DETERMINATION OF THE TRANS FATTY ACID CONTENT OF COMMON PROCESSED FOODS AND THE PLASMA FATTY ACID PROFILE OF LATIN AMERICAN AND CARIBBEAN URBAN POPULATIONS
MULTICENTRIC COLLABORATIVE STUDY SPONSORED BY WHO/PAHO
2010

PROTOCOL

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2009


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I. OVERALL OBJECTIVE

This study is intended to determine the fatty acid profiles of typically consumed foods as well as the plasma and adipose tissue distribution of fatty acids in adults residing in urban areas of Latin American and Caribbean cities, in order to provide information to further current regional and national efforts—such as the PAHO Task Force on Trans Fat Free Americas—towards the elimination of trans fatty acids and the promotion of cardiovascular health interventions.

II. SPECIFIC OBJECTIVES

1. To determine the saturated fatty acid content of several foods typically consumed in various Latin American and Caribbean countries.

2. To determine the cis-unsaturated fatty acid profile of several foods typically consumed in various Latin American and Caribbean countries.

3. To determine the trans-unsaturated fatty acid profile of several foods typically consumed in various Latin American and Caribbean countries.

4. To determine the plasma concentration of cis and trans saturated and unsaturated fatty acids in Latin American and Caribbean adults.

5. To determine the adipose tissue concentration of cis and trans saturated and unsaturated fatty acids in a subsample of Latin American and Caribbean adults.

III. RATIONALE

Cardiovascular disease (CVD) accounts for 2 out of every 3 deaths in Latin America and for nearly half of all deaths in people under 70 years of age (1,2). In addition to early death, CVD causes complications, sequelae and disability, which affect functionality and limit productivity. Moreover, the associated financial and social costs undermine the resources of socialized healthcare systems (1).
According to the Pan-American Health Organization, between 1970 and 2000, ischemic heart disease mortality rates increased in Ecuador and other Latin American countries while they declined in Canada and the United States. These results have been linked to unfavorable changes that are taking place in most Latin American countries, such as the adoption of sedentary lifestyles and unhealthy diets (1,2).

Undoubtedly, an unhealthy diet is an important environmental factor for the early development of CVD (3). The harmful effects of saturated fatty acids (SatFA) and industrial trans fatty acids (TransFA) as well as the cardioprotective effect of cis-unsaturated fatty acids have been demonstrated (4-11). Several Latin American governments have taken steps to eliminate industrial trans fats and replace them with cis-unsaturated fatty acids in an effort to promote health and support the World Health Organization’s Global Strategy on Diet, Physical Activity and Health (4).

In this context, studies geared towards the evaluation of trans fatty acid content in industrially processed foods and the determination of their consumption are relevant for public health. Nevertheless, determining fatty acid intake using standard methods is quite complex. Also, many methods fail to accurately measure typical individual intakes due to daily variations in food consumption.

Therefore, fatty acid biomarkers are widely used as predictors for chronic disease development. The use of biomarkers simplifies epidemiological studies because individual fatty acids can be measured in readily available tissues (e.g., adipose tissue, plasma and erythrocytes) where measured fatty acids come from different lipidic components. Thus, in adipose tissue, fatty acids are mostly derived from triacylglycerols; in erythrocytes they come from phospholipids, and in plasma, from a combination of triacylglycerols, cholesterol esters and lipoprotein phospholipids (11-14). However, the metabolic characteristics of these biological specimens vary greatly (11-14) as some tissues can better reflect dietary consumption while others are better markers of physiological properties.

Adipose tissue is considered the best biomarker for the study of long-term fatty acid intake due to its low turnover rate and lack of responsiveness to acute disease. Various studies conducted in Latin America and several European countries indicate a low rejection rate (13-15) and no collateral effects associated to adipose tissue sampling. The technique for sampling
adipose tissue is less invasive than a routine venipuncture as it does not compromise blood vessels or other soft tissue.

Red blood cells are medium-term biomarkers of fatty acid intake (months) while plasma is useful for short term intake (weeks). Both biomarkers are easy to obtain and their fatty acid contents have shown good correlation with fatty acid intake and adipose tissue concentration (11-14). Nevertheless, Baylin et al (14) and Campos et al (unpublished data) have shown that plasma has better correlations with adipose tissue concentrations of alpha-linolenic acid, docosahexanoic acid (DHA) and eicosapentanoic acid (EPA).

The fatty acid profile of the population and the TransFA content of typically consumed foods are unknown in most Latin American and Caribbean countries. Hence, this project is particularly relevant for the development of plans, public policies and strategies aimed at preventing CVD and promoting a healthy dietary culture in all population groups. In addition, it will allow for the strengthening and modernization of the legal framework with regards to food and nutrition, so that the availability of heart-healthy foods can be ensured. Lastly, the identification of foods high in SatFA and TransFA will allow for appropriate nutritional interventions geared towards preventing the development of CVD at an early age.

IV. TYPE OF STUDY

This is an exploratory, multicentric transverse study. The multicentric study will include the collaboration of several research centers, laboratories and other supporting centers and it will be conducted following the same principles. This means there will be only one protocol, one principal investigator and one final report. Participating investigators in the multicentric study are strictly bound by these rules. After the publication of the general results, each national investigator will be able to use the data generated in his or her city to conduct new analyses.
V. METHODOLOGY

A. Study Sites

The United Nations Human Settlements Programme, UN-HABITAT, in its report “State of the World’s Cities 2008/2009”, indicated that 77% of the Latin American and Caribbean population lives in the great metropolitan areas, usually the capital cities (16). Meanwhile, the Pan-American Health Organization, in its 2002 report “Health in the Americas”, indicated that the greatest population trends in Latin America and the Caribbean take place in the capital cities (17). According to the population databases of the International Center for Tropical Agriculture (CIAT), the United Nations Environment Programme (UNEP), the Center for International Earth Science Information Network (CIESIN), Columbia University and the World Bank (2005) (18), the most populated cities in each sub-region are:

<table>
<thead>
<tr>
<th>City</th>
<th>Population</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>South America</strong></td>
<td></td>
</tr>
<tr>
<td>Buenos Aires, Argentina</td>
<td>11 655 100</td>
</tr>
<tr>
<td>Sao Paulo, Brazil</td>
<td>10 057 700</td>
</tr>
<tr>
<td>Lima, Peru</td>
<td>7 603 500</td>
</tr>
<tr>
<td>Bogotá, Colombia</td>
<td>6 620 500</td>
</tr>
<tr>
<td>Río de Janeiro, Brazil</td>
<td>6 029 300</td>
</tr>
<tr>
<td>Santiago de Chile, Chile</td>
<td>5 032 500</td>
</tr>
<tr>
<td>Caracas, Venezuela</td>
<td>1 763 100</td>
</tr>
<tr>
<td>Quito, Ecuador</td>
<td>1 648 100</td>
</tr>
<tr>
<td>La Paz, Bolivia</td>
<td>1 517 800</td>
</tr>
<tr>
<td>Montevideo, Uruguay</td>
<td>1 449 900</td>
</tr>
<tr>
<td>Asuncion, Paraguay</td>
<td>546 800</td>
</tr>
<tr>
<td><strong>Mesoamerica</strong></td>
<td></td>
</tr>
<tr>
<td>Mexico City, Mexico</td>
<td>8 589 600</td>
</tr>
<tr>
<td>San Salvador, El Salvador</td>
<td>1 760 700</td>
</tr>
<tr>
<td>Tegucigalpa, Honduras</td>
<td>1 186 400</td>
</tr>
<tr>
<td>Managua, Nicaragua</td>
<td>1 106 600</td>
</tr>
<tr>
<td>Guatemala City, Guatemala</td>
<td>1 090 300</td>
</tr>
<tr>
<td>Panama City, Panama</td>
<td>430 700</td>
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</tbody>
</table>
There are 23 cities in Latin America and the Caribbean where the majority of the population is concentrated, but due to limited financial resources to conduct a study of such magnitude, only 40% of the cities (n=9) were selected to comprise the sample. The cities were selected using probability proportional to size sampling (18). The selected cities were: Buenos Aires, Rio de Janeiro, Santiago, Bogota, Caracas, Quito, Mexico City, Guatemala City, San Jose (Costa Rica), Kingston and San Juan (Puerto Rico).

B. Procedure Homologation

A relevant aspect of multicentric studies is the homologation of procedures to select samples for biomarker assessment, target population characteristics and techniques to determine plasma and adipose tissue fatty acids. It is also necessary to homologate the procedures that will be used for food selection, sampling techniques and collection techniques. Finally, it is essential to homologate the analytical techniques to determine fatty acids in food and biological samples and the reporting of results.

Given that the homologation of analytical techniques is critical to ensure the validity of result comparisons among the various participating countries, participant laboratories shall demonstrate high internal consistency in the application of analysis techniques for the determination of fatty acids in food and biological samples. In addition, laboratories must have participated in at least two rounds of external performance assessments that can guarantee the quality of their determinations. These requirements are intended to obtain high quality, reliability and reproducibility data.
Prior to the study, INCIENSA’s laboratory will organize an external assessment of the laboratories interested in analyzing food and biological samples. To this end, each laboratory will get a certified sample to quantify and identify each one of the fatty acids contained in it. Results will be sent to INCIENSA to obtain error percentages and variability coefficients. Those laboratories with the highest accuracy and precision will be selected to conduct the analyses for this study.

C. Study Stages

The study will take place from August, 2009 to December 2010. Detailed methodology guidelines for each stage are described in the next sections.

D. First Stage: Determination of fatty acid contents of processed foods typically consumed in Latin America and the Caribbean (2009 – 2010)

D.1 Objectives

D.1.a General Objective

To determine the fatty acid profile of several foods typically consumed in various Latin American and Caribbean countries.

D.1.b Specific Objectives

1. To determine the concentration of cis and trans saturated fatty acids in spreadable fats, edible oils, cookie products, snacks, fast-food from transnational chains and local restaurants, and bakery products.

2. To determine the concentration of cis and trans unsaturated fatty acids in spreadable fats, edible oils, cookie products, snacks, fast-food from transnational chains and local restaurants, and bakery products.

D.2 Methodology
D.2.a Participant Characteristics

Study participants shall have the following characteristics:

1. **Socio-economic status:** Individuals from the most heavily populated socio-economic level will be selected to participate in the study, in accordance to the guidelines provided by the pertinent local authorities to determine the population’s socio-economic stratification.

2. **Age:** The sample will include subjects from 20 to 60 years of age, as this age group represents nearly 30% of the population of the different countries in the region. Also, this population group has a relatively low prevalence of chronic non-communicable diseases. All participants in this age group must be in good health (see item D.2.a.iv).

3. **Residence:** Urban residents will be selected, as currently over 80% of the Latin American and Caribbean population lives in urban areas. The areas (districts, towns, provinces, etc.) selected will be those where people from the most heavily populated socio-economic level reside. Therefore, this study is an approximation of the situation of the fatty foods market and its potential impact on the population. It is not a representative sample of each city, nor does it intend to find links between food consumption and plasma or lycopite fatty acid distribution.

4. **Health status:** Individuals with no chronic diseases (diabetes, hypertension, heart disease, cancer) or any other conditions requiring a modified dietary pattern will be included in the study.

5. **Children in public schools:** Individuals with children in public schools will be included, as the children will be the link between the investigators and the adults.

D.2.b Ethical Considerations

In compliance with the ethical principles expected of any research involving human subjects, “Informed Consent” shall be obtained prior to taking any biological sample (Schedule 1). In accordance to the Belmont Report guidelines (19), the informed consent shall include a detailed explanation of the study objectives, the voluntary nature of the participation, the nature and scope of the study’s benefits and risks and the choices and rights of the participants. After
reading and discussing the “Informed Consent” with the investigator, subjects that wish to volunteer for the study shall indicate it in writing by signing the informed consent form in the presence of a witness. The principal investigator will keep the original form and provide the participant with a copy.

In addition, in keeping with the Belmont Report guidelines, participants shall be treated ethically, their decisions shall always be respected and the necessary conditions to ensure their physical and emotional well-being shall be provided during the study. Moreover, all participants, regardless of race, socio-economical level, sexual orientation, age, gender or level of education, shall have equal opportunity to participate in the study and receive its benefits (i.e., the results of the fatty acid profile).

At all times, the participants’ decision to volunteer as research subjects and to express their opinions freely at their convenience shall be respected. Also, the confidentiality of collected information shall be safeguarded and the customs and lifestyles of all participants shall be respected.

Given their leading roles, access to the study results shall be considered a right of the participants at all times.

D.2.c Study Participants

Since this is an exploratory study, a sample of 250 subjects (50% male and 50% female) is considered appropriate for each of the 9 Latin American and Caribbean capital cities.

D.2.c.i) Participant Selection

The following procedure will be followed to select study participants:

1. Identify the city’s most heavily populated socio-economic level and the districts that typically house this sector, in accordance with the guidelines provided by the pertinent local authorities to determine the population’s socio-economic stratification.

2. Identify the districts where most of this population is concentrated and randomly select 2 districts or subdivisions. To favor and simplify field logistics, randomly select the first district, and then randomly select the second from neighboring districts.
3. Randomly select 5 secondary schools (both public and private) from the total of existing schools in each of the selected districts.

4. Randomly select one class per grade level and give an invitation letter to every student for delivery to their parents or guardians at home (Schedule 2). The letter shall describe the requirements to participate in the study and include a card where candidate adults can indicate their interest or refusal to volunteer for the study (Schedule 2). Distribute up to 30 cards per each selected class (approximately 1400-1800 invitation letters).

5. Collect all returned cards and select those with affirmative replies. Sort this group by participant gender. Keep all cards for your records.

6. Randomly select 25 men and 25 women from the affirmative replies.

7. Send all the necessary information with the students so that the adults will show up on the date and time arranged by you. Preferably, obtain samples for all the subjects from each particular school on the same day.

D.2.d Informed Consent

Before taking blood or adipose tissue samples, each participant shall voluntarily sign the informed consent form in the presence of a witness (Schedule 1). The witness must not be related in any way to the research group.
D.2.e  Sampling Procedures

Blood samples will be obtained from 250 participants. Adipose tissue samples will be obtained from a subsample of at least 50 participants. For each subject, collect the information requested on the Participant Data form (Schedule 3).

D.2.e.i)  Plasma Sampling Procedure

To obtain plasma samples, subjects must be fasting for 12 hours. The blood sample will be obtained by venipuncture of the antecubital fossa and collected into 10 mL Vacutainer-EDTA tubes containing 0.1% Ethylenediaminetetraacetic acid, as per the guidelines of the National Committee for Clinical Laboratory Standards (20).

D.2.e.ii)  Adipose Tissue Sampling Procedure

Samples will be obtained following the methodology previously described by Beynen and Katan (13). When making the appointment for the sample collection, ask the subject to wear light, comfortable clothing such as sweatpants (trousers are preferred to skirts or dresses). All blood and adipose tissue sampling procedures must be conducted at a comfortable and private location.

1. Label the syringe with the subject’s ID number using a black marker and underline the number. Put gloves on.

2. Ask the subject to lower trousers or skirt slightly to bare the upper half of the left buttock. Have the subject lie face down on an examination table or bed.

3. Press an ice pack to the sampling site for 30 seconds. Disinfect the skin with an alcohol swab. Assemble needle and syringe and have it ready next to the subject.

4. Instruct the subject to tense his or her muscles so that muscle and fat tissue are clearly recognized. Using two fingers and the thumb of the left hand, grab a skinfold from the upper outer quadrant of the buttock.

5. Insert the needle-syringe assembly at a 45 degree angle. Pull the vacuum in the syringe.
6. Gently push the needle back and forth 3 times in a fanning motion or until sample is visible in the syringe. Make sure that the angle is not too shallow. The sample will be collected between the needle and the syringe.

7. After collection, seal the sampling site with a small band-aid. Instruct the subject not to touch the area where the sample was collected from.

8. IMMEDIATELY after collection, cover the needle and wrap the needle-syringe assembly with tin foil. Put inside a plastic bag and inside a cooler at 4 ºC to transport to the laboratory.

The sampling procedure takes about 4 minutes per person. Obtaining adipose tissue samples requires no prior preparation. Samples must be obtained in a private, aseptic area, by experienced health professionals or specialized technicians.

D.2.c.iii) Processing Plasma and Adipose Tissue Samples

Plasma samples must be centrifuged within 4 hours of collection, at 1430 G for 20 minutes at 4 ºC, in order to separate plasma from red blood cells. Store separated plasma at -80 ºC until all samples are collected, or process immediately.

Adipose tissue samples shall be processed as follows:

1. As soon as possible and within 3 hours of collection, process the sample for storage. Sample must remain cold at all times.

2. Put gloves on and clean the area where sample will be processed. Get rid of any supplies or materials that are not related to the procedure, particularly plastic.

3. Clean a Petri dish with 70% ethanol and place on crushed ice. Using a Pasteur pipette, remove the fat biopsy from the syringe and needle and place on the dish. Compact with a spatula.

4. Open two Wheaton vials containing the hexane/isopropanol solution and place on the rack.
5. Using a needle, collect a 2 mg piece of fat and place in the Wheaton vial containing the hexane/isopropanol solution. Stir. Discard needle. Repeat with second vial.

6. IMMEDIATELY close the vials TIGHTLY.

7. Using the spatula and Pasteur pipette, collect the remaining adipose tissue (if any) and place it into a cryovial. Seal under N₂.

8. Store samples at -80 °C until ready for transportation on dry ice.

D.2.f Analytical Method for the Extraction and Methylation of Plasma Fatty Acids

The analytical method for the extraction and methylation of fatty acids from plasma samples is described in Schedules 4 and 5.

D.3 Result Reporting

The plasma and adipose tissue distribution of the various fatty acids shall be reported as a percentage of total fatty acids. Use the enclosed Excel worksheet.

E. Second Stage: Determination of fatty acid profile in Latin American and Caribbean populations using biomarkers

E.1 Objectives

E.1.a Overall Objective

To determine plasma and adipose tissue fatty acid distribution of the Latin American and Caribbean population.

E.1.b Specific Objectives

1. To determine plasma fatty acid distribution in men and women between 30 and 40 years of age, residing in Latin American and Caribbean countries.

2. To determine adipose tissue fatty acid distribution in men and women between 30 and 40 years of age, residing in Latin American and Caribbean countries.
E.2 Methodology

E.2.a Samples

Foods included in the study are those included in these categories: spreadable fats (margarines), edible oils, pastries, cookie products (plain cookies, filled cookies, dipped or covered cookies), fast-foods (French fries) from the three leading transnational fast-food chains (McDonald’s, Burger King and Kentucky Fried Chicken), fast-foods from local restaurants or food stands, snacks (fried chips, extruded corn, puffed corn and others) and most widely consumed bakery products in the various Latin American and Caribbean countries.

E.2.b Prepackaged Product Samples and Sampling Sites

1. Identify the various kinds of spreadable fats (margarines), edible oils, plain cookies, filled cookies, dipped or covered cookies, snacks (fried chips, extruded corn, puffed corn and others) and bakery products that are frequently consumed by the population. Some resources that can be used to obtain this information are food consumption studies, product listings from supermarkets and other stores where the population typically shops for groceries, and inventories from the food industry chambers.

2. Identify the points of purchase where the population typically shops for the pre-packaged foods that are relevant to the study and purchase the samples in accordance to the following sampling plan.

E.2.b.i) Sampling Plan for Prepackaged Products

Samples will be obtained at points of purchase. Lot size will be the total quantity of product available on shelf. Unless there is evidence to the contrary, lots will be assumed to be homogeneous. Sample size depends on lot size. To determine sample size, follow the sampling plan on Table 1.
Table 1. Sampling Plan S4-AQL. 2.5, per lot size

<table>
<thead>
<tr>
<th>Lot Size</th>
<th>Sample Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up to 150</td>
<td>5</td>
</tr>
<tr>
<td>151-200</td>
<td>20</td>
</tr>
<tr>
<td>201-10000</td>
<td>32</td>
</tr>
<tr>
<td>10001-35000</td>
<td>50</td>
</tr>
<tr>
<td>35000-50000</td>
<td>80</td>
</tr>
<tr>
<td>Over 50000</td>
<td>125</td>
</tr>
</tbody>
</table>

Source: Reference #19

E.2.b.ii) Sampling Procedure for Prepackaged Products

Since it is probable that the lots of relevant products available on shelf at the points of purchase will have less than 150 units, 5 units of each relevant product will be selected. Where lots are larger than 150 units, proceed according to Table 1.

Each set of 5 units from the same point of purchase will be homogenized to obtain a composite sample. In the end there will be 3 composite samples per each group of relevant foods.

ii.a) Spreadable fat (margarine) samples

Samples of the 3 most frequently consumed types of spreadable fats must be obtained. Each sample is composed of 5 units, ideally from different lots. One unit equals one pack, one stick or another standard presentation of the product. Total: 3 composite samples.

To prepare composite sample #1, obtain 5 units of the product. Units must be identical, i.e., they must have the same weight and be of the same variety (regular, soft, light). Purchase each unit in its original packaging and do not cover it or wrap it with any type of paper or other grease-absorbing material. Place each unit on a separate, airtight bag.

Take the units to a location where work can be done without the sample being contaminated. Homogenize the 5 units according to the “Sample Homogenization Procedure”.
Label one 10 cm\(^3\) glass vial with Teflon-lined cap with the product’s generic and brand names. For example: Spreadable fat/Numar Regular/S-1.

Analyze the sample according to the instructions in section “Analytical Methodology” or freeze at -20 °C for later analysis. Prepare the other two composite samples in a similar fashion.

**ii.b) Edible oil samples**

Samples of the 3 most frequently consumed types of edible oils must be obtained. Each sample is composed of 5 units, ideally from different lots. One unit equals one pack, one stick or another standard presentation of the product. Total: 3 composite samples.

To prepare composite sample #1, obtain 5 units of the product. Units must be identical, i.e., they must have the same weight and be of the same variety (100% sunflower oil, 100% soybean oil, soybean/palm oil, and others). Purchase each unit in its original packaging and do not cover it or wrap it with any type of paper or other grease-absorbing material. Place each unit on a separate, airtight bag.

Take the units to a location where you will be able to work without contaminating the sample. Homogenize the 5 units according to the “Sample Homogenization Procedure”. Label one 10 cm\(^3\) glass vial with Teflon-lined cap with the product’s generic and brand names. For example: Edible oil/Capullo/S-1.

Analyze the sample according to the instructions in section “Analytical Methodology” or freeze at -20 °C for later analysis. Prepare the other two composite samples in a similar fashion.

**ii.c) Cookie-product samples**

(i) Plain cookies (sweet, salted or oil-sprayed with no fillings or coverings):

Samples of the 3 most frequently consumed types of cookies must be obtained. Each sample is composed of 5 units, ideally from different lots. One unit equals one pack, one stick or another standard presentation of the product. Total: 3 composite samples.

To prepare composite sample #1, obtain 5 units of the product. Units must be identical, i.e., they must have the same weight and be of the same variety (sweet, salted, oil-sprayed).
Purchase each unit in its original packaging and do not cover it or wrap it with any type of paper or other grease-absorbing material. Place each unit on a separate, airtight bag.

Take the units to a location where you will be able to work without contaminating the sample. Homogenize the 5 units according to the “Sample Homogenization Procedure”. Label one 10 cm³ glass vial with Teflon-lined cap with the product’s generic and brand names. For example: Plain cookie/Maria/S-1.

Analyze the sample according to the instructions in section “Analytical Methodology” or freeze at -20 °C for later analysis. Prepare the other two composite samples in a similar fashion.

(ii)  Cream-filled cookies:

Samples of the 3 most frequently consumed types of cream-filled cookies must be obtained. Each sample is composed of 5 units, ideally from different lots. One unit equals one pack, one stick or another standard presentation of the product. Total: 3 composite samples.

To prepare composite sample #1, obtain 5 units of the product. Units must be identical, i.e., they must have the same weight and be of the same variety (fried, baked, salted, others). Purchase each unit in its original packaging and do not cover it or wrap it with any type of paper or other grease-absorbing material. Place each unit on a separate, airtight bag.

Take the units to a location where you will be able to work without contaminating the sample. Homogenize the 5 units according to the “Sample Homogenization Procedure”. Label one 10 cm³ glass vial with Teflon-lined cap with the product’s generic and brand names. For example: Filled cookie/Cremita/S-1.

Analyze the sample according to the instructions in section “Analytical Methodology” or freeze at -20 °C for later analysis. Prepare the other two composite samples in a similar fashion.

(iii)  Covered cookies:

Samples of the 3 most frequently consumed types of covered cookies must be obtained. Each sample is composed of 5 units, ideally from different lots. One unit equals one pack, one stick or another standard presentation of the product. Total: 3 composite samples.
To prepare composite sample #1, obtain 5 units of the product. Units must be identical, i.e., they must have the same weight and be of the same variety (fried, baked, salted, others). Purchase each unit in its original packaging and do not cover it or wrap it with any type of paper or other grease-absorbing material. Place each unit on a separate, airtight bag.

Take the units to a location where you will be able to work without contaminating the sample. Homogenize the 5 units according to the “Sample Homogenization Procedure”. Label one 10 cm³ glass vial with Teflon-lined cap with the product’s generic and brand names. For example: Covered cookie/Chiky/S-1.

Analyze the sample according to the instructions in section “Analytical Methodology” or freeze at -20 °C for later analysis. Prepare the other two composite samples in a similar fashion.

**ii.d) Snack-product samples (fried chips, extruded corn, puffed corn, others)**

Samples of the 3 most frequently consumed types of snacks must be obtained. Each sample is composed of 5 units, ideally from different lots. One unit equals one pack, one stick or another standard presentation of the product. Total: 3 composite samples.

To prepare composite sample #1, obtain 5 units of the product. Units must be identical, i.e., they must have the same weight and be of the same variety (fried, baked, salted, others). Purchase each unit in its original packaging and do not cover it or wrap it with any type of paper or other grease-absorbing material. Place each unit on a separate, airtight bag.

Take the units to a location where you will be able to work without contaminating the sample. Homogenize the 5 units according to the “Sample Homogenization Procedure”. Label one 10 cm³ glass vial with Teflon-lined cap with the product’s generic and brand names. For example: Snack/Chirulitos/S-1.

Analyze the sample according to the instructions in section “Analytical Methodology” or freeze at -20 °C for later analysis. Prepare the other two composite samples in a similar fashion.

**E.2.c Non-Prepackaged (Fast-Foods) Product Samples and Sampling Sites**

In order to homologate the concept of fast-food, this will be understood as an eating style where
foods are prepared and served for rapid consumption, without the use of cutlery. These restaurants offer no table service; people must stand in line to order and pay for their food. Food is served immediately to be consumed on site or somewhere else.

E.2.c.i) Sampling plan for non-prepackaged products

Samples will be obtained at 5 different points of purchase. Lot size will be the total quantity of product available at each point of purchase. Unless there is evidence to the contrary, lots will be assumed to be homogeneous. For sampling purposes, sample size will be a small serving of French fries or one unit of unfilled, phyllo-dough pastry product.

E.2.c.ii) Transnational fast-food chain samples (McDonald’s, Burger King, Kentucky Fried Chicken, others)

For this particular case, fast-foods (see definition in section 5.4) are sold in transnational restaurant chains.

ii.a) Selection of Sampling Sites

Use the information collected on the participant survey (Schedule 3) during the first stage of the study.

1. List the fast-food chains mentioned by the subjects.

2. Select the three (3) most frequently mentioned transnational fast-food chains.

3. Transnational fast-food restaurants must be located in the districts where the first stage of the study took place or, if not available, in the nearest district.

4. Select the product samples following the procedure detailed below and pay before leaving the sampling site.

ii.b) Sample selection and preparation

Obtain three composite samples from each one of the three selected transnational fast-food chains. A composite sample is comprised of 5 units. A small serving of French fries equals one unit.
(i) Transnational fast-food chain No. 1

To prepare composite sample #1, obtain 5 small servings of French fries at 5 different restaurants of the same transnational fast-food chain (one per restaurant). Units must be identical, i.e., they must have the same weight and characteristics (fried, baked, salted, others). Purchase each unit in its original packaging and do not cover it or wrap it with any type of paper or other grease-absorbing material. Place each unit on a separate, airtight bag.

Take the units to a location where you will be able to work without contaminating the sample. Homogenize the 5 units according to the “Sample Homogenization Procedure”. Label one 10 cm³ glass vial with Teflon-lined cap with the product’s generic and brand names. For example: Fast-food/McDonald’s/S-1.

Analyze the sample according to the instructions in section “Analytical Methodology” or freeze at -20 °C for later analysis. Prepare the other two composite samples from this fast-food transnational chain in a similar fashion. Preferably, obtain samples every other day.

(ii) Transnational fast-food chain No. 2 and 3

To obtain and prepare composite samples for the other two transnational fast-food chains, follow the procedure detailed above.

E.2.c.iii) Local fast-food restaurant samples

For this particular case, fast-foods (see definition in section 5.4) are sold in local restaurants/food stands that are not related in any way to the transnational fast-food chains.

iii.a) Selection of Sampling Sites

Use the information collected on the participant survey (Schedule 3) during the first stage of the study.

1. List the fast foods that were most frequently mentioned by the subjects.
2. Select the three (3) most frequently mentioned fast-foods.
3. Identify the local restaurants/food stands where said fast-foods are sold.
4. The local restaurants/food stands must be located in the districts where the first stage of the study took place or, if not available, in the nearest district.

5. Select the five (5) most frequently mentioned restaurants/food stands.

6. Obtain product samples following the procedure detailed below and pay before leaving the sampling site.

   iii.b) Sample selection and preparation

   Obtain three composite samples for each one of the three selected fast-foods. A composite sample is comprised of 5 units. One unit is the typical serving of the particular frequently consumed fast food.

   (i) Local fast-food restaurant/food stand No. 1

   To prepare composite sample #1, obtain 5 units (one unit is a typical serving of the selected fast-food) at 5 different restaurants/food stands (one per restaurant). Units must be identical, i.e., they must have the same weight and characteristics (fried, baked, salted, others). Purchase each unit in its original packaging and do not cover it or wrap it with any type of paper or other grease-absorbing material. Place each unit on a separate, airtight bag.

   Take the units to a location where you will be able to work without contaminating the sample. Homogenize the 5 units according to the “Sample Homogenization Procedure”. Label one 10 cm$^3$ glass vial with Teflon-lined cap with the product’s generic and brand names. For example: Fried food/Enyucado/S-1.

   Analyze the sample according to the instructions in section “Analytical Methodology” or freeze at -20 °C for later analysis. Prepare the other two composite samples in a similar fashion.

   To sample different lots, purchase the units for each of the three composite samples on different dates. Preferably, obtain samples every other day.

   (ii) Local fast-food restaurants/food stands No. 2 and 3

   To obtain and prepare composite samples for the other two local fast-food restaurants/food stands, follow the procedure detailed above.
E.2.c.iv) Bakery samples

iv.a) Selection of Sampling Sites

Use the information collected on the participant survey (Schedule 3) during the first stage of the study.

1. List the bakery products that were most frequently consumed or mentioned by the subjects.
2. Select the three (3) most frequently mentioned bakery products.
3. Identify the local stores where said bakery products are sold.
4. The local stores must be located in the districts where the first stage of the study took place or, if not available, in the nearest district.
5. Select the five (5) most frequently mentioned stores.
6. Obtain product samples following the procedure detailed below and pay before leaving the sampling site.

iv.b) Sample selection and preparation

Obtain three composite samples for each one of the three selected bakery products. A composite sample is comprised of 5 units. One unit is the typical serving of the particular frequently consumed bakery product.

(i) Bakery product No. 1

To prepare composite sample #1, obtain 5 units of the bakery product at 5 different bakeries (one per store). Units must be identical, i.e., they must have the same weight and characteristics (baked, regular, filled, others). Purchase each unit in its original packaging and do not cover it or wrap it with any type of paper or other grease-absorbing material. Place each unit on a separate, airtight bag.

Take the units to a location where you will be able to work without contaminating the sample. Homogenize the 5 units according to the “Sample Homogenization Procedure”. Label
one 10 cm³ glass vial with Teflon-lined cap with the product’s generic and brand names. For example: Pastry/Media luna/S-1.

Analyze the sample according to the instructions in section “Analytical Methodology” or freeze at -20 °C for later analysis. Prepare the other two composite samples in a similar fashion.

To sample different lots, purchase the units for each of the three composite samples on different dates. Preferably, obtain samples every other day.

(ii) Bakery product No. 2 and 3

To obtain and prepare composite samples for the other two bakery products, follow the procedure detailed above.

E.2.d Food Sample Homogenization Procedure

E.2.d.i) Clear liquid, sediment-free samples

Shake the original container vigorously.

E.2.d.ii) Butter, lard, margarine, fats

Soften at room temperature. Process in a food homogenizer. Do not let the sample become warm. If the product is too hard and does not soften at room temperature, homogenize in a mortar.

E.2.d.iii) Fast food

Process in a food homogenizer. Do not let the sample become warm.

E.2.d.iv) Processed foods and others

Snacks, cookies and pastries must be homogenized using a mortar.

E.2.e Analytical Methodology for total fat extraction, extraction and methylation of fat, and identification and quantification of fatty acids in foods

The analytical method for total fat extraction, methylation fat extraction and identification and quantification of fatty acids in foods is described in Schedules 6 and 7.
E.3 Result Reporting

The fatty acid content of foods will be reported in grams of fatty acids per 100 grams of food. Use the enclosed Excel worksheet.

F. Important Considerations

F.1 Quality Control of Analytical Determinations

In order to maintain quality control for the analytical determinations, INCIENSA’s laboratory will arrange for an external assessment of the participant laboratories before and during the study. To this end, each laboratory will receive a certified sample to quantify and identify each one of the fatty acids contained in it. Results will be sent to INCIENSA to obtain error percentages and variability coefficients. Those laboratories with the lowest accuracy and precision shall suspend sample analyses until errors are corrected and satisfactory error percentages and variability coefficients are attained.

F.2 Laboratory Inability to Process Samples

If no laboratory in a participant country is able to process the food or biological samples according to the required methodology, the country coordinator will have to make arrangements with the principal investigator to be assigned a laboratory where the analytical determinations can be made and to receive detailed instructions regarding sample delivery. The country coordinator will be responsible for looking into any national rules and regulations regarding the shipment of food and biological samples to other countries.

F.3 Progress Reports

Each country coordinator will send quarterly reports to the principal investigator detailing how the project is advancing, any limitations for sample collection or analysis, and/or to align with activity timelines. A template for the progress report is described on Schedule 6.

G. Data Analysis

Statistical analysis will be performed using SPSS version 10.0 for Windows (SPSS, Chicago, IL, USA). Fatty acid contents will be expressed as average plus or minus standard
deviation per 100 grams of food. Plasma fatty acid distribution will be described as a percentage of total fatty acids. Data will be calculated for each city, age group and gender.

To determine variations in fatty acid content within the same food group and between different cities, variance will be analyzed using the general linear model (GLM). Variations in fatty acid averages between different cities will be determined using the Tukey multiple comparison test. Similarly, variations in plasma fatty acid distributions within subjects and between the different cities as well as within age groups and genders will be determined using multifactorial GLM analysis of variance (ANOVA) followed by post-hoc multiple comparisons using the Tukey test.

H. Final Report

Once all the information is collected, the multicentric study lead team will prepare a final report where cis and trans saturated and unsaturated fatty acid contents in the different food categories as well as plasma and adipose tissue fatty acid distributions will be compared among the participant countries.
### VI. BUDGET

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<td>• Supelco 47791</td>
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<td><strong>TOTAL PER COUNTRY</strong></td>
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A. **Total Project Cost**

In general terms, a budget of US$13,335 per country will be required to conduct the two stages of the study. Therefore, the project budget is **US$120,000** for direct costs (not including staffing costs).

From June 2009 to December 2010, INCIENSA will contribute US$70,000 to cover the part-time salary of the principal investigator and the fees for the person that will conduct the fatty acid analyses. In addition, the other participant countries will contribute approximately US$25,000 to cover the salaries of the country investigators associated to the study.
### VII. TIMELINE

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<td>First external assessment of analytical determination quality</td>
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<tr>
<td>Preparation of scientific paper</td>
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- **S**: January, **O**: February, **N**: March, **D**: April, **J**: May, **F**: June, **M**: July, **A**: August
- **M**: September, **J**: October, **A**: November, **S**: December
VIII. REFERENCES


INSTITUTE (name of the institute conducting the study in the country)
Pan-American Health Organization

Informed Consent

The (name of the institute conducting the study in the country) institute, in partnership with other public health institutes of Argentina, Brazil, Chile, Colombia, Venezuela, Ecuador, Mexico, Guatemala, Costa Rica, Jamaica and Puerto Rico, is being sponsored by the Pan-American Health Organization to conduct a study to find out the types of fats contained in some frequently consumed foods, as well as what types of fats are circulating in the bloodstream or stored in the buttock fat of people living in some Latin American and Caribbean cities.

Knowing what kind of fats are circulating in your bloodstream or stored in your buttock fat is very important because it tells us which fats you are eating and what potential risks this could pose to your heart's health. Therefore, we are conducting this study so that the health authorities of the various countries can develop programs that will allow people to have healthier hearts.

Participants in this study are men and women between the ages of 35 and 45, residing in urban areas, who do not suffer from any type of disease such as diabetes, high blood pressure or any heart conditions, and whose diet has not been modified by a physician's or dietitian's advice.

To conduct the study, we will need to draw a blood sample using a NEW syringe. This procedure will be performed by experienced physicians or microbiologists. We will also need to take a sample of the fat stored in your buttocks, using a NEW syringe. The buttock fat sample is obtained using a procedure that is similar to getting an injection, except instead of injecting a substance, a tiny bit of fat from the upper portion of the buttock is drawn. Usually, taking blood and fat samples has no harmful side effects. However, sometimes bruises can occur at the injection site. In the case of fat samples, the injection site could become reddish because some ice will be applied to numb the skin and lessen any discomfort that may occur.

Blood samples will be stored at very low temperatures and will be analyzed in your country, specifically at (name of the institute conducting the study in the country) Institute. Test results will be provided to you along with a brief explanation and some dietary recommendations to maintain a healthy heart.
Blood and fat test results, as well as your personal information (age, gender, area of residence) will be kept in strictest confidence. You will be identified with a code number from 001 to 250 to prevent your name from ever being used in the database containing the information from all the participating countries.

You have the right to refuse to participate and to withdraw from this study at any time you deem it necessary, with no detriment to your present or future medical care.

Sir or Madam, if you agree to participate in this study, please sign below.

I, ____________________________, identification card number ____________________________, have understood the above explanation about the study that the (name of the institute conducting the study in the country) Institute wants to conduct. I accept to voluntarily participate in this study to: (MARK AN “X” BESIDE THE TYPE OF SAMPLE OR SAMPLES YOU AGREE TO PROVIDE)

1. _______ Provide the blood sample only.
2. _______ Provide both the blood sample and the buttock fat sample.

I agree to provide the sample I have indicated above to have it analyzed for the type of fats it contains.

I am aware that I can refuse to participate in this study at any time I deem necessary with no detriment to my present or future medical care. I further understand that any information about my identity is confidential and will never be mentioned in any database or anywhere else.

I have also been informed that for any question regarding this study, I may contact ________________, ________________, investigator with _____________________________ Institute, at telephone ____________________________ between the hours of _____ am and _____ pm. (INCLUDE THE INFORMATION FOR THE COUNTRY INVESTIGATOR IN EACH COUNTRY).

_________________________________________  ____________________________
Participant’s name                                   Participant’s signature

_________________________________________
Witness signature
Name and signature of Country Investigator

Note: Give copy to participant
Dear Sir or Madam,

The (name of the institute conducting the study in the country) Institute, in partnership with other public health institutes of Argentina, Brazil, Chile, Colombia, Venezuela, Ecuador, Mexico, Guatemala, Costa Rica, Jamaica and Puerto Rico, is being sponsored by the Pan-American Health Organization to conduct a study to find out the types of fats contained in some frequently consumed foods, as well as what types of fats are circulating in the bloodstream or stored in the buttock fat of people living in some Latin American and Caribbean cities.

Knowing what kind of fats are circulating in your bloodstream or stored in your buttock fat is very important because it tells us which fats you are eating and what potential risks this could pose for your heart’s health. Therefore, we are conducting this study so that the health authorities of the various countries can develop programs that will allow people to have healthier hearts.

To conduct the study, we need 250 men and women to participate. Participants must be between the ages of 35 and 45; residing in urban areas of the country’s capital city; healthy, that is, not suffering from high blood pressure or diabetes mellitus, and not following any special diet to lose weight or for any other medical reason.

For the study, we will need to draw a blood sample using a **NEW** syringe. This procedure will be performed by experienced physicians or microbiologists. We will also need to take a sample of the fat stored in the buttocks, using a **NEW** syringe. The buttock fat sample is obtained using a procedure that is similar to getting an injection, except instead of injecting a substance, a tiny bit of fat from the upper portion of the buttock is drawn.

Usually, taking blood and fat samples has no harmful side effects. However, sometimes bruises can occur at the injection site. In the case of fat samples, the injection site could become reddish because some ice will be applied to numb the skin and lessen any discomfort that may occur.
Blood samples will be analyzed at _________________ Institute. Test results will be provided to you with along a brief explanation. Blood and fat test results, as well as your personal information will be kept in strictest confidence.

We are currently inviting other people to participate in this study. Out of all the persons that agree to participate in the study, we will randomly select two hundred and fifty (250) individuals (125 men and 125 women). Should you be selected, we will contact you.

**If you are selected to participate, you will receive a copy of the study results along with their explanation.**

Thanks in advance for your cooperation.

Sincerely, [Institute seal]

____________________
Country Investigator’s name
Business telephone
Business hours
E-mail address
Sir/Madam: If you agree to participate in this study, kindly complete this card and send it with your child/representative to _____________________ School. Soon we will be sending you information about the date, time and place where you need to show up to provide the sample.

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I, _________________________________________________, have clearly understood the above explanation about the study that the (name of the institute conducting the study in the country) Institute wants to conduct. I accept to voluntarily participate in this study to: (MARK AN “X” BESIDE THE TYPE OF SAMPLE OR SAMPLES YOU AGREE TO PROVIDE)

1. _______ Provide the blood sample only.
2. _______ Provide both the blood sample and the buttock fat sample.
3. _______ I don’t want to participate.

Please answer the following questions:

1. Has a doctor told you that you have high blood pressure? Yes ____ No ____
2. Has a doctor told you that you have diabetes? Yes ____ No ____
3. Has a doctor told you that you have some form of cancer? Yes ____ No ____
4. Has a doctor told you that you have heart disease? Yes ____ No ____
5. Are you on any kind of special diet to lose weight or for any other medical reason? Yes ____ No ____

We are currently inviting other people to participate in this study. Out of all the persons that agree to participate in the study, we will randomly select two hundred and fifty (250) individuals (125 men and 125 women). Should you be selected, please let us know how we may contact you:

I can be contacted at:
Home phone number: _____________________
Work phone number: _____________________
Mobile or cell phone number: ____________________
E-mail address: ________________________________
Other: _____________________________________________________________________________
SCHEDULE 3

Participant Data

IDENTIFICATION CODE: ________________________________  DATE: ______________________

A. PERSONAL INFORMATION

Gender: Male _____ Female _____
Age: _____ years
Weight: _____ kg
Height: _____ cm
Education: _____ years of formal education

B. FOOD INTAKE

1) What kind of oil/brand do you use for home cooking? (The investigator will list the types of oils available in the city and let the participant mention the types/brands he/she uses at home).

   a. Soybean oil: _____ Brand: __________________________________________
   b. Corn oil: _____ Brand: __________________________________________
   c. Sunflower oil: _____ Brand: ______________________________________
   d. Canola oil: _____ Brand: __________________________________________
   e. Olive oil: _____ Brand: ____________________________________________
   f. Other: _____ Brand: ______________________________________________

2) What kind of margarine do you use frequently at home? (The investigator will list the types of margarines available in the city and let the participant mention the types/brands he/she uses at home).

   a. __________________ Brand: _______________________________________
   b. __________________ Brand: _______________________________________
   c. __________________ Brand: _______________________________________
   d. __________________ Brand: _______________________________________
   e. __________________ Brand: _______________________________________
   f. Other: _________________ Brand: __________________________________

3) What kind of snacks do you or your family eat more frequently? (The investigator will list the types of snacks available in the city and let the participant mention the types/brands he/she uses at home).

   a. __________________ Brand: _______________________________________
   b. __________________ Brand: _______________________________________

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4) How frequently do you buy these foods?
   a. Weekly: ______
   b. Monthly: ______
   c. Other (specify): ______________________________

5) Where do you usually buy these foods?
   a. Store name: ________________________________________________
   b. Location: ________________________________________________

6) What kind of pastries do you or your family eat more frequently? (The investigator will list the main types of pastries available in the city and let the participant mention the types/brands he/she uses at home).
   a. ________ Brand: ____________ Bought at: _________________________
   b. ________ Brand: ____________ Bought at: _________________________
   c. ________ Brand: ____________ Bought at: _________________________
   d. ________ Brand: ____________ Bought at: _________________________
   e. ________ Brand: ____________ Bought at: _________________________
   f. Other: ________ Brand: ____________ Bought at: _________________________

7) What kind of bakery products do you or your family eat more frequently? (The investigator will list the main types of bakery products available in the city and let the participant mention the types/brands he/she uses at home).
   a. ________ Brand: ____________ Bought at: _________________________
   b. ________ Brand: ____________ Bought at: _________________________
   c. ________ Brand: ____________ Bought at: _________________________
   d. ________ Brand: ____________ Bought at: _________________________
   e. ________ Brand: ____________ Bought at: _________________________
   f. Other: ________ Brand: ____________ Bought at: _________________________

8) What kind of fast foods do you or your family eat more frequently? (The investigator will list the main types of fast foods available in the city and let the participant mention the types/brands he/she uses at home).
C. INTAKE FREQUENCY

1) How frequently do you consume the products named above: *(Show the options to the participant)*.

<table>
<thead>
<tr>
<th>Food</th>
<th>Every day</th>
<th>1-2 times per week</th>
<th>3-5 times per week</th>
<th>1-2 times per month</th>
<th>Once per month</th>
<th>Never</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Margarine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pastries</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bakery Products</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Snacks</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fast-foods from transnational restaurant chains</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fast-foods from domestic restaurant chains</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
SCHEDULE 4

Methodology for the Extraction and Methylation of Fatty Acids in Plasma Samples

Each composite sample will be analyzed in triplicate.

1. Aliquot 150 μL plasma into 16 mm test tube.
2. Add 6 mL hexane with BHT and 4.5 mL iso-propanol.
3. Vortex for 10 min at motor speed of 5.
4. Centrifuge test tube at 2000 rpm for 5 minutes.
5. Take 4 mL from upper layer and place in 12 mm test tube.
6. Evaporate with nitrogen.
7. Prepare methylating reagent while sample is evaporating.

To prepare methylating reagent, use chart below:

<table>
<thead>
<tr>
<th>No. samples</th>
<th>Methanol</th>
<th>Sulfuric Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-6</td>
<td>25 mL</td>
<td>1 mL</td>
</tr>
<tr>
<td>7-12</td>
<td>50 mL</td>
<td>2 mL</td>
</tr>
<tr>
<td>13-18</td>
<td>75 mL</td>
<td>3 mL</td>
</tr>
<tr>
<td>19-24</td>
<td>100 mL</td>
<td>4 mL</td>
</tr>
<tr>
<td>25-30</td>
<td>125 mL</td>
<td>5 mL</td>
</tr>
<tr>
<td>31-36</td>
<td>150 mL</td>
<td>6 mL</td>
</tr>
</tbody>
</table>

8. Add 4 mL methylating reagent to evaporated sample, cap tightly and place in 80 °C water bath for 1 hour. After 5 minutes in water bath, tighten cap to prevent evaporation.
9. Remove samples from water bath. Add 1 mL DI water and 2 mL hexane using dispensers.

10. Vortex test tubes and centrifuge at 2000 rpm for 5 minutes.

11. Remove hexane layer using a Pasteur pipette and place in clean 12 mm test tube.

12. Add 1 mL DI water to double-wash fatty acids.

13. Vortex test tubes and centrifuge at 2000 rpm for 5 minutes.

14. Remove hexane layer using a Pasteur pipette and place in clean 12 mm test tube.

15. Evaporate with nitrogen.

16. Add 200 μL iso-octane to each sample and incubate at room temperature for 5-10 minutes.

17. Place 100 μL of the final solution in gas chromatography vials and set up GC run as per methodology described on Schedule 7.
SCHEDULE 5

Methodology for the Extraction and Methylation of Fatty Acids in Adipose Tissue Samples

Each composite sample will be analyzed in triplicate.

1. Use the adipose tissue sample (approx. 2 mg) stored in teflon-capped glass vials with approx. 1 mL hexane-isopropanol mixture (3:2 V/V)

2. Vortex for 10 min to homogenize.

3. Transfer 200 μL hexane-isopropanol mixture (3:2) to a 12 mm test tube.

4. Place test tube in 50 °C water bath for 10 min. Evaporate with nitrogen.

5. Prepare methylating reagent while sample is evaporating.

To prepare methylating reagent, use chart below:

<table>
<thead>
<tr>
<th>No. samples</th>
<th>Methanol</th>
<th>Acetyl Chloride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less than 20</td>
<td>10 mL</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>20-39</td>
<td>20 mL</td>
<td>1 mL</td>
</tr>
<tr>
<td>40-59</td>
<td>30 mL</td>
<td>1.5 mL</td>
</tr>
<tr>
<td>60-79</td>
<td>40 mL</td>
<td>2 mL</td>
</tr>
</tbody>
</table>

6. Add 0.5 mL methylating reagent to evaporated sample.

7. Cap tightly and place in 80 °C water bath for 1 hour.

8. Remove cap and evaporate methylating reagent.

9. Add 200 μL iso-octane to evaporated sample and incubate at room temperature for 5-10 min until fatty acids are re-dissolved.
10. Place iso-octane/dissolved fatty acids solution in gas chromatography vials and set up GC run as per methodology described in schedule 7.
Total fat will be quantified in accordance to AOAC methods for the specific type of food.

Methodology for Extraction and Methylation of Fat

1. Place 2 mL of hexane:isopropanol (3:2) mixture in 12 mm test tube.

2. Add appropriate amount of food to test tube:
   a. One full Pasteur pipette for oils or very fatty foods
   b. ~2-3 tablespoons of cookies/pastries/fast foods

3. Add 2 mL of 6% sodium sulfate to the test tube.

4. Vortex test tube.

5. Centrifuge sample for 5 min at 2000 rpm or up to 2500 for foods that are more difficult to separate.

6. Using a serological pipette, remove 0.100–0.200 mL from upper layer to use as starting material and place the remainder in a 12 mm test tube. Volumes will vary depending on the type of food being analyzed.

7. Place test tube in a 50 °C water bath for 10 min. Evaporate sample with nitrogen.

8. Prepare the methylating reagent while the sample is evaporating, using the chart below:
<table>
<thead>
<tr>
<th>No. of samples</th>
<th>Methanol</th>
<th>Acetyl Chloride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less than 20</td>
<td>10 mL</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>20 – 39</td>
<td>20 mL</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>40 – 59</td>
<td>30 mL</td>
<td>1.5 mL</td>
</tr>
<tr>
<td>60 – 79</td>
<td>40 mL</td>
<td>2.0 mL</td>
</tr>
</tbody>
</table>

9. Once hexane has evaporated, add 0.5 mL of methylating reagent to test tube. If using 0.5-1 mL of starting material, use 1 ml of reagent.

10. Cap test tube tightly and place in the 50 °C water bath for 1 hour.

11. Remove cap and evaporate methylating reagent from the test tube.

12. Once the methylating reagent is completely evaporated, add 0.200 mL iso-octane and incubate at room temperature for 5-10 min.

13. Label two vials: one with “C” (concentrate) and the other with “A” (analysis)

14. Fill vial A with the appropriate quantity of iso-octane according to the concentration of food (usually 1.0-1.5 mL)

15. Using a Pasteur pipette, transfer the 0.200 mL iso-octane containing the fatty acids (step 12) to vial “C”.

16. Using the same pipette, add the appropriate quantity (approximately half) of the contents of vial “C” into vial “A”.

17. Use the contents of vial “A” to fill the gas chromatography vials.
Identification and Quantification of Fatty Acids

Each composite sample will be analyzed in triplicate.

1. Analysis will be performed on a Hewlett Packard (now Agilent) model 6890 GC-FID gas chromatograph equipped with model 7673 autosampler injector (Palo Alto, CA) and fused-silica capillary cis/trans column (100 m x 0.25 mm i.d., 0.20 μm film thickness) (SP2560, Supelco, Bellefonte, PA), splitless injection port at 240°C; hydrogen carrier gas at a constant flow of 1.3 mL/min.

2. Inject a 1 μL aliquot into the column and run analysis using the following temperature program: 90-170 °C at 10 °C/min, 170 °C for 5 min, 170-175 °C at 5 °C/min, 175-185 °C at 2 °C/min, 185-190 °C at 1 °C/min, 190-210 °C at 5 °C/min, 210 °C for 5 min, 210-250 °C at 5 °C/min and 250 °C for 10 min.

3. Peak retention times and area percentages of total fatty acids will be identified by injecting known standards (NuCheck Prep, Elysium, MN) and analyzed with ChemStation A.08.03 software (Agilent Technologies, Santa Clara, CA). A total of 49 fatty acids will be analyzed.

4. To obtain known standard, proceed as follows:
   a. Dissolve each of the standards from the corresponding supplier (Nucheck or Supelco) with iso-octane so that the solution is **5 mg/mL for split injection and 10 mg/mL for splitless injection**.
   b. From each of the prepared solutions, aliquot the volume indicated on the table below and transfer to a 250 mL flask (33 mL total).
<table>
<thead>
<tr>
<th>Name</th>
<th>Catalogue No.</th>
<th>5 mg/mL volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linoleic Acid Methyl Ester cis/trans Isomer Mix</td>
<td>Supelco 47791</td>
<td>1</td>
</tr>
<tr>
<td>Reference standards (Schedule 2)</td>
<td>Nu-Chek Prep GLC-463</td>
<td>10</td>
</tr>
<tr>
<td>Reference standards Schedule 2</td>
<td>Nu-Chek Prep GLC-481-B</td>
<td>5</td>
</tr>
<tr>
<td>Hexadecanoic methyl ester</td>
<td>Nu-Chek Prep N-16-M</td>
<td>3</td>
</tr>
<tr>
<td>Heneicosanoic methyl ester</td>
<td>Nu-Chek Prep N-21-M</td>
<td>1</td>
</tr>
<tr>
<td>Tricosanoic methyl ester</td>
<td>Nu-Chek Prep N-23-M</td>
<td>2</td>
</tr>
<tr>
<td>6-Octadecenoic methyl ester</td>
<td>Nu-Chek Prep U-44-M</td>
<td>1</td>
</tr>
<tr>
<td>9-Octadecenoic methyl ester</td>
<td>Nu-Chek Prep U-46-M</td>
<td>1</td>
</tr>
<tr>
<td>9-12 Octadecadienoic methyl ester</td>
<td>Nu-Chek Prep U-59-M</td>
<td>4</td>
</tr>
<tr>
<td>5-8-11-14 Eicosatetraenoic (C20:4) methyl ester</td>
<td>Nu-Chek Prep U-71-M</td>
<td>1</td>
</tr>
<tr>
<td>Methyl ester</td>
<td>Nu-Chek Prep U-71-M</td>
<td>3</td>
</tr>
<tr>
<td>Octadecadienoic (Conjugated) (C18:2, 9 cis, 11 trans) methyl ester</td>
<td>Nu-Chek Prep UC-60-M</td>
<td>1</td>
</tr>
<tr>
<td><strong>TOTAL VOLUME</strong></td>
<td></td>
<td><strong>33 mL</strong></td>
</tr>
</tbody>
</table>

c. Dilute to volume with iso-octane for splitless injection. Do not dilute to volume for split injection.

d. Aliquot prepared solution (250 mL) to small 1 mL vials to prevent oxidation and accidental contamination. Cap under nitrogen current and store at -80 °C. Work one vial at a time.
SCHEDULE 8

Quarterly Report

Date: ________________________________  Report No.:______________

Name of Country Coordinator: ____________________________________________________

1. Progress to date: Include items such as protocol approval in your country, food sample collection/analysis, biological sample collection/analysis, analytical determination quality, and others.

______________________________________________________________________________
______________________________________________________________________________
______________________________________________________________________________
______________________________________________________________________________
______________________________________________________________________________
______________________________________________________________________________
______________________________________________________________________________

2. Have you encountered any obstacles to carry on according to the timeline? Yes __ No __
What have the obstacles been?

______________________________________________________________________________
______________________________________________________________________________
______________________________________________________________________________
______________________________________________________________________________
______________________________________________________________________________

3. What have you done to take care of the obstacles?

______________________________________________________________________________
______________________________________________________________________________
______________________________________________________________________________