Crude Fat Methods – Considerations

AAFCO Lab Methods & Services Committee
Crude Fat Best Practices Working Group
January 2014

The crude fat laboratory methods are "defining" or "empirical" methods. The crude fat fraction is defined by the solvent and the extraction conditions (time, temperature, particle size, ratio of solvent to test portion, etc.) and is not specific for the extraction of lipid material.

Lipids are commonly defined as a broad category of non-polar molecules that are sparingly soluble or insoluble in water, but soluble in benzene, chloroform, hexane, methanol and diethyl ether. Lipids may be fatty acids (bound or free) and derivatives, phospholipids, waxes, sterols, tocopherols, carotenoids, cholesterol and similar compounds.

Triglycerides (glycerol backbone with 3 fatty acids) are the main storage form of lipids in plants and animals, and include fats (solid at 20^{o}C) and oils (liquid at 20^{o}C). When these 3 fatty acids are mostly or all saturated, the material tends to be solid, and when the fatty acids are mostly or all unsaturated, the material tends to be liquid. The unique characteristics of lipids are related to their solubility rather than their structural characteristics.

Lipids in Nature are associated with proteins, carbohydrates and other lipids. These associations may be van der Waals interactions (usually lipid-lipid); electrostatic and hydrogen binding (lipids and proteins); and covalent (lipids, carbohydrates and proteins). Due to the different bonds involved in a complex cellular matrix, different chemical and physical treatments are required for lipid extraction.

As with any chemical analysis, representative sampling is essential as the analysis must reflect the entire batch of material and not just the individual sample. Sample preparation typically includes drying, particle size reduction or hydrolysis, solvent extraction, separation of liquid and solid phases, removal of non-lipid components, removal of the solvent, and drying of the extract.

The crude fat analysis is often considered to be a simple procedure. Because of the variety of matrices in plant and animal tissue, and processed animal foods, and the variety of lipid classes, achieving a complete extraction can be quite challenging.

In addition to lipids, crude fat methods can co-extract any other substances which are soluble under the conditions of the method. These may include residual moisture, residual ethanol, pigments, carotenes, urea and other compounds. Due to the nature of these methods, they are not specific to lipids, nor do the extraction conditions ensure that 100% of the lipid material will be extracted.

Samples for crude fat should be dried and ground. Many organic (non-polar) solvents will extract water along with lipid compounds, and this can be a source of error. As a general statement, water content should be less than 8%, and lower water content is preferred as long as the sample is not heat-damaged by over-drying. High temperature drying is not recommended due to increased binding of lipids to proteins and carbohydrates. These bound lipids are not easily extracted with organic solvents. Low temperature or vacuum drying is the preferred drying method. Polar solvents may not penetrate samples with greater than 8% moisture, so samples must be dried before lipid extraction, and this sample preparation step is described in most extraction methods. Diethyl ether is hygroscopic and when saturated with water, efficiency of lipid extraction is reduced. Samples with higher levels of moisture and/or endogenous water often results in elevated (and erroneous) crude fat content. Ground samples have an increased surface area, which allows for better penetration of solvent throughout the sample and better efficiency of extraction. Floating of particles may be a problem with certain feed samples that are ground too fine unless appropriate measures are taken during the extraction process. This may include the use of Dacron bags or covering the sample with cotton or similar material that has already been through the extraction process and will not contribute to lipid residue. The solvent should drip onto and uniformly penetrate (minimal or no channeling) through the sample.

SOLVENT EXTRACTION

Most older lab methods involve solvent extraction and weighing of the lipid residue after solvent evaporation. For crude fat, diethyl ether is often the preferred solvent as it is relatively non-polar and extracts most non-polar components (triacylglyerols, sterols, tocopherols and similar compounds), but does poorly at extracting the polar lipids, such as glycolipids and phospholipids). “Crude fat” is often synonymous with “ether extract” and generally refers to “free” lipids that can be extracted into less polar solvents such as petroleum ether or diethyl ether. “Bound” lipids require more polar solvents for extraction.

Choice of solvents is based on solvent characteristics. The considerations should include use of high solvent power (non-polar solvents) for lipids; and use of a low solvent power (polar solvents) for proteins, amino acids and carbohydrates. In some instances, this may be a single solvent, or may be a combination of 2 or more solvents in specific ratios. A single solvent is generally preferred to minimize fractionation and time requirements for phase/solvent separation for lipid fraction isolation. A single solvent system usually allows for more complete recovery of the solvent if desired and/or necessary from an environmental standpoint. There is no single solvent that is ideal for all samples.

The selected solvent(s) should have a relatively low boiling point to allow low temperature evaporation and leave no residue. Ideally, the selected solvent(s) should be safe to use (non-flammable and non-toxic in both liquid and vapor states). Waste disposal, either by evaporation into the air or liquid disposal into wastewater should

always be considered. The solvent(s) should easily and thoroughly penetrate the sample to provide for more complete lipid extraction. The solvent(s) should also be relatively inexpensive and be non-hygroscopic.

The commonly used organic (polar) solvents include petroleum ether, diethyl ether, chloroform, methanol, ethanol, isopropanol, *n*-butanol, acetone, acetonitrile, isopropyl ether, dioxane, tetrahydrofuran, dichloromethane, pentane, hexane, benzene, cyclohexane, iso-octanol, or mixtures of these solvents. Carcinogenicity, toxicity, flammability, hygroscopicity and cost must all be considered in choosing a solvent.

Petroleum ether (pet ether) is a commonly used solvent due to its relatively low cost compared to other organic solvents. It is less hygroscopic than diethyl ether, is less flammable than diethyl ether, and is more selective for hydrophobic lipids than diethyl ether. Many labs use petroleum ether as a single solvent while other labs use a specific blend (ratio) of petroleum ether and diethyl ether for lipid extraction. Petroleum ether generally dissolves more non-polar lipids than diethyl ether and has less potential for peroxide formation.

Petroleum ether is also known as benzine, Painter's naphtha, petroleum naphtha, naphtha ASTM, petroleum spirits, X4 or Ligroin. Chemically, it is not an ether like diethyl ether (ethers contain the R-O-R' functional group), but is intermediate between the lighter naphtha and the heavier kerosene. Specific gravity may vary between 0.6 and 0.8 depending on its composition. Petroleum ether can be divided into several fractions during petroleum distillation based on boiling temperature ranges. The petroleum ether distillation fraction from 60 to 80^{o}C is often used as a replacement for hexane. Due to various world languages and interpretations, benzine (petroleum ether) should not be confused with benzene or benzyne, nor should it be confused with gasoline. In many languages, the word for gasoline is derived from the word similar to benzine, e.g. "Benzin" (German), "benzine" (Dutch), "benzina" (Italian) or "benzină" (Romanian). Petroleum ether is group of various volatile, highly flammable, liquid hydrocarbon mixtures used chiefly as nonpolar solvents, and is a mixture of alkanes, e.g., pentane, hexane, and heptane, whereas benzene is a cyclic, aromatic hydrocarbon, C_6H_6.

Ligroin is a refined saturated hydrocarbon petroleum fraction similar to petroleum ether and is used mainly as a laboratory solvent. It is mainly C_7 to C_{11} in the form of about 55% paraffins, 30% monocycloparaffins, 12% alkylbenzenes, and 2% dicycloparaffins, and is nonpolar. Generally laboratory grade ligroin boils at 60 to 90 °C.

There are frequently some differences in consistency among suppliers of petroleum ether, some differences in lot to lot consistency, and some differences in consistency from country to country. The wide range of boiling temperatures makes recovery and re-use difficult.

Solubility of lipids in solvents is based on the relative proportion of polar and non-polar groups in the matrix. Lipids with little or no polar groups (triacylglycerides, cholesterol

esters) are highly soluble in hexanes, benzene or cyclohexane, and in more polar solvents such as chloroform, and diethyl ether, but are insoluble in methanol. The solubility of these lipids increase in alcoholic solvents as the carbon chain length of the alcohol increases, so they are more soluble in ethanol and *n*-butanol. The shorter chain fatty acids in the lipids will have greater solubility in the more polar solvents. Polar lipids are sparingly soluble in hydrocarbon solvents, but dissolve readily in more polar solvents such as methanol, ethanol or chloroform. Oil-soluble flavors, vitamins and colors are usually extracted with lipids when less polar solvents are used. Solvents are covered in considerable detail in the *Food Lipids* book (Chapter 5) listed in the references.

Hexanes are usually a better solvent for crude fat for forages than petroleum ether.

HYDROLYSIS METHODS

The acid hydrolysis method is applicable to baked products and pet foods, and facilitates the extraction of fatty acids from glycerides, glycolipids and phospholipids and sterol esters that might otherwise be left un-extracted due to covalent and ionic bonding. However, it can also facilitate co-extraction of additional non-lipid materials. The addition of hydrochloric acid breaks covalent and ionic bonds of lipids to proteins and carbohydrates, so that the lipids commonly bound to these fractions can be extracted. When the crude fat analysis value is lower than expected, especially for any animal food product that has been heat processed or containing ingredients that have been heat processed, acid hydrolysis should be considered as the method of choice. The acid hydrolysis method is also often used when high fat products, such as calcium salts of fatty acids, are analyzed for crude fat and for emulsified fats.

As a point of reference for heat processed animal feeds that should be analyzed by acid hydrolysis, the product temperatures reached during pelleting are generally 160-185^0 F and may not need acid hydrolysis for crude fat determination. Expelled and expanded product temperatures are generally 240-280^{0}F, high temperature extrusion products are generally processed at 280-325^{0}F (dry pet foods), and baking product temperatures are generally 325-400^{0}F. The high temperature extrusion process is usually followed by a dryer, where air temperatures are typically 400-600^{0}F to drive moisture off. Wet pet foods (cans, trays, or pouches) are typically subjected to retort temperatures of 240-260^{o}F, and may require acid hydrolysis analysis after initial drying is accomplished in the sample preparation step. Pet treats are typically a low temperature extrusion process and are usually closer to expeller temperatures (240-280^{0}F) and may or may not go through a dryer. Manufacturing of calcium salts of fatty acids is also a high temperature process, so acid hydrolysis is necessary for crude fat determination. Spray drying temperatures may be 275-325^{0}F, which may justify acid hydrolysis for crude fat. As a very general statement, those products produced with processing temperatures over 200^{0}F may need to be analyzed by an acid hydrolysis method.

Most dairy products and products high in sugar content require alkali pre-treatment with ammonia to break emulsified fats and solubilize proteins before solvent extraction, usually with a blend of diethyl ether and petroleum ether. This is commonly known as the Roese-Gottlieb method.

OTHER METHODS

Near infrared spectroscopy (NIR) may be used as a non-destructive method of determining crude fat. This requires careful calibration and is most accurate when the NIR instrument is calibrated using a consistent validated method and with the specific food matrix be measured. Matrix effects can result in significant errors if not appropriately calibrated.

Nuclear magnetic resonance (NMR) spectroscopy methods have been developed, and require careful calibration with specific methods and matrices for greatest accuracy.

Supercritical fluid extraction using CO_2 and, in some methods combined with ethyl alcohol, have also been developed for non-polar lipids.

FATTY ACID ANALYSIS

If the lipid extracted material is to be further analyzed for fatty acids, care must be taken in solvent choices and methodology to minimize degradation of the lipids. Chloroform, chloroform-methanol, and the less toxic hexane-isopropanol are frequently used for initial extraction. The most common method is to hold the extracted lipid samples in the cold at -20^{o}C until analysis. Other storage options include the addition of an antioxidant, usually BHA. Sample preparation is often with open (low pressure) chromatography using different ratios (step wise) of diethyl ether and hexane, followed by TLC, LC, SPE or HPLC methods of determination of fatty acids or other lipid fractions. Some of the GC methods require very high temperatures and/or derivatization to lower the boiling point for analysis. Since most of these methods require high temperatures, care must be taken to ensure that the lipid compounds are not degraded during chromatographic analysis. As in all methodology, the exact step-by-step method must be followed for greatest accuracy and precision.

If fatty acids are analyzed for verification of variations of crude fat methods, glycerol must also be accounted for. If a lab chooses to verify results in this manner, it must be considered that this is verification of the triacylglyeride level in the matrix, and there may be other lipid fractions (phospholipids, waxes, cholesterol, tocopherols which will vary among matrices) that are not accounted for.

SUMMARY

Even minor modification of any of the crude fat methods yields a slightly different fraction and a slight different result, and is, therefore, yet a different method. Therefore, care must be taken with any empirical method to follow the method exactly as directed. Any variation of a method or a method that is not used within its scope must be validated. Conducting a fatty acid profile and summing of the fatty acids to yield total fat is the ultimate way to evaluate the effectiveness of a fat method on a particular matrix.

Recommendations: Because the analyte is defined by the method, it is crucial to emphasize that fat methods be followed exactly and the method and solvent be identified in the analyte title in a report of analysis. For example, "Crude Fat by AOAC 920.39, Diethyl Ether Extract" or "Crude Fat by AOAC 954.02, Acid Hydrolysis", etc. It is improper and inaccurate to give each of these methods the same analyte name when they target/extract different lipid fractions.

References:

AOCS Lipid Library. http://lipidlibrary.aocs.org/?navItemNumber=695

CyberLipid Center. http://www.cyberlipid.org/index.htm

Food Lipids: Chemistry, Nutrition and Biotechnology. Second Edition, Revised and Expanded. Casimer C. Akoh and David B. Min, editors. Marcel Dekker, Inc. New York. 2002.

Instructor's Manual for Food Analysis, Third Edition. Kluwer Academic/Plenum Publishers. 2003.

Lange's Handbook of Chemistry. 1944. Norbert Lange, editor. Published by Handbook Publishers, Inc., Sandusky, Ohio, USA.

Möller, Jürgen. 2013. Performance of methods for the determination of crude fat/ total fat. FOSS White Paper P/N 1026670, Issue 1.

Moreau, Robert A. 2005. Extraction and Analysis of Food Lipids. Chapter 5 in *Methods of Analysis of Food Components and Additives*. Chemical and Functional Properties of Food Components series. Semih Otles, editor. Published by CRC Press, Taylor and Francis Group, Boca Raton, FL. USA.

Petfood Technology. First Edition. Jennifer L. Kvamme and Timothy D. Phillips, editors. Watt Publishing. Mt. Morris, IL. 2003.

Thiex, Nancy J, Shirley Anderson and Bryan Gildemeister. 2003. Crude Fat, Hexanes Extraction, in Feed, Cereal Grain, and Forage (Randall/Soxtec/Submersion Method): Collaborative Study. JAOAC International 86(5):899-908.