DEVELOPMENT OF A RAPID PREDICTION MODEL FOR TOTAL FAT CONTENT IN CEREAL FOOD PODUCTS USING NEAR-INFRARED REFLECTANCE SPECTROSCOPY

by

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(Under the Direction of Sandra E. Kays)

ABSTRACT

AOAC Method 996.01, used to determine total fat in cereal foods as defined by the U.S. Nutrition Labeling and Education Act (NLEA), is laborious, time consuming and solvent dependent. Near-infrared (NIR) reflectance spectroscopy, a rapid and environmentally benign technique, was investigated as a potential method for prediction of total fat. NIR reflectance spectra (1104-2494 nm) of ground cereal products (n=72) were obtained using a dispersive grating spectrometer and total fat determined by AOAC Method 996.01. Using multivariate analysis, a modified partial least squares model was developed for total fat prediction, having a SECV of 1.12% (range 0.5-43.2%) and multiple coefficient of determination of 0.99. The model was tested with independent validation samples (n=36); all samples were predicted within NLEA accuracy. NIR reflectance spectroscopy, therefore, has considerable potential for determination of total fat in diverse cereal products for nutrition labeling and monitoring.

INDEX WORDS: NIR; cereal foods; nutritional labeling; total fat; AOAC 996.01

DEVELOPMENT OF A NEAR-INFRARED (NIR) REFLECTANCE MODEL FOR THE RAPID PREDICTION OF TOTAL FAT IN CEREAL FOODS[1]

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To those who have seen greater things in me

than I could have ever imagined.

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INTRODUCTION AND LITERATURE REVIEW

Introduction

Fat in processed cereal foods is of interest to consumers, the food industry, health agencies, and regulatory agencies. Although fat is essential to a healthy diet and has important properties in food processing, high fat intake by humans is associated with increased risk of several serious health conditions such as obesity, heart disease and diabetes (Health, 2002). Therefore, quantitation and reporting of fat in food products is critical to enable consumers to make educated food choices. The Nutrition Labeling and Education Act, enacted in 1990, requires that the nutritional content of processed and packaged food be declared on the product label. The mandatory labeling of total fat and saturated fat was included in this regulation and total fat defined as "total lipid fatty acids expressed as triglycerides" (21CFR101.9) (Code of Federal Regulations, 2003a). The accepted method for measurement of total fat in cereal food products is AOAC Method 996.01, which involves hydrolysis of the food matrix to make fat components available for ether extraction. The lipid extract is then saponified and methylated in preparation GC analysis (AOAC, 2002d). Although accurate, the AOAC method is extremely laborious, time consuming and uses hazardous chemicals. Near-infrared (NIR) spectroscopy is a rapid analytical technique that requires little sample preparation and does not require the use or disposal of chemicals (Blanco and Villarroya, 2002). The technique has been used for prediction of several components in cereal products, but studies on the prediction of fat in cereal food products are limited (reviewed by Kays, 2004). A study is needed to determine whether NIR spectroscopy can be used to predict total fat content in cereal food products within the accuracy required by NLEA regulations. To develop a NIR model for this purpose, a wide range of fat contents, cereal grains, and ingredients would need to be included in the data set to reflect the products commercially available and to build as robust a model as possible. The successful

outcome of such a study could eventually lead to development of an official method to determine total fat content by NIR and a general acceptance of NIR techniques for quality assurance by both industry and regulatory agencies.

Nutrition Labeling For Fat

The Nutrition Labeling and Education Act of 1990 amended the Food Drug and Cosmetic Act of 1938 so that nutrition information and its significance in a healthy daily diet would be available to consumers and expressed in a realistic and understandable way. The amendment was made, in part, to increase consumer awareness, consumer nutrition, improve health, and improve consumer confidence in the food industry. The Act covers labeling of almost all processed food products and voluntary compliance for the fresh produce and fish industry. Exclusions for labeling are, among others, foods prepared for immediate consumption that bear no nutrition claim, foods packaged in individual serving containers as received by the merchant, foods packaged with a total surface area less than 12 square inches, or foods shipped in bulk directly to manufactures for further processing (21CFR101.9(j)) (Code of Federal Regulations, 2003a).

Specific information required on the label includes “percent daily value” to enable educated decisions on how a nutrient fits into a healthy diet, statement of a realistic serving size to enable comparisons between similar products, and quantitative labeling of food components (such as calories, fat, saturated fat, cholesterol, sodium, among others) (21CFR 101.9) (Code of Federal Regulations, 2003a). In addition, The NLEA laid the grounds for strict regulations guiding nutrient claims (e.g. “reduced fat”, “low-fat”, “fat-free”) and the full disclosure of ingredients in a food to promote consumer trust (21CFR101.13, 62,73,75 and 21CFR101.9) (Code of Federal Regulations, 2003a).

With the enactment of the NLEA came the need to define label components, including the component "total fat". Total fat is defined by the NLEA as "total lipid fatty acids expressed as triglycerides" (21CFR101.9) (Code of Federal Regulations, 2003a). This definition includes mono-, di-, and triglycerides (but excludes the glycerol molecule), free fatty acids, phospholipid fatty acids, and sterol fatty acids.

Food regulators and manufacturers use the total fat content of a food to determine if a food meets the requirements to list a nutrient claim, such as "fat free" (if the total fat is <0.5g), "low fat" (if ≤ 3g), "reduced fat" (if the total fat is 25% less than a comparable food), and light (if 50% less fat than a reference food) (21CFR101.62(b)) (Code of Federal Regulations, 2003a). Health claims related to total fat content are: "Development of cancer depends on many factors. A diet low in total fat may reduce the risk of some cancers."; "Diets low in saturated fat, cholesterol, and total fat may reduce the risk of heart disease. Heart disease is dependent upon many factors, including diet, a family history of the disease, elevated blood LDL-cholesterol levels, and physical inactivity."; "Low fat diets rich in fiber-containing grain products, fruits, and vegetables may reduce the risk of some types of cancer, a disease associated with many factors."; and "Development of heart disease depends on many factors. Eating a diet low in saturated fat and cholesterol and high in fruits, vegetables, and grain products that contain fiber may lower blood cholesterol levels and reduce your risk of heart disease." (21CFR101.73, 75-77) (Code of Federal Regulations, 2003a). These nutrient and health claims are often displayed on the front panel of a packaged food, are easily seen by consumers and aid in the marketing of the product.

Because of the marketing potential of nutrient and health claims, an important role of regulatory agencies is to prevent falsely assigned claims. The NLEA, thus, sets limits on the accuracy of labeling. For example, the regulations state that, "a food with a label declaration of

calories, sugars, total fat, saturated fat, cholesterol, or sodium shall be deemed to be misbranded under section 403(a) of this act if the nutrient content of the composite is greater than 20% in excess of the value declared on the label. Provided, that no regulatory action will be based on a determination of a nutrient value that falls above this level by a factor less than the variability generally recognized for the analytical method used in that food at the level involved." (21CFR101.9(g)(5)) (Code of Federal Regulations, 2003a). However, "Reasonable deficiencies of calories, sugars, total fat, saturated fat, cholesterol, or sodium under labeled amounts are acceptable within current good manufacturing practice." (21CFR101.9(g)(6)) (Code of Federal Regulations, 2003a).

As a result of the Nutrition Labeling and Education Act, consumers are now able to compare the nutrition and ingredients information of processed and packaged foods and make educated decisions on how a food product fits into a healthy diet. In addition, the opportunity to use nutrition and health claims gives food manufacturers incentives to develop marketable foods to health conscious consumers. Furthermore, consumer confidence in the American food industry and products is enhanced by increased information about products and the involvement of regulatory agencies in dissemination and monitoring of the information.

Nutritional Importance of Fat

Fat is essential to a healthy diet. Fat in the diet has a vital role in metabolic and membrane functions and physiological processes such as storing energy, protecting and insulating the body, aiding intestinal absorption of fat-soluble vitamins, as eicosanoids, and as essential fatty acids (Kritchevsky, 2002). These essential fatty acids are linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) (Chapkin, 2000).

Different fats have beneficial or detrimental health effects depending on the fatty acids present. Gastrointestinal transduction mechanisms produce different vagal afferent firings for short-, medium-, and long-chained fatty acids and this response seems related to stomach emptying rates and obesity (French and Robinson, 2003). Medium chain (C8:0-C12:0) triglycerides are the most appetite satiating and actually promote weight loss because they are more easily absorbed and metabolized. On the other hand, diets too high in n-6 fatty acids, e.g. linoleic acid (18:2n-6), have been linked with an increased risk for heart disease. Sources of 18:2n-6 fatty acids in the diet are cereals and oils of corn, sunflower, safflower, and soybean which are often incorporated into processed cereal food products (Haard and Chism, 1996).

High fat intakes have been associated with several chronic diseases, e.g. type-2 diabetes, arteriosclerosis, cancer, cardiovascular heart disease and increased risk of obesity (Health, 2002). Low fat diets, in some studies, have also been associated with adverse effects such as decreased high-density lipoprotein (HDL) cholesterol (Kris-Etherton et al., 1996), increased fibrinogen (Elmer et al., 1996), and ischaemic stroke (McGee et al., 1985). Thus, although fat is essential to health and body function, optimal fat intake is very important in overall health and in certain disease conditions.

Obesity

Obesity is dependent on several factors such as genetics, diet, and energy output and is becoming an epidemic in the United States (USDA, 2000; Health, 2002). Nearly 64% of Americans over the age of 20 are considered overweight (Health, 2002). This percentage is an increase of 8% from that reported six years previously. The IRS recognizes that obesity is a disease, and treatment, specifically for obesity, can be claimed as a tax deduction (IRS Revenue Ruling 2002-19) (Internal Revenue Service, 2003). A recent Associated Press release reported

that worldwide, noncommunicable diseases, which include obesity related cardiovascular problems and diabetes, kill about 34 million people per year or approximately 60% of deaths (Associated Press, 2004). In addition, children and adolescents are becoming increasingly obese with nearly 127 million dollars per year being spent in obesity-related hospital costs for children and adolescents. Not surprisingly, obese children and adolescents have increased risk for developing impaired glucose tolerance, type-2 diabetes, and cardiovascular problems (Goran et al., 2003; Kavey et al., 2003).

The National Health and Nutrition Examination Survey (2002) investigated the prevalence of four medical conditions as they related to obesity expressed as body mass index (BMI). Body mass index, ((weight (lbs) ÷ height^2 (in^2))× 703), is a calculation used to determine one's health status with regard to overweight or obesity. A BMI of less than 25 is indicative of a healthy weight, whereas 25-29.9 is associated with overweight and a value equal to or greater than 30 is indicative of obesity (Health, 2002). It was reported that both men and women with BMI $\geq$ 40 had a much higher prevalence of type-2 diabetes, cardiovascular heart disease (CVD), hypertension and osteoarthritis than healthy weight men and women (Table 1.1).

Recommendations For Fat Intake

Many chronic diseases faced today, e.g. type-2 diabetes, arteriosclerosis, cholesterol, stroke and cancer can be prevented and/or treated through diet and weight control. Therefore the nutrition labeling of total fat and fat components, e.g. saturated, unsaturated, monounsaturated, and polyunsaturated fat content, is useful in the development of preventative and management diets. The Dietary Guidelines for Americans (USDA, 2000) recommends, based on a daily 2000 caloric intake, that total fat intake should comprise no more than 30% of calories and saturated

fat no more than 10% of calories (USDA, 2000). However, a recent review suggests a more broad and moderate range, 25-35%, of calories from fat in the diet (Kris-Etherton et al., 2002).

1) Type-2 diabetes. The occurrence of type-2 diabetes has been shown to be negatively associated with consumption of polyunsaturated fatty acids (Meyer et al., 2001). Increased consumption of polyunsaturated and monounsaturated fatty acids and corresponding decreased saturated fatty acid intake has been found to result in greater glycaemic control compared to high carbohydrate diets (Coulston et al., 1987). However, other studies have shown no significant effects from the increased consumption of polyunsaturated fatty acids on occurrence of type-2 diabetes (Bonanome et al., 1991; Garg et al., 1992; Abbot et al., 1989). Nevertheless, investigations into the effects of diets high in monounsaturated fatty acids have repeatedly shown favorable lipid and lipoprotein profiles, including lowered very low-density lipoprotein (VLDL) cholesterol (22% less) and triglycerides (19% less) and increased HDL cholesterol (4% greater) (Garg, 1998; Kris-Etherton et al., 2002). These changes in lipid and lipoprotein profiles are particularly beneficial to diabetic patients. The American Diabetes Association recommends tailored diet treatments for individuals. For obese type-2 diabetic patients with acceptable lipid and lipoprotein ratios, a lower fat diet (<30% total fat) is recommended because it favors weight loss, which in turn affects glucose metabolism and insulin sensitivity. However, for those type-2 diabetic patients that have elevated triglycerides, a moderate fat (30-35%) diet with increased monounsaturated and polyunsaturated fat and decreased saturated fat is recommended (American Diabetes Association, 2002).

2) Arteriosclerosis, cholesterol, and stroke. One of the risk factors for arteriosclerosis is also fatty acid composition of the diet. Saturated fats and *trans*-unsaturated fats increase the low-density lipoprotein (LDL) ratio in serum cholesterol but not the triglyceride levels. In addition,

trans-fatty acids have been shown to decrease HDL cholesterol and increase plasma triglyceride levels two to three times greater than the changes observed from consumption of C16:0 and C18:0, which are fatty acids often replaced by *trans* (Wijendran et al., 2003). Monounsaturated fatty acids, however, have been shown to reduce LDL cholesterol. Polyunsaturated fatty acids (PUFA) increase serum LDL concentrations slightly but are beneficial in that they reduce triglyceride levels in the blood (Kratz et al., 2002). Increased intakes of saturated fats, which are often incorporated into cereal food products, have been linked to several cardiovascular problems such as high serum cholesterol and stroke (Renaud, 2001). Often hydrogenated monounsaturated fats (*trans*) are incorporated into foods to replace saturated fats because of improved melting point and plasticity. Because of the health concerns of saturated and *trans*- fat in the diet, the Journal of the American Medical Association recommends substitution of non-hydrogenated unsaturated fats for saturated and hydrogenated unsaturated fats and increased consumption of n-3 fatty acids, fruits, vegetables, nuts, and whole grains (Hu and Willett, 2002).

3) Cancer. Fatty acids are capable of, either directly or indirectly, regulating various signal pathways, the expression of genes, cell metabolism, cell differentiation, growth, and diseases such as cancer (Wahle et al., 2003). Adipose tissue is actually regarded as an important endocrine organ because it secretes inflammatory mediators, which include tumor necrosis factor- alpha and interleukin 6 (Ajuwon et al., 2004). However, in obese individuals these inflammatory mediators are increased and may actually contribute to metabolic disease. The essential long chained polyunsaturated fatty acid, 18:3n-3, can be supplemented to counteract this (Browning, 2003).

Therefore fat is essential in the diet, but its role is complex. Much research is currently being conducted on the roles of the many types of fat in the diet. As more is learned about the

roles of saturated, monounsaturated, and polyunsaturated fats in causing disease and in disease prevention and treatment, consumers will be able to tailor diets more efficiently to their individual needs.

Role of Fat in Food Processing

Fats and oils play an important role in food processing. About 80% of the total annual production of oils and fats, 90.4 million tons, is for human consumption. Of this, 20 million tons are used as a frying medium (Gunstone, 2002). Use in frying allows the transfer of heat to the food causing evaporation of water and cooking of the product. Use in cooking produces flavors and products such as the desirable oxidation product 2, 4-decadienal and the undesirable and controversial acrylamide (Friedman, 2003). Fats and oils aid in food processing, allowing the manipulation of melting behavior and plasticity of baked goods through different blends of oils. Hydrogenated monounsaturated fats are often incorporated into foods to replace saturated fats because of the preferable melting point and plasticity of the hydrogenated oils. The use of fats and oils in baked goods determines the oxidative stability of the product altering the shelf life as well as the nutritive content (Gunstone, 2002). The aeration and texture of cakes, breads and cookies are also affected by the use of fat as during the baking process the fat crystals melt and air bubbles are left in their place. In the ice cream industry, fat stabilizes aerated foam, changing the melting temperature, creaminess, and taste (Gunstone, 2002). Guichard (2002) found, in model food systems, that the addition of fat, in contrast to protein, significantly increased the retention of hydrophobic flavor compounds due to the melting point of the fat. Manipulations in the fat content of model foods also changed the perception of flavor compounds (Guichard, 2002). Therefore, to food processors fats and oils aid in mouth feel/texture, as a flavor carrier, and in achieving the baking properties desired.

Cereals and Processed Cereal Products

Cereal products are essential to a healthy diet and compose the foundation of the United States Department of Agriculture food guide pyramid. According to the Dietary Guidelines for Americans, an essential diet contains six to eleven servings a day of cereals and grains (USDA, 2000).

Cereals are starchy grains of grasses that are used for foods and include wheat, rice, maize, barley, oats, rye, grain sorghum, proso, millet, teff, and Job's tears. Although not technically cereals, the food processing industry often includes buckwheat, amaranth, and quinoa as cereals. The American per capita consumption of flour and cereal products is 200 pounds per year and averages about 10.6 servings a day, however much of this consumption is as refined grains (Putnam et al., 2002). According to a Food Review release, in 2000 there was a 12% increase in calorie consumption in the U.S. from 1985 to 2000. Of this increase, 46% was from grains (mainly refined grains) and 24% from added fats (Putnam et al., 2002).

Few studies have examined the role of cereals in Western diets (Barker et al., 2000; Galvin et al., 2003). A recent study reported the importance of cereal foods in the diet of 1379 Irish adults (Galvin et al., 2003), and the affects of ready-to-eat breakfast cereals on vitamin intakes. The researchers found that despite the small amounts consumed (mean 28.6 grams), breakfast cereals contributed substantially to carbohydrate (8.1%), starch (10.8%), dietary fiber (9.8%), non-starch polysaccharides (10.8%), iron (18%), thiamin (14%), riboflavin (17%), niacin (15%), vitamin B_6 (13%) folate (18%), and vitamin D (10%) intakes. Of significant note is that increased consumption of ready-to-eat breakfast cereals was correlated with greater compliance with dietary recommendations for fat intake. In addition, higher consumption of breakfast cereals was correlated with an increased nutrient density (for many vitamins) and a lower

occurrence of vitamin deficiencies (Galvin et al., 2003). Thus breakfast cereals play an important role in overall nutrition.

Distribution of Fat in Cereal Products

Alone, cereal grains have low fat content, for example rice and oats contain approximately 0.9-3.1% and 4.9-7.9% fat, respectively (Bhatty and Rossnagel, 1980; Mazza, 1988; Morrison, 1978a-b; Lorenz and Hwang, 1986). However, with the exception of rice, cereals undergo a wide range of treatments and processes that often introduce other components with sugar and fat being the most common. As a result, cereal products can be very diverse in fat content. For example, the fat content of breakfast cereals can range from zero (extruded corn cereal) to 24% (coconut almond granola). Boosalis (2000) reported cholesterol, total fat, saturated fat, monounsaturated fat, polyunsaturated fat, and fatty acid distribution for numerous convenience foods, including cereal based convenience foods. Snack bars ranged in fat content from 2.4% (corn cake) to 31.1% (granola bar coated with peanut butter and milk chocolate); dry mixes from 2.5% (plain pancakes, dry mix complete, prepared) to 18.6% (chocolate dry mix pudding type); cookies from 1.55% (oatmeal, commercially prepared, fat free) to 26.4% (chocolate sandwich, crème filling, regular); chips from 15.2% (nacho flavored tortilla chips, light) to 33.4% (corn-based, extruded, plain); and crackers from 0.9% (rye wafers, plain) to 26.7% (wheat sandwich with peanut butter filling) (Boosalis, 2000). Therefore, fat content in cereal products can vary widely, predominantly as a result of processing.

Methods of Fat Analysis

Prior to the enactment of the NLEA, the accepted methods of fat analysis in cereal foods were AOAC Methods 920.39, 945.16, and 922.06. AOAC Methods 920.39 and 945.16, better known as the Soxhlet extraction method, determine crude fat gravimetrically following solvent

extraction (Zou et al., 1999; AOAC, 2002a,c). Although relatively simple, Soxhlet extraction often gives a lower analyte result compared to AOAC Method 922.06, especially for cereal products (Zou et al., 1999; Ranhotra et al., 1996). The method does not include the digestion or hydrolysis of the food matrix, therefore, bound lipids, including naturally occurring or added lipids and emulsifiers, can be excluded from the measurement (Ranhotra et al., 1996). AOAC Method 922.06, another gravimetric method of fat analysis, includes the separation of fat from the food matrix by hydrolysis with hot acid, making available previously bound lipids for ether extraction. The ether extract is evaporated and the analyte weighed to determine crude fat (Zou et al., 1999; AOAC, 2000b). This method tends to overestimate fat content because other components of the food, such as waxes, pigments, and other ether-soluble materials, may be included with the gravimetric measurement (Zou et al., 1999; Ranhotra et al., 1996). A study conducted by Zou and associates found that in cereal products heat treated during processing, fat was not easily recovered using solvents alone. Most cereal foods are processed with heat and, therefore, require acid hydrolysis prior to solvent extraction for more complete recovery of fat. This is particularly so for products that were baked or toasted. Due to the composition of cereal food products and to the NLEA definition of total fat (Code of Federal Regulations, 2003a), a method that includes hydrolysis of the food sample and excludes non-lipid components is required for the measurement of total fat.

AOAC method 996.01 addresses this need and was accepted as an official method of analysis in 1997. The method involves hydrolysis of the cereal sample with hot 8N HCl, liberating fat from the food matrix, and results in a more complete extraction with higher triglycerides than the use of solvents alone. After solvent extraction, evaporation, and saponification of the lipid components, the extract is esterified to form methyl esters of the fatty

acids. This step is very specific and excludes ether soluble non-energy contributing substances. Capillary gas chromatography (GC) is then implemented for the analysis of the fatty acid methyl esters in the determination of the complete fatty acid composition of the sample. Fatty acid composition is then used to determine the total fat content as well as the saturated, polyunsaturated and monounsaturated fatty acid content of the food.

Quantitation of fatty acids during GC analysis is via the internal standard tritridecanoin (C13:0), which is added to the sample matrix prior to the hydrolysis step. The fatty acid C13:0 does not occur in nature and is used as a correction factor for recovery against the individual fatty acid methyl esters of the sample and the individual fatty acid methyl esters of a mixed standard of known concentrations. Therefore, the analysis of total fat by capillary GC eliminates the need for gravimetric analysis of fat and excludes the measurement of non-energy contributing substances and possible human error associated with such measurements.

Disadvantages of AOAC Method 996.01 are that it is very time intensive, taking approximately eight hours to prepare two to three samples in duplicate for GC analysis. GC analysis is then performed in triplicate for each sample duplicate, thus, adding considerable time to the assay. Another disadvantage of AOAC method 996.01 is the requirement for a substantial amount of hazardous chemicals, i.e. diethyl ether (ethyl ether), petroleum ether, and boron trifluoride. Diethyl ether is highly unstable, forming peroxides when exposed to air and light, very volatile and flammable, and mildly irritating to the skin and mucus membranes. If inhaled in high concentrations diethyl ether can lead to narcosis, unconsciousness, or death due to respiratory paralysis. Petroleum ether is also highly volatile and flammable, exploding when introduced to air in conjunction with a flame. It has a defatting effect on the skin and if inhaled in large concentrations can cause headaches, drowsiness, and possibly coma. Finally, boron

trifluoride is a highly flammable chemical, is unstable, and must be stored under refrigerated conditions (2-8°C). Boron trifluoride is toxic if swallowed and harmful if the vapors are inhaled, causing possible irreversible effects. To minimize exposure of the operator to these chemicals, all steps involving them are performed in a fume hood. However, disposal of these chemicals needs to be handled carefully and adds substantial cost to the operation and the environment.

For many reasons, AOAC Method 996.01 is a costly analysis. Large numbers of samples are analyzed for fat content used for quality assurance by the food industry, commercial analytical laboratories, and regulatory agencies. For example, the projected cost to implement AOAC method 996.01, for chemicals alone, is $6.48 per sample (not in duplicate). This does not include labor, laboratory glassware (including rather specific glassware), water baths, steam tables, heating plates, vials, caps, capillary columns, gases, gas chromatograph, and specific hazardous chemical waste disposal. Often, food companies and researchers contract commercial analytical laboratories to perform the nutrition analysis of a food. This too can become expensive. One estimate given by a national analytical laboratory for the analysis of total fat in cereal foods was $150 per sample (in duplicate).

A significant, trend in scientific analysis is to decrease solvent use. This trend may be prompted by environmental concerns, the cost of solvents, and/or the need for disposal of hazardous materials (Stark, 1996). The Pollution Prevention Act of 1990 established a “green chemistry” national policy. This policy aims to prevent or reduce pollution at its source whenever possible through chemical products or processes that reduce or eliminate the use of and generation of hazardous substances thereby protecting human health and the environment (42CFR 133.13101(a)(2)) (Code of Federal Regulations, 2003b). Another trend is to reduce the amount of bench work in manufacturing and monitoring agencies. This may have originated

from reduced funding for staffing, which makes necessary the consolidation of laborious procedures into one piece of equipment (Mindel, 1997) thus increasing the presence of in-line monitoring in industry.

The need for rapid, yet accurate and environmentally benign techniques that reduce bench work has led to the investigation of spectroscopic methods of analysis for fat and other components of foods. Spectroscopic methods require no chemicals, are extremely rapid and are not labor intensive. Therefore, they are being increasingly investigated and used as alternatives to "wet chemistry" methods of analysis of agricultural commodities, foods, and pharmaceuticals (Blanco and Villarroya, 2002).

Near-Infrared (NIR) Reflectance Spectroscopy

Infrared spectroscopy is based on physical and chemical principles. In the 1800s Herschel discovered and coined the term "infrared" (beyond the red) (Osborne, 1981). He wanted to explain which colors of light carried the sun's warmth and found through his research that warmth is carried by waves that are invisible to the human eye and are much longer than those of visible light (Table 1.2). Later in the 1900s, further progress was made in infrared research; Coblentz discovered that compounds with similar chemical groupings also had similar absorption bands in the infrared region (2,500-15,000 nm). For example, Coblentz reported that aldehydes, ketones and all similar compounds with a carbonyl functional group demonstrated fundamental absorption bands around 6,000 nm (Osborne, 1981). The organic bonds -CH, -NH, and -OH also absorb energy at specific wavelengths. The concentration of bonds in the sample is relative to the heights of the peaks in the spectra. Therefore, chemical structures influence absorption at specific wavelengths, thus, providing information about the structure of a compound from its absorption spectra. Chemical bonds each have a vibration that is unique and

will absorb radiation at a particular wavelength. The near-infrared (NIR) region is composed of overtones and combination bands of fundamental vibrations in the mid-infrared (mid-IR) region. However, while the infrared region was being further studied, NIR was hampered until the invention of photoelectric detectors.

Absorption in the NIR region is affected by any property that affects bond strength, such as aromaticity, polar groups, and hydrogen bonding to neighboring atoms. Thus the amount of radiation absorbed can be considered proportional to the number of similar chemical bonds, enabling quantification of materials (Murray, 1986; Osborne, 1981). A spectrum results from the variation between the absorption and the reflected or transmitted energy projected at each wavelength and is expressed as log (1/reflectance) (Osborne, 1993).

Absorptions of the chemical groups CH, NH, and OH are very strong in the mid-IR region making it difficult to analyze constituents quantitatively. However, the constituents do not have strong absorption in the NIR region. Plus, the NIR region has weaker overtones and combination bands and chemical groups in protein, oil, fiber, and moisture can be measured. Therefore, NIR is preferable to mid-IR spectroscopy for the quantitative analysis of food constituents (Osborne, 1981).

Development of a robust NIR calibration that is relevant to the particular samples and component of interest is crucial to the NIR technique. A successful calibration depends on four factors. The first is suitability of the samples selected for construction of the model. Samples should represent the diversity expected in future samples to be analyzed. The second factor is the selection of a good reference method. A reference method should be as accurate and precise as possible since NIR models are only as accurate as the reference method chosen. The third is the development of a mathematical relationship, using chemometrics, between the sample

spectra, generated by the spectrometer, and the sample values for a parameter, obtained from the laboratory reference method. Finally, the fourth factor is adequate validation of the mathematical model using independent test samples (Williams, 2001).

1) Sample selection. First one must select a large number of samples, known as calibration samples. The samples must have a wide range and ideally an even distribution in composition and variation. It is important to choose calibration samples that are relevant to the characteristics of future samples. Nevertheless, selected samples should not to be redundant or the calibration model will be biased (Williams, 2001). Failure to include variation normally encountered may cause independent validation samples to be discarded as global and neighborhood H outliers. The global H outlier statistic gives a value of the difference between the sample and the population mean, while the neighborhood H statistic gives an indication of the distance between the predicted sample value and the predicted value of the nearest sample in the data set. It is important to have an even distribution of values for the analyte in question in order to avoid a skewed model. For example, if many high fat samples are selected and few low fat samples, the NIR prediction model would overestimate the low fat samples and underestimate the higher fat samples. As a rule of thumb, Gaussian, or normal, distribution patterns should be avoided for calibration sample selection. An even distribution along an anticipated range is ideal. For selection of independent validation samples, there are several methods of approach. One method is selection at a different time and date. Another is the selection of calibration and validation samples from a group based on sample number. In general, independent validation samples should not introduce different variation or replicate any calibration samples (Williams, 2001).

2) Reference method accuracy. The accuracy of the constructed model depends on the accuracy of the reference method chosen, due to the assignment of reference values to spectra.

Modern NIR reflectance spectrometers are very precise because of improvements in optical technologies, scanning equipment, chemometrics and software. Near-infrared spectrometers are often more precise than the reference methods available since many reference methods are a century old (Osborne et al., 1993). Therefore, a researcher must be very sure of the accuracy and precision of the reference method chosen keeping in mind the model's future application. For example, there have been several studies on the analysis of fat in cereal products using NIR spectroscopy over the past 20 years. However, of those studies, none is applicable to nutrition labeling in the United States today because the methods used either over estimated or under estimated total fat.

Hildebrand and Koehn discovered that sample preparation could account for up to 60-70% of the overall error in reference analysis (Hildebrand and Koehn, 1944). When samples are ground in preparation for reference and spectral analysis, as well as during sub-sampling, it is vital that the sample be thoroughly mixed. This ensures that sub-samples are representative, especially when the sample is heterogeneous, e.g. granolas. Reference results are often expressed on a dry weight basis as changes in relative humidity can cause changes in moisture content of the sample. In addition, the sample should be stored under appropriate conditions of temperature and humidity. Model success depends upon very careful preparation of the sample and performance of the reference analysis.

3) Chemometric analysis. Once NIR spectra are obtained and laboratory reference values assigned, chemometric analysis is performed for model construction. Chemometric analysis is a mathematical technique in which a computer program explains the nature of results through self-learning algorithms. The availability and decreasing cost of powerful computers coupled with

advanced software for chemometric analysis have enabled the rapid development of NIR models that are versatile, useful and robust over broad spectral regions (Dunmire and Williams, 1990).

Before modeling occurs repetition in the calibration data set and interferences must be minimized. If the calibration data set is large, an algorithm can be employed to determine which samples are spectrally similar and can, thus, be excluded from the calibration model. When samples are difficult to acquire or expensive to analyze, selection of calibration samples for the model can be based on known variations (Kays et al., 1999). Common interferences for calibration models include moisture content and particle size distribution. Minimization of interference due to particle size distribution can be accomplished by pre-treatment of the spectra with a scatter correction such as a multiplicative scatter correction or standard normal variate procedures. Forward or backward stepwise and step-up multiple linear regression, least squares regression, partial least squares regression and artificial neural networks (nonlinear) are multivariate analysis methods that can be used. The multivariate analysis method of choice will depend on the linearity between the NIR data and the reference data and the purpose of the analysis (Williams, 2001; Blanco and Villarroya, 2002).

During multivariate analysis, the sample reference values can first be used to reduce the number of NIR wavelengths to those wavelength segments that best explain the reference values, e.g. peaks at 1212 nm are useful in models for measurement of fat in high fat samples. Second, regression of the reference values and compressed wavelength segments is used to construct a mathematical model for prediction of the component of interest. This is most often performed by partial least squares (PLS) analysis, and sometimes modified PLS. Partial least squares is currently regarded as more robust than other regression techniques and results in lower prediction error. Once a regression technique has been selected, predictive cross-validation is

employed to determine the number of regression factors that is best for the model and will protect against over fitting. The optimal number of factors for the model is that which gives the minimum error between modeled and reference values (standard error of cross validation), the highest multiple coefficient of determination (R^2) and is still conservative in number. Generally, the fewer factors the better as more noise is built into the model with increased numbers of factors. The maximum number of factors that can be used is determined by the number of samples in the calibration (ASTM, 1995).

4) Validation. The final step in the development of a NIR technique is validation and is necessary to determine model accuracy and applicability. Validation of the model is performed through prediction of independent samples. Independent samples included in validation should not be replicates of calibration samples or introduce new variation but be of similar type. Accuracy of the model is expressed as the standard error of performance (SEP), which is the standard deviation of differences between NIR predicted and reference method values for the analyte. In general, to be satisfactory, the SEP should be no more than twice the pooled standard error of the replicated reference method. Several additional statistics are useful in evaluation of the success and usefulness of the NIR model. For the validation data set these statistics are: the mean and standard deviation (SD) of the reference method data and the NIR data, the bias, slope, and coefficient of determination (r^2) of the regression of reference data versus NIR data and the RPD. The means for the reference method values and NIR predicted values should be very similar, as should the standard deviations. Bias is the mean difference between the reference method values for each sample and the NIR predicted values in the validation data set and should be very low compared to the SEP. The slope and coefficient of determination show the relation of NIR predicted values to the reference values and should be close to 1.0. The RPD is the ratio

of the SD to the SEP for the prediction of the independent validation samples. The RPD indicates model applicability. For example, a model with an RPD of 3.1-4.9 is considered fair and suitable for screening; an RPD of 5.0-6.4 is good and suitable for quality control; an RPD of 6.5-8.0 is very good and suitable for process control, and an RPD value of 8.1 or greater is considered excellent and suitable for any application (Williams, 2001).

Advantages of NIR Spectroscopy

There are numerous advantages of near-infrared reflectance spectroscopy as an analytical technique. First, the technique can be non-invasive and non-destructive, thus, samples can be processed or utilized in further applications. Second, near-infrared reflectance spectroscopy is rapid and can be used for in-line process and quality control. Third, minimal sample preparation is required, sometimes merely grinding of the sample is sufficient. Fourth, spectral data are obtained in a matter of seconds. Fifth, no chemicals are required in the analysis, there is no need for specific disposal of hazardous waste, and, thus, the technique is environmentally benign. Additionally, labor cost is drastically cut, thereby reducing overall costs and increasing productivity (Blanco and Villarroya, 2002). Once a NIR calibration is obtained, NIR techniques take minutes to analyze the component of interest. Multiple determinations of components is also possible using NIR spectroscopy in that, from a single spectrum, multiple components can be predicted without additional labor or supplies, thereby, reducing the cost of analysis. For example, it is possible to determine the protein, starch, moisture, fat, and dietary fiber content as well as obtain color information all from one spectrum (Kays, 2004). Recent advances in NIR technology include miniaturization of optical technologies making portable NIR spectrometers possible. Such portable NIR spectrometers can be handheld, carried in a backpack, or even be mounted to a tractor (Blanco and Villarroya, 2002).

Applications of NIR Spectroscopy

Near-infrared (NIR) spectroscopy is a rapid, accurate and environmentally benign technique that can be used both qualitatively and quantitatively. The NIR technique eliminates most human error such as transferring, measuring, weighing, transposing, and other cumulative errors found in traditional "wet chemistry" techniques. All that is required for sample analysis using NIR reflectance spectroscopy is placement of the sample into a NIR spectrum analysis cell (prior grinding of the sample is needed in some cases) for scanning and the prediction of the analyte of interest.

The agricultural sector was the first to explore NIR spectroscopy as a technique (Blanco and Villarroya, 2002). The technique is now used extensively in agriculture in North America and Europe, to predict the protein and moisture content of grain (Frankhuizen, 1992; Williams and Norris, 1987). It is of note that, the Canadian Grain Commission implemented NIR spectroscopy for determining protein in wheat to replace the traditional Kjeldahl method. In 1995, the new technique was evaluated and it was found that the change to NIR spectroscopy saved approximately $2.5 million dollars and 47 tons of caustic waste in one year (Stark, 1996).

Near-infrared spectroscopy has been used for cereal products in such applications as the determination of amino acids in barley, and wheat, ash in flour (flour purity-bran), cellulose and color in flour (bran), dietary fiber in breakfast cereals, fat, β-glucan, wheat hardness, moisture, protein, sedimentation volume of wheat (protein), starch, starch damage, sucrose, and the presence of fungal spores, and water absorption (Osborne et al., 1993; Kays, 2004). Near infrared technology is not exclusively used for quantitation. Degree of cook in extrusion cookers and dough mixing in mixers have also been investigated in "real time", using NIR technology paired with fiber optic probes (Osborne, 1984; Wesley et al., 1998; Alava et al., 2001). Another

novel approach using NIR is its use in conjunction with artificial intelligence, i.e. fuzzy neural networks, to express human quality parameters, such as taste (Champagne et al., 1996; Barton II et al., 1998). A similar application is widely used in Japan to grade both domestic and imported rice. Taste analyzers use NIR measurements of composition and preference sensory scores to correlate into physicochemical parameters of the rice (Champagne et al., 1996). Use of these, so called, fuzzy neural networks and NIR are being considered in other nations for rice grading. However the models are limited to each nation or region's preference and the scale is not universal (Champagne et al., 1996; Barton II et al., 1998).

While AOAC method 996.01 costs approximately $6.48 per sample for reagents, plus costs for labor, glassware, and equipment, the only significant cost in using NIR is the equipment, with a small amount of labor. For example, the NIRSystems 5000, the "workhorse of industry", costs $56,500 and measures spectra within 1100-2500 nm. Another NIR system that many research institutions prefer is the NIRSystems 6500, which costs approximately $60,000 and has an expanded range of 400-2500 nm. If calibration construction is performed with either spectrometer, a special statistical software package is needed and costs an additional $7,200. However, a GC, required for the analysis of total fat in cereal foods using AOAC Method 996.01, would cost approximately $50,000-$60,000.

NIR Analysis of Fat

Near-infrared spectroscopy has been used extensively in a variety of products to predict fat content. For example, the dairy industry uses visible/NIR spectroscopy on-line as a means to determine the health status of livestock and for proximate component analysis, including fat content, of fluid milk and cheese (Jankovská et al., 2003; Albanell et al., 2003; Hernández et al., 2002; Lien et al., 2002; Whyte et al., 2000; Sgorlon et al., 2003). Near-infrared spectroscopy is

used for fat determination in the on-line monitoring and process control of ground beef (Togersen et al., 2003; Anderson and Walker, 2003a,b). The technique has also been applied to fat determination in live fish and cuts of pork, meats and fish (González-Martin et al., 2003; Chan et al., 2002; González-Martin et al., 2002; Cozzolino and Murray, 2002; Shimamoto et al., 2003a,b; Shimamoto et al., 2001; Solberg et al., 2003; Vogt et al., 2002).

One of the earliest studies for the application of NIR spectroscopy for fat determination in cereal foods was conducted by Kaffka et al. (1982). This was part of a project to determine egg content in pasta. Near-infrared reflectance spectra were correlated to fat values obtained by a modified version of the Lidner method. The fat extraction method involves phosphoric acid-alcohol hydrolysis followed by petroleum ether lipid extraction, evaporation, and gravimetric analysis of the residual. Thirty calibration samples of pasta (consisting of varying quantities of wheat flour, white of egg, and yolk of egg) were prepared by the Research Institute of Flour Milling and Baking Industries. Each sample was then packed into three different cells and scanned (1,000-2,638.4 nm) by a computerized spectrophotometer. The spectrophotometer consisted of a Cary Model 14 prism-grating monochromator with optics optimized for near-infrared. A high correlation of 0.9973 for fat with a standard error of $\pm$0.112% was obtained (Kaffka et al., 1986). An independent validation test set was not conducted. However, Kaffka et al. (1982) concluded that NIR reflectance spectroscopy was a potential technique for evaluation of pasta quality.

A multicomponent application of NIR spectroscopy in cereals was investigated by Sato et al. (2001) for the 1996 harvest of buckwheat flour. The parameters of interest were moisture, fat, and protein content. In this study, fat was determined by Soxhlet extraction. It was hypothesized that the NIR model developed could be used to predict the same components in the

harvests of following years. Successful analysis was achieved for the multiple components for the 1996 harvest. However, the prediction of the same components for 1997 was limited in success, and a bias correction was required. Once bias corrected, the model could be used successfully for prediction of moisture, fat and protein for breeding selection (Sato et al., 2001).

Near-infrared spectroscopy has been used in the satisfactory analysis of fat in baked products such as bread (Osborne et al., 1984). Osborne's research demonstrated that a finely ground sample is not always necessary for an accurate reading. When intact bread samples were compared with traditionally analyzed dried and powered samples the NIR results were relatively close. Gravimetric analysis of fat, following acid hydrolysis and solvent extraction, was used as the reference method for fat determination. Multiple correlation coefficients were 0.948 for intact and 0.981 for ground samples and the residual standard deviations were 0.16% for intact and 0.15% for ground samples. Validation samples were predicted with a standard deviation of the difference of 0.18% for intact and 0.17% for ground samples.

Osborne et al. (1983) developed a NIR reflectance model for prediction of fat in cake mixes. The reference method was Soxhlet extraction. A large number of retail samples (n=211) were used with a fat content range of 8.0-25.0%. For calibration development, 112 samples of bread, spongecake, scone, shortbread and shortcrust mixes were scanned with a 12-filter instrument and for model testing 99 additional samples were scanned. A model was developed via multiple linear regression of Soxhlet values and sample spectra (4 wavelength regions). The standard deviation of differences between the NIR and Soxhlet results was 0.62% fat with r=0.996. Testing of the model was performed on another instrument and at a different location. A bias adjustment to the model was required to adjust for the new instrument. However, on

prediction of validation samples there was a loss in accuracy, attributed to a change in the nature of the fat in the validation samples or to a unsatisfactory calibration.

Most NIR (1100-2498 nm) models for prediction of components in cereal foods are sample specific. Osborne (1988) used 44 differing cereal food samples (20 biscuits, 14 breads, and 10 breakfast cereals) with a range in fat content of 0.9-28.3% to develop a model to predict fat. Thirty-nine different cereal food samples (13 biscuits, 16 breads, and 10 breakfast cereals) with a range in fat content of 1.2-27.3% were chosen as independent test samples. The residual standard deviation of the NIR predictions versus the reference values for the calibration data set was 0.92% fat with a correlation of R=0.995. For prediction of the independent validation samples the standard deviation of differences was 1.10%. Wavelengths selected for model development were at 1210, 1388, 1720, 1760, 2306 and 2344 nm. In addition, 1700 was included to compensate for the starch, protein, and sucrose in the food (Osborne, 1988). However, in this study the total fat content may have been overestimated by the reference method (Zou et al., 1999; Ranhotra et al., 1996).

Kays et al. (2000) reported prediction of fat in a diverse set of cereal products. The sample set ranged in fat content from 0.02 - 25.6% and included representative 45 samples for calibration that were selected, by a selection algorithm, from a group of 147 samples. The reference method used was gravimetric analysis of fat following petroleum ether extraction (AOAC Method 945.16) and thereby analyzed crude fat content not total fat. The NIR model developed predicted crude fat with a standard error of cross validation of 1.16% and multiple coefficient of determination (R^2) of 0.98. Independent validation samples (n=72) were predicted with a standard error of performance of 0.96%, a coefficient of determination (r^2) of 0.98, and a slope of 0.98 (Kays et al., 2000). It was concluded that NIR spectroscopy, has potential for

quality monitoring of cereal food products but further studies were needed to develop a model to predict fat content within the accuracy required for U.S. nutrition labeling.

Several studies have been conducted on analysis of fat in cereal foods using NIR reflectance spectroscopy over the last 20 years. None of the studies have used a reference method that accurately measures total fat content and, thus, none of the previous models would be applicable for nutrition labeling purposes. In addition, previous models did not have the wide distribution of fat contents, grain types and ingredients currently available in retail markets. With the use of AOAC Method 996.01 as the reference method, the development of a NIR reflectance model for the determination of total fat in cereal foods, that is sufficiently accurate to meet NLEA specifications, may be possible.

Objective

The objective of this study is to develop a rapid NIR reflectance model for the analysis of total fat in cereal foods that is applicable for nutrition labeling and monitoring in accordance with NLEA guidelines.

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Table 1.1. Prevalence of medical conditions as related to body mass index (BMI) for healthy weight and obese individuals (Health, 2002)

medical condition	occurrence in men with a BMI[a] 18.5-24.9 (%)	occurrence in women with a BMI 18.5-24.9 (%)	occurrence in men with a BMI $\geq$ 40 (%)	occurrence in women with a BMI $\geq$ 40 (%)
type-2 diabetes	2.03	2.38	10.65	19.89
CVD[b]	8.84	6.87	13.97	19.22
hypertension	23.47	23.26	64.53	63.16
osteoarthritis	2.59	5.22	10.04	17.19

[a] BMI (body mass index), [b] CVD (cardiovascular heart disease)

Table 1.2. Approximate ranges of wavelengths found in the electromagnetic spectrum

type of light	wavelength
radio waves	> 30 cm
microwaves	1 mm – 30 cm
far-far-infrared	40,000 nm – 1 mm
far-infrared	6,000 nm – 40,000 nm
mid-infrared	1,300 nm – 6,000 nm
near-infrared	700 nm – 1,300 nm
visible	400 nm – 700 nm
ultraviolet	10 nm – 400 nm
x-rays	0.01 nm – 10 nm
gamma rays	<0.01 nm

CHAPTER 2

DEVELOPMENT OF A NEAR-INFRARED (NIR) REFLECTANCE MODEL FOR THE RAPID PREDICTION OF TOTAL FAT IN CEREAL FOODS[1]

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ABSTRACT

AOAC Method 996.01, used to determine total fat in cereal foods as defined by the U.S. Nutrition Labeling and Education Act (NLEA), is laborious, time consuming and solvent dependent. Near-infrared (NIR) reflectance spectroscopy, a rapid and environmentally benign technique, was investigated as a potential method for prediction of total fat. Near-infrared reflectance spectra (1104-2494 nm) of ground cereal products (n=72) were obtained using a dispersive grating spectrometer and total fat determined by AOAC Method 996.01. Using multivariate analysis, a modified partial least squares model was developed for total fat prediction, having a SECV of 1.12% (range 0.5-43.2%) and multiple coefficient of determination of 0.99. The model was tested with independent validation samples (n=36); all samples were predicted within NLEA accuracy. Near-infrared reflectance spectroscopy, therefore, has considerable potential for determination of total fat in diverse cereal products for nutrition labeling and monitoring.

Introduction

Fat is essential to a healthy diet and has important properties in food processing. A high fat intake by humans, however, is associated with an increased risk of several serious health conditions, such as heart disease, obesity and diabetes (Kris-Etherton et al., 2002; Health, 2002). Accurate nutrition labeling of food products helps consumers make informed food selection decisions and monitor the nutritional quality of their diets and the portions eaten. The Nutrition Labeling and Education Act (NLEA) of 1990 requires that all processed and packaged foods display the total fat and saturated fat contents on the "Nutrition Facts" label (21CFR101.9(c)(2)(i)) (Code of Federal Regulations, 2003). For NLEA purposes, total fat is defined as the sum of all fatty acids expressed as triglyceride equivalents (21CFR101.9(c)(2)) (Code of Federal Regulations, 2003) and is measured, in cereal foods, by AOAC Method 996.01 (AOAC, 2002e; Ngeh-Ngwainbi et al., 1997). This method measures the total fat content of diverse cereal foods more accurately than previous gravimetric methods of fat analysis (Zou et al., 1999). It involves the hydrolysis of fat components and subsequent extraction into ethyl and petroleum ethers, followed by evaporation of the ethers and saponification of the extract. The extract obtained is then esterified and the quantity of individual fatty acid methyl esters is determined by capillary gas chromatography (GC). AOAC Method 996.01 is the method approved for U.S. nutrition labeling of total fat in cereal foods because of the complete and specific measurement of all fatty acids present in the food matrix. Earlier gravimetric methods (AOAC, 2002a,b,d) tended to overestimate or underestimate fatty acids in most cereal foods, depending on whether or not hydrolysis was included in the procedure (Zou et al., 1999; Ranhotra et al., 1996). These methods were, thus, not acceptable for nutrition labeling purposes.

In contrast to earlier methods in which the lipid extract was determined gravimetrically, AOAC Method 996.01 is arduous, time consuming and requires the use of additional hazardous chemicals such as boron trifluoride and hydrochloric acid as well as ethyl and petroleum ethers (Ngeh-Ngwainbi, 1997; AOAC, 2002a,b,d,e; Zou et al., 1999). An accurate technique that is rapid and does not require the use of hazardous chemicals would benefit consumers, industry, academia, monitoring agencies and the environment.

Near-infrared (NIR) reflectance spectroscopy is a rapid analytical technique that requires very little sample preparation, requires minimal labor and does not require the use, or disposal, of chemicals. These advantages parallel trends in industry toward reduction in bench time and consolidation of laborious procedures onto one instrument (Blanco and Villaroya, 2002). Near-infrared spectroscopy is used on a large scale in agriculture for the evaluation of cereal grain quality in the United States, Canada and Europe (Williams and Norris, 1987; Osborne et al., 1993; Blanco and Villaroya, 2002). The technique involves measuring sample absorptions in the NIR region of the electromagnetic spectrum (750-2500 nm). Absorption is affected by any property that affects bond strength such as aromaticity, polar groups and hydrogen bonding to neighboring atoms. A spectrum results from the reflected or transmitted energy at each wavelength and is usually expressed as log (1/reflectance) or log (1/R). The spectra of numerous samples can be modeled against an analyzed component and then, using the model, the component can be predicted in new samples.

In addition to assessing cereal grain quality, NIR has been used to predict several nutritional components in a wide range of processed cereal products (reviewed by Kays, 2004); however, reports on the prediction of fat content in cereal products have been limited. Osborne (1988) used 44 cereal food samples (biscuits, breads and breakfast cereals) to develop a single fat

calibration and tested the calibration using 39 cereal food samples of similar types. Fat was measured gravimetrically after hydrolysis and solvent extraction of the sample, a method that has limitations. Techniques involving the gravimetric analysis of lipid components after acid hydrolysis and solvent extraction, tend to overestimate total fat content in cereal foods, due to extraneous ether-soluble materials, which are included in the gravimetric measurement (Zou et al., 1999; Ranhotra et al., 1996). Kays et al., (2000) used 45 representative cereal product samples in a modified partial least squares model for prediction of crude fat. Near-infrared reflectance spectra were obtained using a dispersive grating monochromator and crude fat measured gravimetrically after Soxhlet extraction with petroleum ether (AOAC Method 945.16) (Kays et al., 2000). Again, the reference method has limitations in that the ether extraction-gravimetric analysis technique, in general, underestimates total fat content and is not accurate for analysis of heat-treated cereal samples (Zou et al., 1999; Ranhotra et al., 1996). In particular, bound lipids are not extracted, including those naturally occurring and those present as a result of processing (e.g. emulsification).

AOAC Method 996.01 extracts lipid components more thoroughly than either of the two reference methods used for the NIR reflectance models described above and provides an analyte that meets the NLEA definition for total fat. The acid hydrolysis step of the procedure allows the release of bound lipids that are subsequently extracted. Although hydrolyzed, non-lipid components can be included in the extract, but the specificity of the esterification and GC analysis in AOAC Method 996.01 prevents non-lipid components being included in the measurement. This allows the complete and specific measurement of total and individual fatty acids. Consequently, AOAC Method 996.01 is the approved method for determination of total, saturated, unsaturated and monounsaturated fat for nutrition labeling of cereal products.

It was proposed that a rapid NIR reflectance model could be developed that is sufficiently accurate for the prediction of total fat for nutrition labeling and monitoring using AOAC Method 996.01 as the reference method. In contrast to previous models, this model will include measurement of all fatty acids in cereal products, as required by U.S. Nutrition labeling regulations. In addition, the range of fat content in the model developed will be considerably wider than in previous models, thereby, reflecting the range of retail products available.

Materials and Methods

Cereal Food Products

Four commercial retailers were the source of cereal samples for the study. The sample sets reflected the range of products available to consumers. Samples encompassed a wide range of fat contents, grain types, and processing methods and included breakfast cereals, crackers, cookies, granola bars, flours, snack chips, pastas, meal kits, sweet and savory snacks, as well as unprocessed whole grains. Cereal grain types incorporated into the study were wheat, oats, corn, rice, millet, buckwheat, and multiple grain mixtures including combinations of rye, barley, triticale, amaranth, and quinoa. Cereal samples also contained a wide range of concentrations for sugar, fat, dietary fiber and protein and a variety of ingredients such as salt, cocoa, honey, dried vegetables, soy, dried fruits, nuts, spices, and herbs. Processing methods used in production of the samples include baking, extrusion, milling, frying, and air puffing. In all, 73 samples were purchased for calibration and 36 independent samples were purchased, at a later date, for validation. The samples purchased for validation were never part of the calibration data set.

Sample Preparation and Storage

Cereal samples with both a low fat (<10% fat) and low sugar (<20% sugar) content, based on nutrition label values, were dry milled to <500 μm with a Cyclotec 1093 cyclone mill

(FOSS North America, Inc., Eden Prairie, MN). However, low fat-high sugar (>20% sugar) samples required the addition of liquid nitrogen to the sample to aid in grinding. On the other hand, high fat (>10%) samples were ground using a household coffee grinder (Kitchen Aid model BC G100WH, Kitchen Aid, St. Joseph, MI.). Immediately after milling, samples were scanned with the NIRSystems spectrometer, to obtain NIR spectra.

Ground samples were stored overnight at –28˚ C in low-density polyethylene bags, and total fat analysis (AOAC Method 996.01) was performed the following day. Following total fat analysis, ground cereal food samples were stored at -70° C in doubled low-density polyethylene bags to retard lipid oxidation. Any repeat sample analyses were performed within 3 weeks. Milled cereal products were found to be stable when stored at –28˚ C and –85˚ C for 3 weeks (Vines and Kays, unpublished data). Aliquots of fatty acid methyl esters in n-heptane formed by saponification and methylation were held, overnight, in Teflon capped glass vials at –28˚ C and used the next day for repeat analysis, if necessary.

Reference Analysis

Total fat of both test samples and NIST SRM 1846 (Infant Formula) was determined by AOAC Method 996.01 (AOAC, 2002e; Satchithanandam et al., 2001). Two grams is the sample size recommended for analysis, but sample size was reduced for samples containing >13% total fat (based on the nutrition label declaration). An internal standard, tritridecanoin (T-3882, Sigma, St. Louis, MO) in chloroform, was added to the sample in the Mojonnier tube immediately after weighing. The sample was digested with hot 8N HCl. The hydrolyzed fat components were extracted into ethyl and petroleum ethers while still in the Mojonnier tube (to minimize transfer loss), and then the ethers were evaporated and the extract saponified and methylated. The fatty acid methyl esters formed were analyzed in parallel with a fatty acid

methyl ester standard (KEL-FIM-FAME-5 Metreya, Inc., Pleasant Gap, PA) using a Hewlet Packard II 5890 Series gas chromatogram (Agilent Technologies Inc., Palo Alto, CA) fitted with an auto sampler and a Restek Rtx® -2330 capillary column (10% cyanopropylphenyl-90% biscyanopropyl polysiloxane, 30m x 0.25 mm ID x 0.2 μm df, Restek Corp., Bellafonte, PA). Total fat was calculated as the sum of individual fatty acids expressed as triglyceride equivalents. The crude fat content for each sample was determined, in duplicate, by AOAC Method 945.16 (AOAC, 2002d), a solvent extraction-gravimetric method, using the Soxtec 1040 Extraction System (FOSS North America, Inc., Eden Prairie, MN) apparatus with petroleum ether as the solvent.

Sample dry matter was determined using a forced air oven (105°C) according to AOAC Method 935.29 (AOAC, 2002c). Laboratory reference values for total fat (triglyceride equivalents) and crude fat were expressed on a dry weight basis.

Spectroscopic Analysis

Ground samples were thoroughly mixed and sub-samples placed in triplicate NIR spectral analysis cells (internal diameter=38 mm, depth=9 mm). Each sample cell was scanned using a dispersive NIRSystems 6500 spectrometer (FOSS North America Inc., Eden Prairie, MN), in reflectance mode, fitted with a spinning cup sampling mechanism. Each cell was scanned 16 times, and the 16 spectra were averaged and transformed to log (1/R). After visual inspection, the spectra of the triplicate sub-samples were averaged.

Development of Calibration for Prediction of Total Fat

Near-infrared reflectance models (wavelength range 1104-2494 nm) were developed using a commercial spectral analysis program (WINISI, FOSS North America Inc., Eden Prairie, MN). Preprocessing of the spectral data consisted of using a normal multiplicative scatter

correction, to remove scattering interferences due to particle size, followed by second derivative processing (gap=8 nm, smoothing interval= 8 nm). The data was centered on the mean spectrum and mean reference value using modified partial least squares (PLS) regression. The PLS regression is modified in that the reference values and reflectance data are scaled at each wavelength to have a standard deviation of 1.0 before each PLS regression term (Shenk and Westerhaus, 1991). One sample was eliminated from the calibration set, because of a high Mahalanobis distance (9.88, typical exclusion is >3.0). The outlier sample was a cookie with high fruit content and a sticky, paste-like texture after milling. A calibration (n=72 samples) was developed for the prediction of total fat content in cereals using modified PLS regression. Several methods of preprocessing were systematically applied to the data. The methods applied were first and second derivative and several scatter corrections [none, normal multiplicative scatter correction, standard normal variate (SNV), detrending and a combination of SNV and detrending]. The preprocessing methods used to develop the calibration were those that were optimum for a minimum error following cross validation (20 cross validation groups). The optimum number of PLS regression terms for the calibration was determined by cross validation and was that which gave the minimum error between predicted and reference values (SECV) on cross validation.

Model Validation

The NIR reflectance model for prediction of total fat content in cereal products was tested using independent validation samples (n=36). Performance statistics used to assess the model were standard error of performance (SEP), coefficient of determination (r^2), slope, bias and RPD. RPD is an indicator of model applicability and is the ratio of the SEP to the standard deviation of the reference values (Williams, 2001). That is, the ratio of the SEP to the AOAC values for total

fat. If the RPD is 8.1 or greater, the model is considered excellent and suitable for most applications.

Applicability of the NIR Calibration for Nutrition Labeling

NLEA regulations for reporting calories, sugars, total fat, saturated fat, cholesterol or sodium state that the product is not in compliance if "the nutrient content of the composite is greater than 20 percent in excess of the value for that nutrient declared on the food label. Provided, that no regulatory action be based on a determination of a nutrient value that falls above this level by a factor less than the variability generally recognized for the analytical method used in that food at the level involved" (21CFR101.9(g)(5)) (Code of Federal Regulations, 2003). For nutrition labeling purposes, amounts of fat "shall be expressed to the nearest 0.5 gram increment below 5 grams and to the nearest gram increment above 5 grams. If the serving contains less than 0.5 gram, the content shall be expressed as zero" (21CFR101.9(c)(2)) (Code of Federal Regulations, 2003).

If the regulation is applied to the NIR predicted values obtained for total fat (the values that would appear on the nutrition label) for the samples of the validation set, the NIR predictions would be in compliance with the NLEA as long as the predictions did not underestimate the actual total fat content of the cereal products by more than 20%. For the purposes of estimating compliance for the model the authors will assume that the AOAC values for total fat content are the actual or real values. Thus, the error of the NIR prediction for total fat for each cereal sample was calculated as follows:

$$\text{prediction error (\%)} = \frac{(\text{NIR derived value} - \text{AOAC derived value})}{\text{AOAC value}} \times 100$$

The prediction error was determined using AOAC and NIR derived values for: percent total fat and g fat/serving (calculated using the serving size stated on the product's nutrition label). Negative values for prediction error will indicate underestimation of the AOAC value by NIR. If the NIR prediction underestimates the AOAC value by more than 20%, the NIR prediction will not meet NLEA requirements. That is, unless the error or variability of the analytical method is greater than the amount by which the NIR prediction underestimates the AOAC value.

Results

Reference Method Results and Cereal Sample Distribution

The ranges of total fat content in the calibration and validation data sets were 0.48-43.17% and 2.06-35.71%, respectively, using AOAC Method 996.01. The standard error of the laboratory method (SEL) (ASTM, 1995), or pooled standard deviation of the repeatability of the reference method, was 0.33% total fat. The distribution of grain types along with range, means and standard deviation for total fat content for each grain type in the calibration and validation data sets is given in Tables 2.1 and 2.2, respectively. The distribution of grain types in the validation data set is very similar to that in the calibration data set.

The range for crude fat content in all samples (n=108), measured by AOAC Method 945.16, was 0-40.0 % and the SEL was 0.29%. Total fat content of cereal samples was higher than crude fat content in 93 of the 108 samples (Figure 2.1) and the overall average of the difference between the two measurements for fat was 1.24 %, indicating that underestimation of total fat by the gravimetric method is consistent and considerable.

Spectra of Cereal Products

Selected spectra from a low, medium, and high fat cereal food sample are shown in Figure 2.2. Bands attributed to absorption by C-H groups in oil can be seen at 1212, 1728, 1760, 2308 and 2346 nm (Williams and Norris 1987). The band at 1212 is associated with the C-H stretch 2nd overtone, 1728 and 1760 nm with the C-H stretch 1st overtone, 2308 nm with C-H stretch and C-H deformation and, finally, at 2346 nm with CH_2 symmetric stretch and $=CH_2$ deformation (Osborne et al., 1993; Williams and Norris, 1987). Peak intensities in these regions, particularly 1212 nm, 1728-1760 nm and 2308-2346 nm, are seen to be typically greater in high fat samples (Figure 2.2, plot A). Other predominant absorption peaks in the spectra of ground cereal product samples are at 1434 nm for O-H stretch 1st overtone in carbohydrates, 1936 nm for O-H stretch and O-H deformation in water and 2104 nm for O-H deformation and C-O stretch in carbohydrates (Osborne et al., 1993; Williams and Norris, 1987).

NIR Reflectance Model for Total Fat

A NIR reflectance model was developed for the prediction of total fat using modified PLS regression. Four factors were used for the model and gave the minimum in cross validation error between predicted and reference values (Figure 2.3). The standard error of cross validation (SECV) between predicted and reference values for the modified PLS model was 1.12% with an R^2 of 0.99 (Table 2.3, Figure 2.4A). When independent validation samples were predicted using the model, one validation sample, a processed, buttered corn product, was both a global H and a neighborhood H outlier. The error between predicted and reference values (standard error of performance, SEP) for the validation data set, excluding the outlier, was 0.94% with an r^2 of 0.99. Inclusion of the outlier gave a SEP of 1.07 and r^2 of 0.99. In order to have a model as relevant as possible, the outlier will not be excluded (Table 2.3, Figure 2.4B). Linear regression

of the NIR predicted values against the AOAC reference values for the model gave an equation of y= 0.9869x + 0.1755 for the calibration and y= 0.998x + 0.1151 for validation. The intercepts and slopes were not significantly different ($p>0.05$) from 0.00 and 1.00, respectively.

Applicability of the model was tested first by examining the RPD. The NIR reflectance model for prediction of total fat has an RPD of 8.43 (Table 2.3), which indicates an excellent model for most applications (Williams, 2001). When applicability was estimated for compliance with U.S. nutrition labeling regulations, it was found that all the samples were predicted within the accuracy required by nutrition labeling legislation. Only one of the samples, a whole grain puffed cereal, was underestimated by more >20% (23%) by the NIR model. However, the sample had a very low fat content, <0.5 g fat/ serving as determined by both AOAC Method 996.01 and the NIR model. The label declaration for <0.5 g/serving is zero, therefore, the AOAC and NIR determinations were in agreement for this sample after rounding. Only one cereal product sample was overestimated by the NIR model by >20% (the NIR predicted value was 8.95% and the AOAC value was 6.81% total fat), but according to nutrition labeling regulations, reasonable overstatements of total fat on the label "are acceptable within good manufacturing practice" (21CFR101.9(g)(6)) (Code of Federal Regulations, 2003).

PLS Loadings

The NIR model, using four factors, explained 98.8% of the spectral variation. Pearson correlation coefficients for total fat for factors one, two, three and four were 0.928, 0.258, 0.191, and 0.132, respectively. The modified PLS loading for factor one had high variation in the regions of 1212, 1386, 1728, 2304, and 2346 nm (Figure 2.5). These wavelengths are attributed to absorption by C-H stretch 2nd overtone (1212), C-H stretch (1386), C-H stretch 1st overtone (1728 and 1760 nm) and CH_2 symmetric stretch and $=CH_2$ deformation (2346) (Osborne et al.,

1993; Williams and Norris, 1987; Murray and Williams, 1987). In the second loading (not shown) the greatest variation was at 1434 nm, which is attributed to absorption by O-H groups in carbohydrate and/or possibly water (not shown). However, the major influences in the model appear to be from conformations in oil found in the first factor.

Discussion

A NIR model for prediction of total fat in cereal foods has been developed using a calibration data set with a wide range of grain types, ingredients and processing methods. A broad range of fat and sugar contents and a variety of other ingredients, such as salt, cocoa, honey, vegetables, soy, fruits, nuts, spices and herbs were included in the calibration data set, so that the model would be robust to the range of products available in the marketplace. The model was tested using an independent set of validation samples, which also contained a wide range of fat, sugar and fiber content and also a wide variety of flavor/spice ingredients and other additives. Linear regression of the NIR predicted values versus the AOAC values for total fat in the validation data set gave a low standard error of performance (SEP) and bias accompanied by a coefficient of determination and slope very close to one. These results indicate the potential for precise determination of total fat by NIR throughout the range of fat contents, and the high RPD value of 8.43 indicates excellent potential for the model for most applications, including quality and process control (Williams, 2001). When assessed by nutrition labeling standards, all predictions for samples were found to be within the accuracy required by the NLEA (21CFR101.9(g)(5)) (Code of Federal Regulations, 2003). Thus, the model has excellent potential for nutrition labeling purposes.

The range in total fat content in the validation samples is broad, however, it is not as broad as that in the calibration data set. This is because additional commercial samples in the

36-43% range were not available at the four retail stores used as the source of samples without repetition. In fact, cereal products with >36% total fat content are quite rare. Although the accuracy of the model has not been tested in the 36-43% range, the high multiple coefficient of determination and low SECV for the model indicate good potential for predicting total fat accurately at these levels.

The first modified PLS factor was the most highly correlated to total fat content. Interpretation of the loading plot for the first modified PLS factor indicated that C-H groups in lipids are the most important in development of the model. Influences from water or carbohydrate are not seen in the first factor but are present in the factor two loading, which is of lesser importance. Therefore, influences for the model are predominantly from C-H groups present in oil with minor influences from water or carbohydrate.

After a NIR reflectance model for total fat prediction in cereal products is constructed, all that is needed by the food processor, monitoring agency, or researcher is to grind and pack the sample into spectral analysis cells and to scan with the NIR spectrometer. If the scanning is done in triplicate cells for each sample, the time involved is <10 minutes per product. In comparison, the traditional method of analysis, AOAC Method 996.01, can take up to 8 hours for extraction of the lipid and preparation for GC analysis. Additional time is required for GC instrumental analysis, which needs to be done in triplicate for each replicate of the sample analyzed. Therefore near infrared reflectance spectroscopy is a very rapid, relevant and useful technique for prediction of total fat for the food production industry and monitoring agencies, where efficiency and productivity are critical.

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Table 2.1. Range, mean, and standard deviation (SD) of total fat, expressed as triglycerides, in cereal food products in the calibration data set

cereal grain	n[a]	range (%)	mean (%)	SD (%)
wheat	28	2.0-42.9	17.3	10.6
oats	6	4.8-23.4	13.0	7.9
corn	7	0.5-38.1	20.7	13.8
rice	6	1.4-25.8	11.6	10.1
millet	0			
buckwheat	0			
multiple[b]	25	2.3-43.2	15.5	9.9

[a] n= number of samples; [b]cereal grains present in multiple grain products (followed by occurrence) for the calibration data set: wheat (19), oats (18), corn (8), rice (15), rye (7), barley (10), millet (1), triticale (3), buckwheat (2), amaranth (1), and quinoa (1)

Table 2.2. Range, mean, and standard deviation (SD) of total fat, expressed as triglycerides, in cereal food products in the validation data set

cereal grain	n[a]	range (%)	mean (%)	SD (%)
wheat	10	2.1-32.5	19.0	8.58
oats	5	6.9-27.0	17.5	7.4
corn	4	21.4-35.7	25.9	6.8
rice	0			
millet	1	4.3		
buckwheat	1	5.9		
multiple[b]	15	2.7-32.5	15.6	9.1

[a] n= number of samples; [b]cereal grains present in multiple grain products (followed by occurrence) for the validation data set: wheat (13), oats (12), corn (4), rice (8), rye (3), barley (6), millet (3), triticale (2), buckwheat (1), amaranth (0), and quinoa (0)

Table 2.3. Statistics for the prediction of % total fat, expressed as triglycerides, in cereal food products by near-infrared reflectance spectroscopy[a]

method	calibration					validation							
	n	mean (%)	SD (%)	SECV (%)	R^2	n	mean (%)	SD (%)	SEP (%)	r^2	bias (%)	slope	RPD
AOAC	72	16.19	10.39	---	---	36	17.39	9.02	---	---	---	---	---
NIR	72	16.18	10.31	1.12	0.99	36	17.47	9.07	1.07	0.99	-0.08	0.99	8.43

[a]AOAC Method 996.01 (AOAC); near-infrared spectroscopy (NIR); sample population (n); standard deviation (SD); standard error of cross-validation (SECV); multiple coefficient of determination (R^2); standard error of performance (SEP); coefficient of determination (r^2); ratio of standard deviation of the reference values to the SEP (RPD).

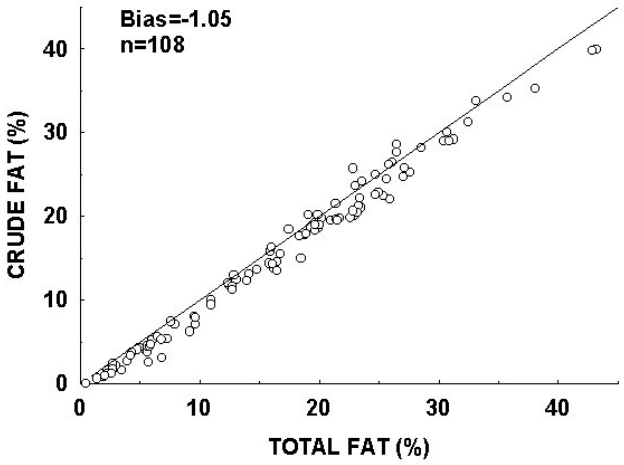

Figure 2.1. Plot of total fat determined by AOAC Method 996.01 versus crude fat determined by AOAC Method 945.16 in cereal food products (n=108) with the line of equality superimposed.

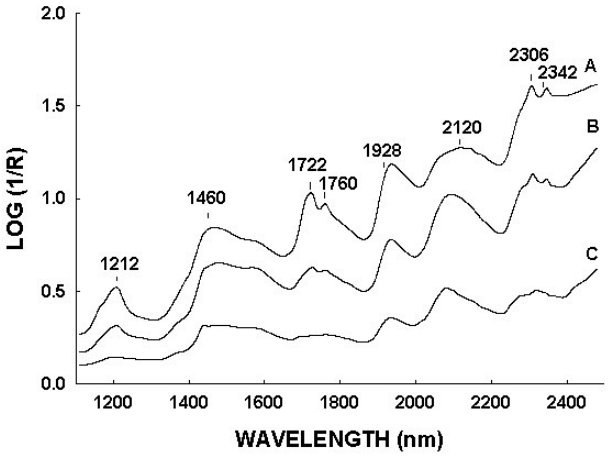

Figure 2.2. Log (1/R) spectra for three retail cereal food product samples in the calibration data set. A contains 43% total fat, B contains 16% total fat and C contains <1% total fat.

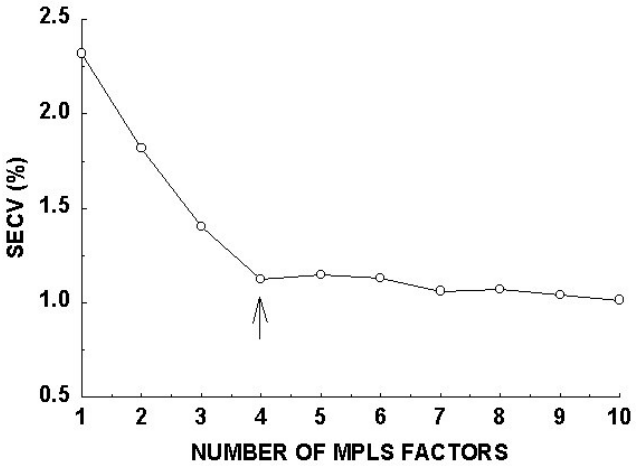

Figure 2.3. SECV versus number of modified PLS factors for the NIR model to predict total fat in cereal food products.

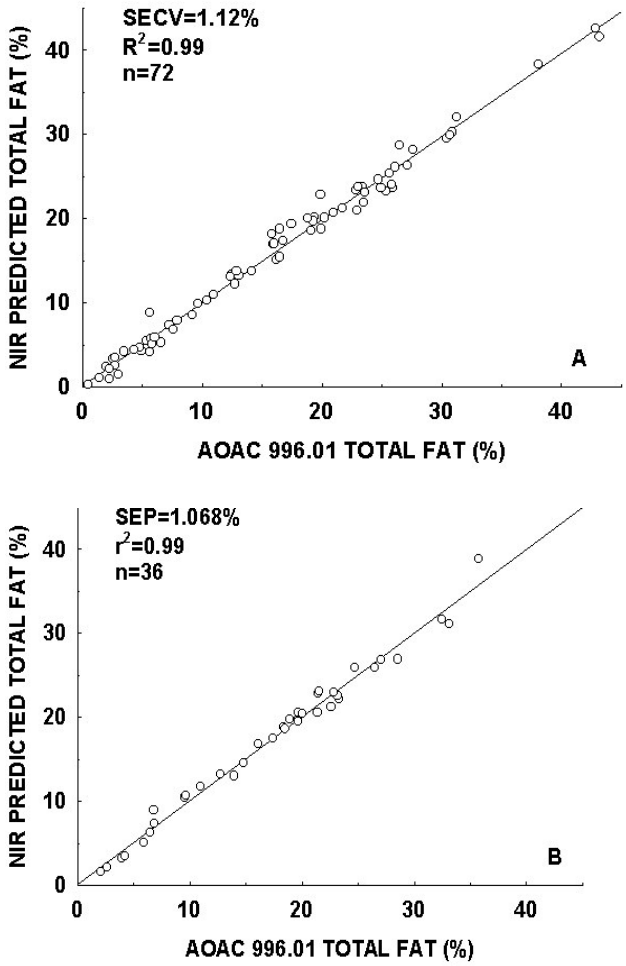

Figure 2.4. Calibration (A) and validation (B) plots of AOAC determined total fat versus NIR predicted total fat in cereal food products.

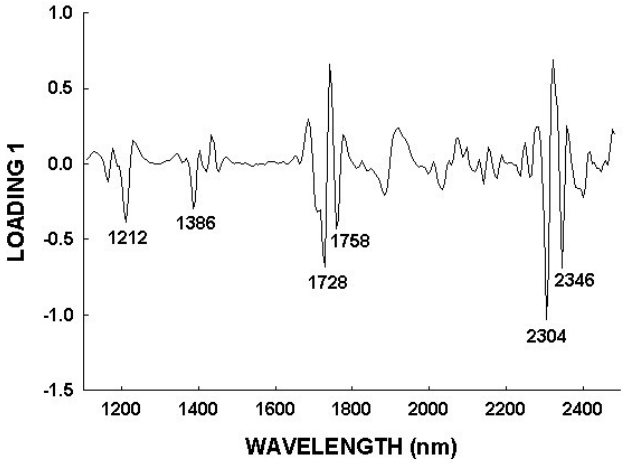

Figure 2.5. Loading spectrum for modified PLS factor 1 of the model to predict total fat in cereal food products.

CHAPTER 3

SUMMARY AND CONCLUSIONS

This thesis investigated the potential of near-infrared (NIR) reflectance spectroscopy as a technique for the analysis of total fat in diverse cereal food products. In addition, the accuracy of the technique was assessed for use in U.S. nutrition labeling. The current method of analysis for total fat in cereal foods, AOAC Method 996.01, is laborious, time consuming, requires the use of and disposal of hazardous chemicals, is costly, and is labor intensive. On the other hand, a NIR technique for the prediction of total fat in cereal food products would be beneficial, as NIR techniques are rapid, accurate, environmentally benign, and require minimal labor input.

A model was developed for prediction of total fat using a cross-section of the cereal food products commercially available. The products selected (n=72) represented a wide variety of cereal food types, cereal grains and major ingredients, and a wide range of total fat, sugar, total dietary fiber, flavors and other additives. This diversity was selected in order to make the model robust to variations in composition encountered in retail cereal products. When the model was used to predict total fat in independent samples (n=36) (range 2.06-35.71% total fat) the standard error of prediction (1.07%), coefficient of determination (0.99) and RPD value (8.45), indicate that the model is suitable for most applications, including quality and process control.

Using NLEA guidelines for nutrition labeling, it was found that all the independent samples were predicted within the required accuracy. One sample, a low fat (2.06%) air puffed whole grain breakfast cereal, had a prediction error >20% (23% error). However, the total fat per serving was <0.5 g for both the AOAC Method 996.01 reference method and NIR model, thus the product would be labeled 0 grams total fat in both cases. The NIR model for prediction of total fat can thus be used for, screening, monitoring, process, quality control, and nutrition labeling.

Evaluation of Pearson correlation coefficients for total fat for the factors in the model indicated that factor one was the most important. The modified PLS loading for factor one demonstrated substantial absorption peaks in the regions of 1212, 1386, 1728, 2304, and 2346 nm. These wavelength regions are associated with absorption by CH groups in oil. Influences from water or carbohydrate are present in the second factor, but because this factor is of lesser importance the influence is minimal.

Once a NIR reflectance model for total fat is constructed, all that is needed by the food manufacturer, monitoring agency, or researcher is to grind and pack the sample into spectral analysis cells and scan with the NIR spectrometer. The time required for triplicate NIR spectral analysis is a matter of minutes, no chemicals are used, and thus there is minimal labor input. This is preferable to the traditional "wet chemistry" method of analysis using AOAC Method 996.01, which is very lengthy, costly, arduous, and requires the use of chemicals. In conclusion, near-infrared reflectance spectroscopy can be used for the prediction of total fat in cereal food products within the accuracy required for U.S. nutrition labeling, thus, benefiting food manufacturers, consumers, monitoring agencies, and researchers alike.

APPENDICES

Appendix A

CEREAL FOOD PRODUCTS SAMPLE SETS

Calibration Sample Set

Low Fat Low Sugar

- Wheat Bran: Bob's Red Mill Natural Foods Inc., Milwaukie, OR
- Stone Ground White Rice Flour: Bob's Red Mill Natural Foods Inc., Milwaukie, OR
- Instant Oatmeal-Regular Flavor: Quaker Oats Co., Chicago, IL
- Amaranth Flakes Multi-Grain Cereal: Arrowhead Mills Inc., Uniondale, NY
- Original Frosted Mini-Wheats: Kellogg USA Inc., Battle Creek, MI
- Complete Wheat Bran Flakes: Kellogg USA Inc., Battle Creek, MI
- 100% Whole Wheat Stone Ground Sesame Cracker: Ak-mak Bakeries, Sanger, CA
- Bulk-Bin Pop Corn Kernels: Earth Fare Inc., Ashville, NC
- Tabouli: Fantastic Foods Inc., Napa, CA
- Puffed Brown Rice Cereal: Nutritional Food Products, Mecca, CA
- Strawberry Mini-Wheats: Kellogg USA Inc., Battle Creek, MI
- Nature's Burger Mix: Fantastic Foods Inc., Napa, CA
- Uncle Sam Cereal: U.S. Mills Inc., Needham, MA

Low Fat High Sugar

- Low Fat Granola with Raisins: The Kroger Co., Cincinnati, OH
- Fruity Pebbles: Kraft Foods North America Inc., Rye Brook, NY
- Instant Oatmeal-Maple & Brown Sugar: The Kroger Co., Cincinnati, OH
- Oatmeal Crisp-Almond: General Mills Sales Inc., Minneapolis, MN
- Frosted Flakes: Kellogg USA Inc., Battle Creek, MI

- Honey Puffed Kashi: Kashi Co., La Jolla, CA
- Fig Newtons: Kraft Foods North America Inc., East Hanover, NJ
- Hot Cereal Cranberry Orange Oatmeal: Fantastic Foods Inc., Napa, CA
- Original Kashi Good Friends: Kashi Co., La Jolla, CA
- Raisin Bran Total: General Mills Cereals LLC, Minneapolis, MN
- Back to Nature Soy Granola: Organic Milling Corp., San Dimas, CA
- Wheaties Energy Crunch: General Mills Cereals LLC, Minneapolis, MN

High Fat Low Sugar

- Toasteds Crackers-Wheat: Keebler Co., Elmhurst, IL
- Great Grains-Crunchy Pecans; Kraft Foods North America Inc., Rye Brook, NY
- Saltines-Wheat: The Kroger Co., Cincinnati, OH
- Mini Ritz Crackers-Original: Kraft Foods North America Inc., East Hanover, NJ
- Ritz Crackers: Kraft Foods North America Inc., East Hanover, NJ
- Wheat Thins-Harvest Crisps 5-Grain: Kraft Foods North America Inc., East Hanover, NJ
- Wheat Thins-Original: Kraft Foods North America Inc., East Hanover, NJ
- Triscuit- Original: Kraft Foods North America Inc., East Hanover, NJ
- Wheatables-Honey Wheat: Keebler Co., Elmhurst, IL
- Original Oat Bran Graham Crackers: Health Valley Co., Irwindale, CA
- Original Rice Bran Crackers: Health Valley Co., Irwindale, CA
- Baked Wheat Crisps: The Kroger Co., Cincinnati, OH
- Breadshop's Gourmet Orange Almond Granola: Breadshop Natural Foods, Irwindale, CA
- Breadshop's New England Supernatural Cereal: Breadshop Natural Foods, Irwindale, CA
- Golden Temple Cranberry Orange Crunch Granola: Golden Temple, Eugene, OR

- All Natural Rich Baked Crackers: Hain Celestial Group Inc., Uniondale, NY
- Toasted Corn-Bulk: Phoenix Market and elsewhere, Athens, GA
- Salted Sesame Sticks-Bulk: Earth Fare Inc., Ashville, NC
- Ramen Noodle Soup-Beef: Maruchan Inc., Irvine, CA
- Giant Goldfish-Pizza: Pepperidge Farm Inc., Norwalk, CT
- Quakes Rice Snacks-BBQ: Quaker Oats Co., Chicago, IL
- StoveTop One Step Stuffing-Chicken: Kraft Foods Inc., Glenview, IL
- Torengos-Splash of Salsa: Proctor & Gamble, Cincinnati, OH
- Flavor Originals Vegetable Thins: Kraft Foods North America Inc., East Hanover, NJ
- Cheetos Crunchy: Frito-Lay Inc., Plano, TX
- Flavor Originals Twigs: Kraft Foods North America Inc., East Hanover, NJ
- El Sabroso Guacachip Tortilla Chips; Snak King, City of Industry, CA
- Bearitos Tortilla Chips-with Blue Corn: Hain Celestial Group Inc., Melville, NY
- Garden of Eatin' Garden Grains: Hain Celestial Group Inc., Melville, NY
- Tex Mex Mix: New England Natural Bakers, Greenfield, MA

High Fat High Sugar

- Golden Vanilla Wafers: Keebler Co., Elmhurst, IL
- Grahams-Honey: Keebler Co., Elmhurst, IL
- Duchy Originals-Lemon: Walkers Shortbread Ltd., Aberlour-on-Spey, Scotland
- Lite n' Crunchy Granola-Bulk: Phoenix Market and elsewhere, Athens, GA
- Lemon Almond Biscotti: Pamela's Products Inc., Ukiah, CA
- Honey Gone Nuts Granola-Bulk: Earth Fare Inc., Ashville, NC
- Ginger Man: Pepperidge Farm Inc., Norwalk, CT

- Chocolate Chips: Famous Amos Chocolate Chip Cookie Co. LLC, Elmhurst, IL
- Chewy Dipps- Peanut Butter: Quaker Oats Co., Chicago, IL
- Chocolate Chunk-Sausalito: Pepperidge Farm Inc., Norwalk, CT
- Oreo Chocolate Sandwich Cookies: Kraft Foods North America Inc., East Hanover, NJ
- Peanut Butter Dreamers: The Kroger Co., Cincinnati, OH
- Save the Forest Nut Granola: New England Natural Bakers, Greenfield, MA
- Mini Rainbow Chips Deluxe: Keebler Co., Elmhurst, IL
- Carob Hazelnut Cookies: Pamela's Products Inc., Ukiah, CA
- Mint Milano: Pepperidge Farm Inc., Norwalk, CT
- Champion Chip Cookies-Espresso: Newman's Own Organics, Aptos, CA
- Key Lime White Chocolate Cookies: Brent & Sams Cookies Inc., Little Rock, AR

Validation Sample Set

Low Fat Low Sugar

- Cheerios: General Mills Cereals LLC, Minneapolis, MN
- Grape-Nuts Cereal: Kraft Foods North America Inc., Rye Brook, NY
- 10 Grain Pancake & Waffle Mix: Bob's Red Mill Natural Foods Inc., Milwaukie, OR
- Puffed Kamut Cereal: Arrowhead Mills Inc., Hereford, TX
- Whole Grain Millet Flour: Arrowhead Mills Inc., Hereford, TX
- Buckwheat Flour: Arrowhead Mills Inc., Hereford, TX

Low Fat High Sugar

- Müeslix-with Raisins, Dates & Almonds: Kellogg USA Inc., Battle Creek, MI
- Hamburger Helper-Philly Cheesesteak: General Mills Sales Inc., Minneapolis, MN
- Post Selects-Banana Nut Crunch: Kraft Foods North America Inc., Rye Brook, NY

- Breadshop's Raspberry 'n Cream Granola: Breadshop Natural Foods, Irwindale, CA
- Wheatables-Original: Keebler Co., Elmhurst, IL
- Wheatsworth: Kraft Foods North America Inc., East Hanover, NJ
- Cinnamon Grahams: The Kroger Co., Cincinnati, OH
- Garden Vegetable Snack Crackers: The Delicious Frookie Co. Inc., Des Plaines, IL
- TLC-Original 7 Grain: Kashi Co., La Jolla, CA
- Wild Blueberry Granola: Golden Temple, Eugene, OR
- Coconut Almond Granola: Golden Temple, Eugene, OR
- Super Nutty Granola: Golden Temple, Eugene, OR
- Pecan Shortbread: Pamela's Products Inc., Ukiah, CA
- Walkers Shortbread Rounds: Walkers Shortbread Ltd., Aberlour-on-Spey, Scotland
- Twistini-Cheese: Haddon House Food Products Inc., Medford, NJ
- Raspberry Heritage Granola: Nature's Path Foods Inc., Delta, British Columbia
- Sandies Swirl-Caramel Pecan Shortbread: Keebler Co., Elmhurst, IL
- Fiber Rye Crispbread: Wasa North America, Saddle Brook, NJ
- Bearitos Tortilla Chips-White Corn: Hain Celestial Group Inc., Melville, NY
- Bearitos Buttery Flavor Popcorn: Hain Celestial Group Inc., Melville, NY
- Garden of Eatin' Pico de Gallo Tortilla Chips: Hain Celestial Group Inc., Melville, NY
- Ole' Corn Tostadas: Ole' Mexican Foods Inc., North Cross, GA

High Fat High Sugar

- Cracklin' Oat Bran: Kellogg USA Inc., Battle Creek, MI
- 100% Natural Granola-Oats, Honey & Raisins: Quaker Oats Co., Chicago, IL
- Supernatural with Almonds & Raisins Granola: Breadshop Natural Foods, Irwindale, CA

- Oatmeal Raisin: Famous Amos Chocolate Chip Cookie Co. LLC, Elmhurst, IL
- Duchy Originals-Orange: Walkers Shortbread Ltd., Aberlour-on-Spey, Scotland
- Ginger Zing Granola with Cashews: Nature's Path Foods Inc., Delta, British Columbia
- Coconut Chips Deluxe: Keebler Co., Elmhurst, IL
- Honey & Maple Waffles: Shady Maple Farm Ltd., Mississauga, Ontario

Appendix B

DETERMINATION OF TOTAL FAT AS TRIGLYCERIDES IN CEREAL PRODUCTS

References:

AOAC. 996.01. Fat (Total, Saturated, Unsaturated, and Monounsaturated) in Cereal Products: Acid Hydrolysis Capillary Gas Chromatographic Method. 32.2.02A. AOAC 17th edition: Revision 1. Editor W. Horwitz. Arlington, VA, 2002.

AOAC. 935.29. Moisture in Malt: Gravimetric Method. 27.3.06. AOAC 17th edition: Revision 1. Editor W. Horwitz. Arlington, VA, 2002.

AOAC Method 996.01 was modified slightly according to the FDA ACNA protocol.

Principle:

Total lipid extract of a test sample is achieved through digestion with hot HCl. Hydrolyzed fat components are then available for extraction using mixtures of ethyl and petroleum ethers. Once ethers are evaporated, the extract is then saponified and methylated. Fatty acid methyl esters are determined by capillary gas chromatography. Total fat is then calculated as the sum of individual fatty acids expressed as triglycerides. Saturated, unsaturated, and monounsaturated fats are calculated as the sum of individual fatty acids.

Reagents

ACS grade chemicals were used unless noted.

Ethanol (95%-100%)

Chloroform (HPLC Grade)

Diethyl ether

Petroleum ether

n-heptane (GC grade)

Boron trifluoride (BF_3) 14% in methanol (store at 4°C)

Triglyceride internal standard solution: 5 mg/mL $C_{13:0}$ tritridecanoic acid in chloroform. Needs to be accurate. Tare 10 mL volumetric flask fitted with glass funnel. Weigh 0.5 g tritridecanoin into funnel. Remove and move flask fitted with glass funnel to fume hood. Using a capillary pipette, rinse tritridecanoin into flask with chloroform (HPLC grade). Remove funnel and carefully adjust volume to 10 mL. Using a capillary pipette, pipette internal standard

solution into two 5 mL vials fitted with Teflon caps. This amount of solution is for approximately 10 samples.

8N HCl: 25 parts 37.7% concentrated HCl to 11 parts double distilled H_2O. For 500 mL, add 347 mL concentrated HCl to 153 mL double distilled H_2O in a 500 mL graduated cylinder. Pour into 1 L glass stock bottle, and store at room temperature.

~0.5N NaOH in Methanol: Weigh 10 g NaOH and place in a 500 mL Erlenmeyer flask, add approximately 200 mL HPLC/GC grade methanol. Swirl to dissolve, adjust volume to 500 mL with methanol. Pour into 1 L glass stock bottle, and store at room temperature.

Saturated NaCl: Weigh 142 g NaCl into a tared 600 mL beaker. Add 400 mL double distilled H_2O. Place beaker on a hot plate and stir with heat (low setting) to dissolve. Allow to cool, filter and store in a 1 L glass stock bottle. (N.B. 1 g NaCl dissolves in 2.8 mL H_2O or 2.6 mL boiling H_2O). Store at room temperature.

FAMEs standard solution: In 1 ml heptane: 0.3 mg/ml methyl octanoate, 0.5 mg/ml methyl decanoate, 1.0 mg/ml methyl dodecanoate, 0.5 mg/ml methyl tridecanoate, 0.5 mg/ml methyl tetradecanoate, 0.3 mg/ml methyl 9(Z)-tetradecenoate, 0.3 mg/ml methyl pentadecanoate, 2.0 mg/ml methyl hexadecanoate, 1.0 mg/ml methyl 9(Z)- hexadecenoate, 0.5 mg/ml methyl heptadecanoate, 1.0 mg/ml methyl octadecanoate, 0.4 mg/ml methyl 9(E)-octadecenoate, 3.0 mg/ml methyl 9(Z)-octadecenoate, 2.0 mg/ml methyl 9,12(Z,Z)-octadecadienoate, 0.3 mg/ml methyl eicosanoate, 1.0 mg/ml methyl 9,12,15(Z,Z,Z)-octadecatrienoate, 0.3 mg/ml methyl 11(Z)-eicosenoate, 0.3 mg/ml methyl docosanoate, 0.3 mg/ml methyl 13(z)-docosenoate

Apparatus

Grinding mill: Tecator Cyclotec 1093, FOSS North America Inc., Eden Prairie, MN
Kitchen Aid BCG100 WH blade coffee grinder, St. Joseph, MI

Enclosed balance: Mettler- Toledo AE 163, Columbus, OH

50 ml test tube rack

Mojonnier fat extraction flasks

Weighing paper

Small Scoop

Labeling Tape

Glass pipettes: pasteur, capillary, 1 ml volumetric, and 10 ml graduated and volumetric

Neoprene stoppers: size "1"

Two water baths: (1) 80 ± 2°C (2) ambient temperature

Glass beads

Glass wool

Glass funnel

Flat-bottom boiling flasks: 250 mL capacity with 24/40 mouth and corresponding glass stoppers

Beakers: with corresponding sized watch glasses for covers (100 mL- 500 mL)

Steam table

Nitrogen tank

Condenser: water-cooled with 40-50 cm jacket and 24/40 joint

50 ml mixing cylinders: with corresponding glass stoppers

Glass vials: with corresponding Teflon lined septa (5ml and autosampler GC vials)

Gas chromatograph: capillary split/splitless injection system and flame ionization detector
Hewlett Packard 5890 series II, Agilent, Palo Alto, CA

GC column: 30m x 0.25mm internal diameter capillary column composed of 0.2 μm nonbonded 90% biscyanopropyl and 10% polysiloxane

Aluminum weighing pans

Metal trays

Forced air oven: set at 104°C ± 0.5°C

Rubber-coated tongs

Procedure:

1. Extraction of Fat

Caution: All steps with the exception of the water bath incubations should be conducted in the fume hood. Gloves and goggles are required at all times.

1) Equilibrate water bath to 80 ± 2°C.

2) Weigh 2 $\pm$ 0.0001 g sample into Mojonnier tube. If % fat is greater than 13%, reduce weight of sample accordingly. Prepare each test sample in duplicate. Tap sample down into reservoir at base of tube to ensure optimal wetting. Keep lower chamber of Mojonnier tube horizontal. Add 2 mL ethanol to wet the sample. Swirl until thoroughly wetted. Accurately, add 1 mL C13:0 triglyceride internal standard solution using a glass volumetric pipette, then add 10 mL 8N HCl rinsing down the sides of the tube during addition (do not allow reservoir contents to reach above constriction at bottom of stem). Swirl until thoroughly mixed. Place tubes in a 50 mL test tube rack.

3) Fit each Mojonnier tube with a size "1" neoprene rubber stopper to prevent spurting of hot HCl. Place rack in the 80°C H_2O bath for 40 minutes. During sample digestion, swirl and vent tubes every 15 minutes to prevent stoppers popping off.

4) Set out the glassware for subsequent steps, i.e. six 250 mL flat-bottom boiling flasks with two glass boiling beads in each; ground glass stoppers for flasks; labels for flasks; three beakers with watch glass covers and label one each for diethyl ether, petroleum ether, and the third 1:1 diethyl ether and petroleum ether mixture; six glass funnels with plugs of glass wool packed firmly enough in stem of funnel to allow free passage of ether into the flat round bottom flasks containing boiling beads. (A small amount of glass wool can be packed into funnel stem using a small spatula.)

5) Fill a container large enough to hold test tube rack with tap H_2O.

6) Remove rack from H_2O bath and immediately place in container filled with ambient temperature H_2O and cool (about 10-15 minutes).

7) Remove rack from H_2O and add enough ethanol to each Mojonnier tube so that the liquid contents reach the constriction just below the bottom of the stem.

8) Add 30 mL diethyl ether * (see note at end of step) and fit again with neoprene stopper. (*Caution: Sample must be cool at this point to keep pressure from building and blowing acid out when tube is shaken. Wear protective eyewear and gloves. Avoid grasping tube with bare hands*). After addition, for the first shake roll gently back and forth but do not touch the stopper with tube contents, vent tube to release pressure. Repeat three times. Then shake back and forth two times, allowing contents to touch stopper, vent pressure each time. Then stopper and shake vigorously for 1 minute. Release pressure. Add 30 mL petroleum ether. Shake and release pressure as before with the 30 mL addition of diethyl ether. Shake vigorously for 1 minute again. Then return tube back to rack ensuring that the tube tilts at about a 45° angle and sample is suspended in solvent. Let set 10-15 minutes until settling is complete and two distinct layers are present. Decant top aqueous layer into the filter funnel fitted with glass wool and filter into the flat-bottom boiling flask. Great care must be taken to decant only the top aqueous layer. Rinse lip of tube with 1:1 diethyl ether: petroleum ether to rinse residual fat into the filter funnel to allow filtering into the flask.

* Test for peroxidation of diethyl ether: Dispense 10 mL of H_2O in a scintillation vial, add a small scoop of potassium iodide and 2 drops of 2N HCl then 5 mL diethyl ether. Shake well. If contents turn an orange color then peroxides are present (positive). Discard diethyl ether if test is positive.

9) Repeat step 8 two more times, except for each repetition use only 15 mL each of diethyl ether and petroleum ether.

10) While waiting for the third sedimentation, prepare beakers for the methylation step, i.e. set up four beakers covered with watch glasses and labeled methanolic NaOH, BF_3, n-heptane, or saturated NaCl solution. Set out six 50 mL mixing cylinders fitted with ground glass stoppers.

11) On a steam table in a ventilation hood, evaporate ethers to almost dryness slowly from the 250 mL flat-bottom boiling flasks with glass boiling beads.

12) At almost dryness, remove flask from the steam table and flush with nitrogen gas (via Pasteur pipette connected to tubing from a tank of compressed UHP nitrogen) to complete the evaporation process. Ensure that the flow of gas is not so strong that the sample is sputtering. Use a clean Pasteur pipette for each sample or flask. Stopper immediately with ground glass stopper (possible stopping point but completion of extraction should be on same day). (N.B. Do not overexpose extracts to air. Complete fat analysis as soon as possible, preferably on the same day of extraction if possible.)

2. Saponification and Methylation

Caution: Use a fume hood, wear gloves and safety glasses. Also perform methylation for one sample at a time.

1) Using a glass 10 mL graduated pipette, add 10 mL methanolic NaOH solution to flask containing extracted fat.

2) Attach flask to water-cooled condenser and heat on a heating plate set on medium low. Reflux 10 minutes (refluxing is when a drop is first seen from the condenser into the flask and the sample begins to boil and condenses on the sides). (N.B. Ensure that heating plate is set so that there is about one drop per second.)

3) Add 10 mL BF_3 in methanol reagent with a glass graduated pipette from top of condenser. Continue to reflux 4 additional minutes. (N.B. Flash point of BF_3 in methanol is 11°C and vapors are extremely harmful. Keep beaker, containing BF_3 in methanol, covered with a larger inverted beaker.)

4) Accurately add 10 mL n-heptane with a glass volumetric pipette through top of condenser. It is very important that this amount be accurate so tilt solvent pipette so it does not drip. Reflux 1 more minute. Leave the pipette in the condenser to allow residual n-heptane to drain.

5) Remove flask and condenser from heat. Cool for 10 minutes so that all the heptane drains down into the flask. Remove condenser and stopper flask immediately.

6) Roll and shake contents of flask to ensure through mixing. Quickly pour contents of flask into a 50 mL mixing cylinder. Stopper both the flask and cylinder with ground glass stoppers. Rinse flat-bottomed boiling flask with 10 mL saturated NaCl solution. Swirl and roll to ensure mixture and pour into cylinder. Cap. Transfer label from flask to cylinder. Mix contents of cylinder well by rotating your wrist like a centrifuge. Invert cylinder 5 times and swirl each time.

7) Let mixture settle for 15 minutes. While taking great care not to touch the sides, uncap and transfer approximately 1 mL aliquots of upper layer to two vials using a capillary pipette. Seal vials tightly with Teflon lined septum and caps. One vial will be for GC analysis and the second for repeat analysis if necessary.

8) Repeat steps 1-7 for each remaining sample.

3. Capillary Gas Chromatographic (GC) Analysis

1) Set the GC instrument according to AOAC Method 996.01 parameters. That is, a gas chromatograph equipped with a capillary split/ splitless injection and flame ionization detector. The capillary column specification for this method is a 30m x 0.25mm internal diameter capillary column composed of 0.2 μm nonbonded 90% biscyanopropyl and 10% polysiloxane. System settings for the method should be an injector temperature of 250°C, detector temperature of 275°C with gas flows of 34 mL/min for USP hydrogen, 300 mL/min for breathing quality air with a split ration of 100:1. The carrier gas should be helium at a linear velocity of 21 cm/s at 175°C. The temperature program for the method should be an initial temperature of 120°C held for 4 minutes and then increased 5°C/min until the final temperature of 230°C is reached and held for 5 minutes.

2) Inject heptane and observe trace.

3) Inject 1 μL FAMEs standard solution of known concentration into GC column. Observe chromatogram for any chromatographic artifacts. Make sure that all FAMEs in standard have been eluted from capillary column.

4) Inject 1 μL of FAMEs in n-heptane from laboratory-extracted samples into GC column. Repeat for each sample.

5) Repeat step 4.

6) Repeat step 4

7) Input peak areas from the GC trace into constructed spreadsheet to determine total fat content (fresh weight basis). *Equations used for construction of this spreadsheet can be found in Appendix C.*

8) Average total fat percentage for the triplicate GC runs for each sample. Calculate duplicate average. Determine dry weight (see 4 below) and calculate results on a dry weight basis.

4. Dry Weight Determination

Note: Perform measurement on each sample in duplicate.

1) Label aluminum weighing pans for each sample. Place labeled pans and three unlabeled pans in a metal tray and place in the forced air oven (104°C $\pm$ 0.5°C).

2) Dry pans for at least one hour.

3) After drying, "hot weigh" the pans as follows. Using rubber-coated tongs, remove one unlabeled pan from the oven and place on the balance until weight equilibrates. Return pan to oven. Repeat with remaining unlabeled pans. This will equilibrate the balance chamber temperature.

4) Remove one labeled pan at a time from the oven and weigh. Record weight.

5) Repeat step 4 for each remaining pan.

6) Allow scale to return to ambient temperature.

7) Thoroughly mix sample ensuring proper distribution of heterogeneous components. Weigh 3 $\pm$ 0.001 g of sample into corresponding labeled pan. Record weight.

8) Dry overnight in forced air oven (104°C $\pm$ 0.5°C).

9) After equilibrating the balance, as in step 3, carefully remove each labeled pan containing dried sample from oven and record the weight while "hot" as in step 4.

10) Calculate percent dry matter for each sample (average of duplicates) and calculate percent total fat on a dry weight basis.

Appendix C

EQUATIONS FOR TOTAL FAT AND FATTY ACID DETERMINATION

- Individual fatty acid response factor (R_i):

$$R_i = \frac{Ps_i \times Ws_{C13:0}}{Ps_{C13:0} \times Ws_{is}}$$

Ps_i=peak area of a fatty acid methyl ester (FAME), *i,* in injected standard mixture
$Ws_{C13:0}$= mg of $C_{13:0}$ FAME in FAME standard mixture
$Ps_{C13:0}$= peak area of $C_{13:0}$ FAME in injected FAME standard mixture
Ws_{is}= mg of a FAME, *i,* in standard FAME mixture

- Amount of each fatty acid (g), expressed as methyl esters, in test sample (F_{ME}):

$$F_{ME} = \left[\frac{Pt_i}{Pt_{C13:0}} \times \frac{Wt_{C13:0} \times 1.006}{R_i} \right] \times 1000$$

Pt_i= peak area of a fatty acid methyl ester (FAME), *i,* in injected test sample
$Pt_{C13:0}$= peak area of internal standard $C_{13:0}$, in injected test sample
$Wt_{C13:0}$= mg of internal standard $C_{13:0}$, in test sample

- Amount of each fatty acid (g), expressed as triglycerides, in test sample (F_{TG}):

$$F_{TG} = F_{ME} \times f_{TG}$$

f_{TG} = theoretical conversion factor constant (Table 1)

- Amount of each fatty acid (g), expressed as fatty acids, in test sample (F_{FA}):

$$F_{FA} = F_{ME} \times f_{FA}$$

f_{FA} = theoretical conversion factor constant (follows equations in this appendix)

- Percentage total fat, expressed as triglycerides, in test sample (Total Fat (%)):

$$\text{Total Fat} = \left[\Sigma \left[\frac{F_{TG}}{W} \right] \right] \times 100$$

W= weight (g) of test sample

- Percentage saturated fat, expressed as fatty acids, in test sample (Saturated Fat (%)):

$$\text{Saturated Fat} = \left[\Sigma \left[\frac{\text{saturated } (F_{FA})}{W} \right] \right] \times 100$$

- Percentage unsaturated fat, expressed as fatty acids, in test sample (Unsaturated Fat (%)):

$$\text{Unsaturated Fat} = \left[\Sigma \left[\frac{\text{unsaturated } (F_{FA})}{W} \right] \right] \times 100$$

- Percentage monounsaturated fat, expressed as fatty acids, in test sample (Monounsaturated Fat (%)):

$$\text{Monounsaturated Fat} = \left[\Sigma \left[\frac{\text{monounsaturated } (F_{FA})}{W} \right] \right] \times 100$$

- Percentage dry matter (DM (%)):

$$\text{DM} = \left[\frac{W_{DS} - W_{PE}}{W} \right] \times 100$$

W_{DS}= weight (g) of test sample and aluminum weigh pan dried
W_{PE}= weight (g) of empty aluminum weigh pan empty dried
W= initial weight (g) of test sample

- Prediction error of NIR Reflectance Spectroscopy (PE (%)):

$$\text{PE} = \left[\frac{NIR_P - AOAC_D}{AOAC_D} \right] \times 100$$

NIR_P= percentage total fat predicted by NIR reflectance spectroscopy
$AOAC_D$= percentage total fat determined by AOAC reference method

Theoretical conversion factors for the determination of each fatty acid expressed as triglycerides and/or fatty acid.

Fatty Acid	$f/_{TG}$	$f/_{FA}$
8:0	0.9915	0.9114
10:0	0.9928	0.9247
12:0	0.9937	0.9346
14:0	0.9945	0.9421
14:1	0.9944	0.9417
15:0	0.9948	0.9453
16:0	0.9950	0.9481
16:1 cis	0.9950	0.9477
17:0	0.9953	0.9507
18:0	0.9955	0.9530
18:1 cis or trans	0.9955	0.9527
18:2	0.9954	0.9524
18:3	0.9954	0.9520
20:0	0.9959	0.9570
20:1	0.9959	0.9568
22:0	0.9962	0.9604
22:1	0.9962	0.9602

REFERENCE:

AOAC. 996.01. Fat (Total, Saturated, Unsaturated, and Monounsaturated) in Cereal Products: Acid Hydrolysis Capillary Gas Chromatographic Method. 32.2.02A. AOAC Official Methods of Analysis 17th edition: Revision 1. Editor W. Horwitz. Arlington, VA, 2002.

Appendix D

COMPARISON OF FAT VALUES (%)

#	sample	method[a]		difference
		AOAC 996.01 FAT%	AOAC 945.16 FAT%	
405	Frosted Flakes	0.478	0.001	0.477
302	Stone Ground White Rice Flour	1.412	0.628	0.784
410	Raisin Bran Total	1.987	0.832	1.155
704	Puffed Kamut Cereal	2.056	0.940	1.118
305	Original Frosted Mini-Wheats	2.253	1.185	1.068
406	Honey Puffed Kashi	2.320	1.641	0.679
306	Complete Wheat Bran Flakes	2.583	1.915	0.668
702	Grape-Nuts Cereal	2.690	1.272	1.418
311	Strawberry Mini-Wheats	2.711	1.691	1.020
309	Tabouli	2.776	2.375	0.401
310	Puffed Brown Rice Cereal	3.024	2.144	0.880
409	Original Kashi Good Friends	3.486	1.619	1.867
703	10 Grain Pancake & Waffle Mix	3.946	2.697	1.249
705	Whole Grain Millet Flour	4.278	3.376	0.902
402	Fruity Pebbles	4.366	3.692	0.674
403	Instant Oatmeal-Maple & Brown Sugar	4.779	4.105	0.674
401	Low Fat Granola with Raisins	4.939	4.256	0.683
412	Wheaties Energy Crunch	5.401	4.184	1.217
411	Back to Nature Soy Granola	5.627	3.772	1.855
301	Wheat Bran	5.636	4.499	1.137
304	Amaranth Flakes Multi-Grain Cereal	5.699	2.594	3.105
308	Bulk-Bin Pop Corn Kernels	5.811	4.397	1.414
706	Buckwheat Flour	5.905	4.683	1.222
408	Hot Cereal Cranberry Orange Oatmeal	6.072	5.247	0.825
801	Müeslix-with Raisins, Dates & Almonds	6.486	5.598	0.888
313	Uncle Sam Cereal	6.561	5.501	1.060
407	*Fig Newtons* *(excluded from calibration, NIR outlier)*	*6.662*	*0.727*	*5.935*
918	Fiber Rye Crispbread	6.815	5.300	1.515
701	Cheerios	6.888	3.109	3.779

404	Oatmeal Crisp-Almond	7.287	5.378	1.909
303	Instant Oatmeal-Regular Flavor	7.618	7.419	0.199
312	Nature's Burger Mix	7.937	7.068	0.869
307	100% Whole Wheat Stone Ground Sesame Cracker	9.222	6.242	2.980
902	Post Selects-Banana Nut Crunch	9.546	7.999	1.547
916	Raspberry Heritage Granola	9.661	7.880	1.781
522	StoveTop One Step Stuffing-Chicken	9.680	7.105	2.575
505	Wheat Thins-Harvest Crisps 5-Grain	10.955	9.993	0.962
907	Garden Vegetable Snack Crackers	10.959	9.438	1.521
510	Original Rice Bran Crackers	12.353	12.014	0.339
502	Great Grains-Crunchy Pecans	12.498	11.747	0.751
908	TLC-Original 7 Grain	12.761	11.259	1.502
503	Saltines-Wheat	12.774	11.582	1.192
509	Original Oat Bran Graham Crackers	12.880	12.968	-0.088
514	Golden Temple Cranberry Orange Crunch Granola	13.083	12.420	0.663
906	Cinnamon Grahams	13.976	12.355	1.621
602	Grahams-Honey	14.140	13.139	1.001
903	Breadshop's Raspberry 'n Cream Granola	14.821	13.633	1.188
521	Quakes Rice Snacks-BBQ	15.838	14.368	1.470
517	Toasted Corn-Bulk	15.934	15.849	0.085
604	Lite n' Crunchy Granola-Bulk	15.987	16.349	-0.362
1001	Cracklin' Oat Bran	16.114	14.266	1.848
516	All Natural Rich Baked Crackers	16.210	13.773	2.437
507	Triscuit- Original	16.466	14.589	1.877
607	Ginger Man	16.497	13.553	2.944
512	Breadshop's Gourmet Orange Almond Granola	16.785	15.460	1.325
508	Wheatables-Honey Wheat	17.468	18.496	-1.028
1002	100% Natural Granola-Oats, Honey & Raisins	18.374	17.646	0.728
901	Hamburger Helper-Philly Cheesesteak	18.498	14.952	3.546
519	Ramen Noodle Soup-Beef	18.812	17.830	0.982
910	Wild Blueberry Granola	18.901	17.863	1.038
605	Lemon Almond Biscotti	19.084	20.165	-1.081
506	Wheat Thins-Original	19.314	18.748	0.566
606	Honey Gone Nuts Granola-Bulk	19.415	18.898	0.517
915	Twistini-Cheese	19.661	19.012	0.649
912	Super Nutty Granola	19.691	18.308	1.383

1003	Supernatural with Almonds & Raisins Granola	19.699	19.277	0.422
511	Baked Wheat Crisps	19.894	20.199	-0.305
601	Golden Vanilla Wafers	19.980	18.630	1.350
1009	Honey & Maple Waffles	20.054	19.021	1.033
513	Breadshop's New England Supernatural Cereal	20.214	19.724	0.490
501	Toasteds Crackers-Wheat	20.989	19.478	1.511
1006	Duchy Originals-Orange	21.389	21.558	-0.169
919	Bearitos Tortilla Chips-White Corn	21.405	19.611	1.794
922	Ole' Corn Tostadas	21.572	19.469	2.103
520	Giant Goldfish-Pizza	21.690	19.771	1.919
904	Wheatables-Original	22.614	19.856	2.758
603	Duchy Originals-Lemon	22.811	25.726	-2.915
1007	Ginger Zing Granola with Cashews	22.813	20.660	2.153
612	Peanut Butter Dreamers	22.946	20.043	2.903
618	Key Lime White Chocolate Cookies	23.036	23.618	-0.582
905	Wheatsworth	23.180	20.456	2.724
1004	Oatmeal Raisin	23.275	21.250	2.025
613	Save the Forest Nut Granola	23.422	22.148	1.274
611	Oreo Chocolate Sandwich Cookies	23.476	21.059	2.417
2504	Ritz Crackers	23.557	24.149	-0.592
921	Garden of Eatin' Pico de Gallo Tortilla Chips	24.714	22.562	2.152
609	Chewy Dipps- Peanut Butter	24.740	24.941	-0.201
617	Champion Chip Cookies-Espresso	24.944	22.849	2.095
529	Bearitos Tortilla Chips-with Blue Corn	25.382	22.405	2.977
608	Chocolate Chips	25.643	24.420	1.223
615	Carob Hazelnut Cookies	25.844	26.180	-0.336
530	Garden of Eatin' Garden Grains	25.930	22.071	3.859
614	Mini Rainbow Chips Deluxe	26.088	26.426	-0.338
610	Chocolate Chunk-Sausalito	26.452	27.640	-1.188
914	Walkers Shortbread Rounds	26.476	28.638	-2.162
911	Coconut Almond Granola	27.039	24.682	2.357
504	Mini Ritz Crackers-Original	27.157	25.817	1.340
527	El Sabroso Guacachip Tortilla Chips	27.638	25.211	2.427
1008	Coconut Chips Deluxe	28.560	28.202	0.358
526	Flavor Originals Twigs	30.401	29.013	1.388
616	Mint Milano	30.707	30.052	0.655

524	Flavor Originals Vegetable Thins	30.861	28.993	1.868
523	Torengos-Splash of Salsa	31.246	29.180	2.066
917	Sandies Swirl-Caramel Pecan Shortbread	32.500	31.223	1.277
913	Pecan Shortbread	33.109	33.752	-0.643
920	Bearitos Buttery Flavor Popcorn	35.707	34.155	1.552
525	Cheetos Crunchy	38.065	35.295	2.770
518	Salted Sesame Sticks-Bulk	42.866	39.820	3.046
531	Tex Mex Mix	43.171	39.974	3.197

[a] average of duplicates