. With the Waters Synapt G2Si QToF, Progenesis can import MS/MS data generated by DIA, but not MS/MS data generated by the unsupported DDA mode. DDA MS/MS data from other MS/MS instruments can be imported into Progenesis. Without the MS/MS spectra, Progenesis relies largely on accurate mass for identifications resulting in incorrect isomers being assigned to lipids. Lipids species with fatty acids that are known to be low in the food supply such as 16:1 were assigned to identifications of highly abundant lipids. Manual inspection of the raw chromatograms and spectra in the MassLynx software was required to correct these misidentifications. This is not a feasible approach for widescale lipidomic profiling of the food supply, so another MS/MS instrument should be considered or improvements to the lipid identification software is necessary. The experience of observing high abundant food lipids being identified as containing fatty acids that are known to be very low in the food supply indicates that the software is not using all the potential resources available for making the identifications. It would be interesting if mass spectrometry-based machine learning techniques [117] could be developed to take advantage of the existing food fatty acid databases to improve food lipidomic identifications and enable large scale profiling.

8.3 Conclusion

In conclusion, the macrolipidomic profiling of meals provided insight into daily intakes of fatty acyl specific lipids, but it appears to be a premature approach at this time. Future studies determining lipidomic profiles of food should target the profiling of food items with nutrient profiles documented in nutritional databases such as the Canadian Nutrient File and the USDA National Nutrient Database. As such the fatty acid data in these databases can be used to screen the lipidomic analysis results. Additional fatty acid analyses could then be done when the patterns of fatty acids in the nutrient database and the lipidomic profiling did not match to reduce the analytical burden of also completing gas chromatography on every food item undergoing lipidomic profiling. Moving forward, lipid identification systems need to improve, and they should be integrated with database systems for compiling the food lipidomic data. FooDB is a new comprehensive resource on food constituents, chemistry and biology has been recently released [118], but lipidomic profiles of food items within it are limited and it appears that integration with the Canadian Nutrient File has not been considered at this time. Overall, food lipidomic profiling is possible. The search for food authenticity, quality, and safety markers has improved over the past decade due to the development of lipidomics [119]. The specificity and sensitivity of mass spectrometry-based methods have been officially recognized by international quality system control agencies, and the application of multi-stage ion analysis has become mandatory by global regulations to identify fraud in food processing. However, these applications are targeted towards specific compounds and not comprehensive lipidomic profiling. Advancements and assessments of methods and lipidomic data processing must continue and thorough examination of the stability of food lipids at the lipidomic level are

required before widescale profiling of the food supply can be attempted. If this can be done, lipidomic approaches have broad potential for advancing nutrition and food science research. Lipidomic data will provide a better understanding of fat in the food supply and can give deeper insight into molecular composition and biological processes, provide a better understanding of the effect of processing on the dietary value of food, and improve food traceability and quality control resulting in a safer and healthier food supply.

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