METHODS AND MATERIALS

3. Methods and Materials

The present investigation was undertaken to study the “Composition of processed foods, status of food labeling and its utility towards healthy food choices.” This chapter outlines the experimental design and discusses the methods and materials used to fulfill the stated objectives. The study was carried out in four phases,

Phase I: Situational Analysis- Processed Packaged Food Consumption among Consumers

A situational analysis was carried out to assess the frequency and amount of processed packaged foods consumed by subjects (n=807, ≥15 years) from Urban Vadodara alongwith their socio-demographic profile using semi-structured questionnaire.

Phase II: Market survey

Based on the results of Phase-I, Market Survey of processed packaged foods was carried out in supermarkets (n=4) and grocery stores (n=4) in Vadodara to,

- Enlist processed packaged foods across the brands having nutrition labeling.
- Examine the food labels for various components of nutrition labeling namely, symbols and logos, nutrient claims, health claims, ingredients list, allergen declaration, Nutrition Facts Panel (NFP), information on colors, flavors and preservatives, manufacture and best before date and other miscellaneous information.
- Examine food labels of processed packaged foods for their compliance with FSSA and Codex Standards.
- Identify processed packaged foods having multiple sources of “nutrients of concern”

A total of 1,020 processed packaged foods of various national and local brands were enlisted. They were categorized into 29 food categories and further into 10 food groups based on the major constituting ingredient as given in the Table 3.1.

Table 3.1: Processed Packaged Foods Examined for Food Labeling

S.No.	Food groups	Food categories	Number of products
1	Wheat and oats based products	a. Cornflakes, oats and muesli	46
		b. Noodles, pasta and macaroni	66
2	Bakery products	a. Salty Biscuits	18
		b. Sweet biscuits	88
		c. Sweet cream wafers	31
3.	Confectionery	a. Chocolates	39
		b. Cakes	6
4	Fruit based products	a. Canned fruits	6
		b. Jam, marmalades and jellies	38
5	Milk based products	a. Butter and cheese	18
		b. Spreads and dips	17
6	Drinks	a. Malted beverages	27
		b. Soft drinks	13
		c. Energy drinks	27
		d. Juices	37
		e. Squashes	15
7	Ready-to-cook/eat products	a. Ready to cook foods	95
		b. Ready to use spice mixes	99
		c. Ready to make cake and ice cream	16
		d. Ready to eat sweets	27
		e. Soups	41
8	Food adjuncts	a. Pickles	18
		b. Papads	14
		c. Chutneys	6
		d. Ketchups and sauces	49
9	Snacks	a. Namkeens and savories	107
		b. Chips	32
		c. Popcorn	10
10	Baby Foods	a. Cereal and milk based baby foods	14
TOTAL			1020

Phase III: Nutrient analysis of selected processed packaged foods

Nutrient analysis of the shortlisted processed packaged foods was carried out by standardized procedures for the following nutrients,

a) Sodium
b) Potassium
c) Total sugars
d) Total Dietary fibre
e) Fatty acid profile

- Total fat
- Saturated fat
- Mono-unsaturated fat
- Poly-unsaturated fat
- Trans fat

The reported values of these nutrients on NFP were compared with the analyzed values to estimate the percent variation between the two. The percent variation between reported versus analyzed values was either declared as over-reporting (reported values >10% of the analyzed values) or under-reporting (reported values <10% of the analyzed values). The percent variation was calculated by dividing the difference in reported and analyzed values by reported values and multiplying by 100. If the variation was found to be more than 10%, then those products were categorized as "Over-reported" or "Under-reported." Few products did not report certain nutrients which were analyzed and thus termed as "not reported."

Phase IV: Consumer Awareness and Capacity Building on Food Labeling

Consumer awareness and capacity building survey was carried out in three sub-phases,

Sub-phase (a): Consumer awareness and practices survey

Consumer awareness and practices survey was carried out to assess the consumer's knowledge, attitude and practices regarding selection of processed packaged foods. The aim of the survey was to assess the ability of consumers to comprehend various components of food labels namely, symbols and logos, nutrient claims, health claims, ingredients list, allergen declaration, NFP, information on colors, flavors and preservatives, manufacture and best before date and other miscellaneous information.

Sub-phase (b): Development of intervention tools and capacity building of consumers

An education session was conducted for consumers to enhance awareness regarding the importance of food labels and to build capacity on the use and interpretation of food labels in healthy food selection. Two educational tools i.e. 1x1

meter colored poster on “A B C of Food Label” and 15-page booklet on “How to read nutrition labels: A step wise guide” was developed for the same.

Sub-phase (c): Impact evaluation after intervention

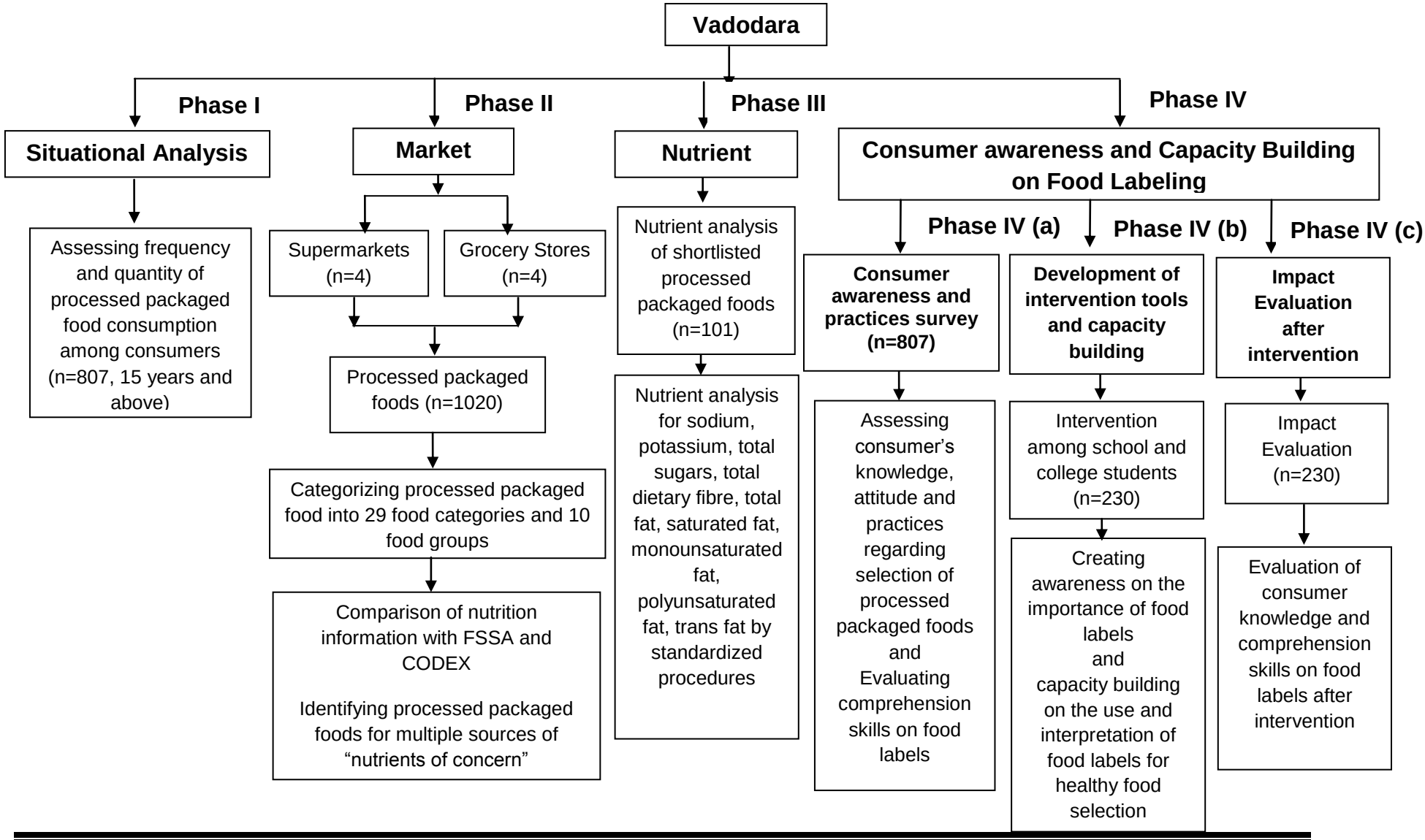
This phase dealt with assessing consumer’s knowledge and comprehension skills for food labels after capacity building.

Table 3.2: Tools and techniques

Study Phases	Parameter	Method
Phase I: Situational analysis	Height and weight Socio-demographic profile, frequency and amount of processed food consumption	Anthropometry Interview Method using Semi-structured Questionnaire
Phase II: Market Survey	Examination of Food labels	Standardized Proforma
Phase III- Nutrient analysis of selected processed packaged foods	a) Sodium b) Potassium c) Total Sugars d) Total Dietary Fibre e) Fatty Acid Profile • Total fat • Saturated fat • Mono-unsaturated fat • Poly-unsaturated fat • Trans fat	a) AOAC 969.23 b) AOAC 969.23 c) Total sugars- DNSA method d) AOAC 985.29 e) AOAC 996.06
Phase IV-Consumer awareness and Capacity Building on Food Labeling	(a) Consumer awareness	Interview Method using Semi-structured Questionnaire
	(b) Development of intervention tools and capacity building of consumers	Interactive Education session using 1x1 meter Poster Self-reading material (15- page Booklet)
	(c) Impact evaluation after intervention	Semi-structured Questionnaire Experimental Label 1 and Label 2

The study has been approved by Departmental Medical Ethical committee (Ethical Clearance number: F.C.Sc/FND/ME/50 dated: 30th September 2010)

Figure 3.1: Schematic Diagram of Experimental Design



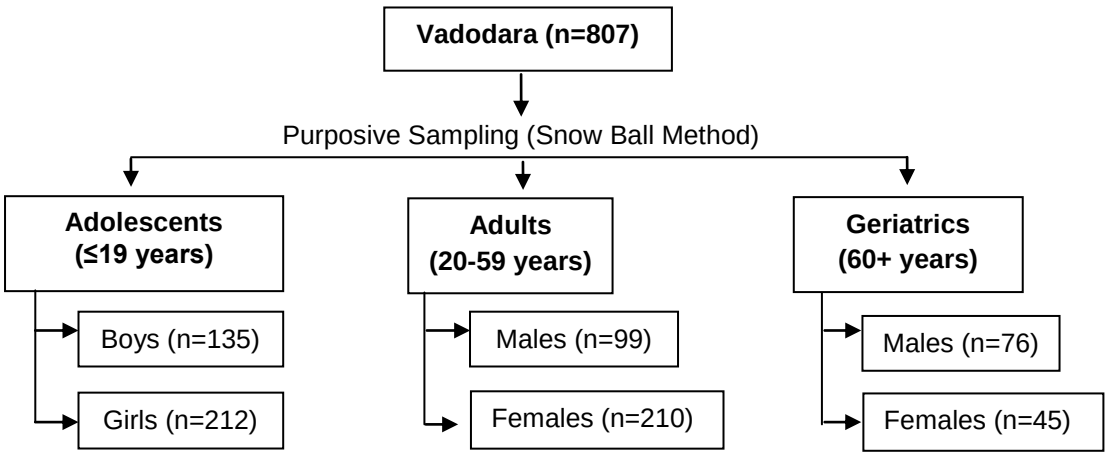
Phase I: Situational Analysis- Processed Food Consumption among Consumers

- The situational analysis aimed at eliciting information on frequency and amount of processed food consumed by subjects aged ≥15 years from Urban Vadodara. Written informed consent (Annexure I) was taken from the subjects who agreed to participate in the study. A total of 807 subjects were enrolled and they were categorized according to gender and age groups. The socio-demographic characteristics and processed packaged food consumption data was collected using semi-structured questionnaire (Annexure II). The semi-quantitative food frequency questionnaire was used to elicit the information on brands, quantity and frequency of consumption of processed packaged foods in different generic categories as given in Table 3.1.

- Inclusion criteria for situational analysis
  - Consumers who could read and understand English.
  - Consumers who agreed to participate in the study and gave informed written consent.

- Exclusion criteria for the consumer survey
- Consumers who were unable to read and understand English.
- Consumers who did not gave their consent to participate in the study.

Figure 3.2: Schematic Diagram Representing Sampling Design for Consumer Enrollment



The semi-structured questionnaire aimed at eliciting the following socio-demographic information of the consumers,

- Name
- Age
- Gender
- Educational Qualification
- Family income
- Profession
- Marital Status
- Height (using fiber glass tape)
- Weight (using bathroom scale)
- Medical condition (if any)
- Allergy (if any)
- Family type
- Household composition

Height and weight was taken to calculate the Body Mass Index (BMI) which was calculated with the following formula:

BMI = Weight (in kg) / Height (m^2)

Table 3.3: Classification of BMI according to Asia Pacific criteria, 2004

Presumptive Diagnosis	BMI (kg/m^2)
Obese	≥ 25
Overweight	23 - 24.9
Normal	18.5 - 22.9

Phase II: Market Survey

The store managers of supermarkets and grocery stores were approached for their written consent to carry out the survey. Four supermarkets and four grocery stores agreed and participated in the survey. Each product that was packaged and contained nutrition information was photographed. The pictures taken were

thoroughly examined for the food labeling. A total of 1,020 processed packaged foods of various national and local brands were examined, categorized into 29 food categories and further into 10 food groups based on the major constituting ingredient (Table 3.1). All the processed packaged foods were studied in detail using a standardized proforma (Annexure III) for the following information,

a. General Information:

- Name of the product
- Pack Size
- Serving Size
- Number of servings
- Manufacture and Best Before Date
- Kinds of Nutrition Facts Panel (Per 100g, Per serving, % Daily Value).

b. Nutrition Facts Panel (NFP) Information

• Energy	• Calories from Fat
• Total Fat	• Saturated fatty acids(SFA)
• Monounsaturated fatty acids(MUFA)	• Polyunsaturated fatty acids(PUFA)
• Cholesterol	• Trans fatty acids(TFA)
• Carbohydrates	• Sugar
• Protein	• Vitamin A
• Vitamin C	• Other Vitamins (if any)
• Iron	• Calcium
• Other minerals (if any)	• Footnote

c. Symbols and Logos

• Vegetarian symbol	• Non-vegetarian symbol
• Fruit Product Order (FPO)	• Healthy Choice Logo
• Smart Choice Logo	• Indian Standards Institute certification (ISI)
• International Organization for Standardization (ISO)	• Agricultural Marketing Logo (AGMARK)
• Hazard Analysis Critical Control Point certification (HACCP)	

d. Nutrient claims

e. Health claims

f. **Ingredients list**
g. **Allergen declaration**
h. **Manufacture and best before date**
i. **Information about colors, flavors and preservatives**
j. **Other miscellaneous information (batch number, manufacturer's address, etc.)**

PHASE III: Nutrient Analysis of Selected Processed Packaged Foods

This phase dealt with the chemical analysis of 101 shortlisted processed packaged foods to compare the nutrients (sodium, potassium, total sugars, total dietary fibre, total fat, saturated fat, monounsaturated fat, polyunsaturated fat, trans fat) reported on NFP with the analyzed values by standardized procedures.

- Inclusion criteria for nutrient analysis of processed packaged foods:
  - Most frequently consumed foods (based on the situational analysis of frequency of processed packaged food consumption)
  - Products reporting nutrient or ingredient claims related to calories/energy, salt/sodium, sugar, fat and fatty acids, trans fat or fibre.
  - Products reporting health claims related to heart, bones, brain, blood pressure, etc.

- Exclusion criteria for nutrient analysis:
  - Products that do not contain any nutrient or ingredient claims.
  - Products that do not contain any health related claims.

Shortlisted products (n=101) were chemically analyzed as per the standard procedures given below:

1. Sodium (AOAC 969.23)
2. Potassium (AOAC 969.23)
3. Total sugars- DNSA method
4. Total Dietary fibre (AOAC 985.29)
5. Fatty acid profile (AOAC 996.06)
   - Total fat
   - Saturated fat

- Mono-unsaturated fat
- Poly-unsaturated fat
- Trans fat

Sodium, potassium, total sugars, and total dietary fiber analysis was carried out in the laboratory of Department of Foods and Nutrition, The M.S. University of Baroda, Vadodara, Gujarat. Fatty acid profile was carried out at Analytical and Environmental Services Laboratory (AESL) (Registration No. T-2128), GIDC, Vadodara, Gujarat. AESL is accredited under National Accreditation Board for Testing and Calibration Laboratories, India.

PHASE IV: Consumer Awareness and Capacity Building on Food Labeling

Consumer awareness and capacity building was carried out in three sub-phases as discussed below:

Sub-phase (a): **Consumer Awareness and Practices Survey**

Consumer awareness and practices survey was carried out to assess the consumer's knowledge, attitude and practices regarding selection of processed packaged foods and comprehension of food labels. The data was collected using a pre-tested semi-structured questionnaire (Annexure IV). The previously enrolled 807 consumers from "Phase I- Situational Analysis: Processed packaged food consumption among consumers" filled the questionnaire for the following information,

Part I: Knowledge, Attitude and Practices

This part of the questionnaire contained questions regarding consumer's preferences for processed packaged foods purchase, their understanding and use of nutritional facts panel and ingredients list.

Part II: Awareness and Use of logos and Quality Symbols

This part of the questionnaire aimed at eliciting information on the use, awareness and influence of quality symbols while making food purchase decisions. It contained seven quality symbols namely AGMARK, Smart choice, Vegetarian logo, Non-vegetarian logo, HACCP, FPO and Healthy Choice. The consumers had to identify the symbol and answer whether that symbol had any influence on their purchase decisions.

Part III: Comprehension Skills

This part of the questionnaire dealt with assessing consumer's ability to comprehend the information given on NFP. They were shown four NFPs from four distinct products. All four NFPs were different from each other with respect to the format and the depth of the information provided by them about the nutrients. Consumers had to categorize the NFPs as healthy, less healthy or unhealthy based on the reported nutrients and their amounts, giving reasons for the same. The questionnaire also aimed at eliciting information on consumer's understanding on various terminologies used on NFP.

Table 3.4: Description of four NFPs

Nutrition Facts Panels (NFPs)	Kind of Information given
NFP 1	• Per 100 g
NFP 2	• Per 100g • Micro nutrients are listed with their functions • Two different panels for macro and micro nutrients
NFP 3	• Per 100 g and Per serving
NFP 4	• Per 100g, per serving and % Daily Value • Two different panels for "per 100g" and "Per serving and % daily value"

The data obtained with the help of semi-structured questionnaire was coded and statistically analyzed using Excel 2007 and SPSS-16 software.

Sub-phase (b): Development of Intervention Tools and Capacity Building of Consumers

This phase dealt with the development of Nutrition Health Education (NHE) material which was used as a reference material for guiding consumers in making healthy processed packaged food selection. Two educational tools namely, 1x1 meter colored poster on "A B C of Food Labels" (Annexure V) and a 15 page colored, pictorial booklet on "How to Read Nutrition Labels: A Step Wise Guide" (Annexure VI). The booklet contained information related to food label understanding and comprehension. It consisted of simple information from "What a nutrition label is", "how does it look like" to "calculating nutrients by using formulae" in order to make

healthy choice by the consumers. The booklet also gave few thumb-rules to quickly differentiate between healthy and unhealthy foods. Quick tips at the concluding page of the booklet summed up the contents of booklet in few points.

Capacity building and intervention was purposively done among school and college students (n=230) as the consumption of processed foods was found to be the highest in this group during "Phase I- Situational Analysis: Processed Food consumption among consumers." The other reason for selecting this group for intervention was food habits can be formed and modified easily at this stage. They can adopt healthy food habits and continue throughout their life-time. Therefore, this phase involved imparting nutrition education to 230 school and college students (aged 15-22 years) regarding the importance of reading and understanding food labels. Nutrition education sessions were conducted to introduce the concepts of logos and quality symbols, nutrient claims, health claims, ingredients list, allergen declaration, NFP, manufacture and best before date and other miscellaneous information. They were also taught about the significance of the order of ingredients in the ingredients list, the ingredients to be avoided and alternative names for harmful ingredients, how to read and interpret NFP information, importance of various nutrients in health and fitness. Queries were answered in the post session.

Sub-phase (c): Impact Evaluation after Intervention

To assess the impact of intervention on knowledge and comprehension skills of the school and college students on nutrition labels, a self administered, pre-tested semi-structured questionnaire (Annexure VII) was given to the students (n=230) post intervention. The questions in the questionnaire were based on the two experimental food labels (Annexure VIII-a and Annexure VIII-b) which were enclosed with the same. The experimental food labels represented the nutrition labels of two different products. The students had to answer the questions pertaining to logos, quality symbols, health and nutrition claims, allergen information, information about preservatives, colors and flavors, ingredients list and NFP by studying the dummy food labels in detail. The filled-in questionnaires were coded and statistically analyzed using Microsoft Excel 2007 and SPSS-16 software. The results of pre and post-intervention were compared to evaluate the change in knowledge and comprehension skills among the intervention group.

The following section of "Methods and Materials" chapter gives in detail the standardized procedures used to carry out the nutrient analysis.

1. Estimation of Sodium and Potassium

(Flame Photometry Method, AOAC 969.23)

Principle

Flame photometry relies on the principle that an alkali metal salt drawn into a non-luminous flame will ionize, absorb energy from the flame and then emit light of a characteristic wavelength as the excited atoms decay to the unexcited ground state. The intensity of emission is proportional to the concentration of the element in the solution.

Materials Required

- Equipment/Apparatus: Weighing balance, muffle furnace, desiccators, hot air oven, borosilicate crucibles, water bath, automatic pipettes (200µl-1000µl).
- Glass wares: Graduated beakers, funnels, borosilicate crucibles, volumetric flasks, measuring cylinder.
- Others: Whatman ashless filter paper No.44

Reagents

- De-ionized water,
- Sodium Standards (Sigma Aldrich-Fluka Analytical; 1000 mg/l ± 4mg/l, product Code 11116500)
- Potassium Standards (Sigma Aldrich-Fluka Analytical; 1000 mg/l ± 4mg/l, product Code 101280125)
- Concentrated Hydrochloric acid.

Standardization

The standard solutions of sodium (1000mg/l) and potassium (1000mg/l) were procured from Sigma Aldrich-Fluka Analytical to prepare the working standard solutions. The procured standard solutions were diluted with de-ionized water to obtain a series of working standard solutions of sodium and potassium as given in Table 3.5 and Table 3.6.

Table 3.5: Preparation of Working Standard of Sodium

Standard Solution	De-ionized Water	Sodium
100 µl	100 ml	1 ppm
200 µl	100 ml	2 ppm
300 µl	100 ml	3 ppm
400 µl	100 ml	4 ppm

Table 3.6: Preparation of Working Standard of Potassium

Standard Solution	De-ionized Water	Potassium
200 µl	100 ml	2 ppm
400 µl	100 ml	4 ppm
600 µl	100 ml	6 ppm
800 µl	100 ml	8 ppm

Each working standard solution was aspirated in Flame Photometer to plot standard graphs as given in Annexure IX and Annexure X.

Procedure

Estimation of sodium and potassium involves following steps:

I. **Moisture Determination (AOAC 925.09)**

- About 5g of the ground sample was weighed in a crucible and dried in an oven to 105 degrees Celsius and cooled in a desiccator. The process of drying, cooling and weighing was repeated till a constant weight was achieved. The final weight due to the loss of moisture was calculated as given below,

$$\text{Moisture \%} = \frac{\text{Loss in weight}}{\text{Initial weight of the sample}} \times 100$$

$$= \frac{W_1 - W_2}{W_1 - W} \times 100$$

Where,

W = Weight of the crucible

W_1 = Weight of the crucible + weight of the sample

W_2 = Weight of the crucible + weight of the dried sample

II. **Ash Determination (AOAC 900.02A)**

- The dried sample obtained from moisture determination was weighed accurately and heated first over a low flame till all the material was completely charred, followed by heating in a muffle furnace for about 10-12 hours at about 600 degree Celsius.
- It was then cooled in a dessicator and weighed. To ensure complete ashing, the crucible was again heated in the muffle furnace for half an hour, cooled and weighed. This was repeated until two similar consecutive weights were obtained and the ash was almost white or greyish white in color.

$$\text{Ash \%} = \frac{\text{Weight of ash}}{\text{Weight of the sample taken}} \times 100$$

$$= \frac{W_3 - W}{W_1 - W} \times 100$$

Where,

W= Weight of the crucible

W_2 = Weight of the crucible + weight of the sample after drying

W_3 = Weight of the crucible + weight of the sample after ashing

III. **Sample preparation and determination**

- The ash was moistened with 1 ml of de-ionized water and 5 ml of concentrated hydrochloric acid was added to it. The mixture was then evaporated to dryness on a boiling water bath.
- Another 5 ml of concentrated hydrochloric acid was added and the solution was evaporated to dryness as before. After this, 4 ml of hydrochloric acid and

1 ml of de-ionized water was added and the solution was warmed over boiling water bath for 5 minutes.

- This solution was then filtered into a 100 ml volumetric flask using Whatman No. 44 filter paper and the volume was made upto 100 ml using de-ionized water.
- The prepared sample solutions were aspirated in Flame Photometer (Model: Elico CL 361) and readings were recorded.

  ***Note*:** Further dilutions were prepared for the sample solutions when required.

Calculations

$$\text{Sodium/Potassium (mg/100g)} = \frac{\text{Concentration obtained from standard graph (ppm)} \times \text{volume make-up (ml)} \times \text{dilution (if any)} \times 100}{1000 \times \text{sample weight (g)}}$$

Quality Control

- Prior to taking the sample readings, diluted HCl was aspirated for 15 minutes and then de-ionized water for another 15 minutes to make the nebulizer inlet tube free from any ions.
- A blank was always run to set zero before sample analysis.
- Calibration was done each time before taking sample readings by aspirating the blank solution and standard solutions after every 10 samples.
- All the samples were analyzed in duplicates for precision.

2. Estimation of Total Dietary Fiber

(Enzymatic –Gravimetric Method, AOAC 985.29 using TDF-100ASigma Kit)

Dietary fiber is a mixture of complex organic substances and was initially defined as remnants of plant cells resistant to hydrolysis by the alimentary enzymes of man. This definition was modified to include hemicelluloses, celluloses, lignins, pectins, gums, non digestible oligosaccharides and waxes. This broader definition acknowledges the significance if fiber as a chemical and physiological component of the diet.

Principle

Samples of dried, fat-free foods are gelatinized with heat stable α-amylase and then enzymatically digested with protease and amyloglucosidase to remove the protein and starch present in the sample. Ethanol is added to precipitate the soluble dietary fiber. The residue is then filtered and washed with ethanol and acetone. After drying, the residue is weighed. Half of the samples are analyzed for protein and the others are ashed. Total dietary fiber is the weight of the residue less the weight of the protein and ash.

Reagents

- α-Amylase, Heat Stable; Product Code A3306
- Protease; Product Code P3910
- Amyloglucosidases; Product Code A9913
- Celite, Acid Washed; Product Code C8656
- Petroleum ether; Product Code 18, 451-9
- Ethyl Alcohol, ACS reagent; Product Code 45,984-4
- Acetone, ACS reagent; Product Code 32,011-0
- Sodium Phosphate, Diabasic, anhydrous; Product Code S0876
- Sodium Phosphate, Monobasic, anhydrous; Product Code S0751
- Sodium Hydroxide, 1.0 N; Product Code 930-65
- Hydrochloric Acid, 1.0 M HCl; Product Code 920-1

Apparatus

1. Fritted crucible-porosity #2 (coarse 40-60 microns)
2. Vacuum source: A vacuum pump or aspirator equipped with an inline double vacuum flask was used to prevent contamination in case of water backup.
3. An air oven capable of operating at 105 ^{0}C or a vacuum oven set at 70 ^{0}C.
4. Desiccators
5. Muffle Furnace
6. Boiling water bath
7. Constant temperature water bath adjustable to 60 ^{0}C with either a multi station shaker or multi station magnetic stirrer to provide agitation of the digestion flasks during enzymatic hydrolysis.
8. Thermometer
9. Beakers-400 ml or 600 ml tall form

10. Analytical balance capable of weighing to 0.1 mg.
11. pH meter-standardized at pH 4.0 and pH 7.0.

Preparation of Crucibles

Crucibles were washed thoroughly, heated for an hour at 525 ^{0}C and then cooled. Crucibles were soaked, rinsed in water and then dried in air. To each crucible, 0.5 grams of Celite was weighed and dried at 130 ^{0}C to a constant weight (one hour or more). Crucibles were cooled in desiccators and weighed to nearest 0.1 mg. Crucible weight was recorded as "Celite + Crucible Weight" or W_1 and stored in desiccators until required.

Sample preparation

- Food samples were de-fatted with petroleum ether when the fat content of the sample was greater than 10%.
- Loss of weight due to the fat removal was recorded and appropriate correction to the final percentage of dietary fiber was made. Fat was removed from all the food samples when the fat content was unknown.
- Food samples were homogenized and dried overnight in an air oven at 105 ^{0}C (70 ^{0}C in vacuum oven). Samples were cooled in desiccators and dry milled in mesh or grounded in a mortar. Samples which could not be heated were freeze dried before milling. Dried samples were stored in desiccators until analysis was carried out.

Reagents

De-ionized water was used to prepare the following solutions,

1. 78% Ethanol

   In a one liter volumetric flask, 207 ml de-ionized water was poured. The volume was diluted with 95% ethanol. Mixed and brought to volume again with 95% ethanol whenever required and mixed.

2. Phosphate Buffer, 0.08 M, pH 6.0

   104g of Sodium Phosphate Dibasic (Na_2HPO_4) (Product Code S 0876) and 8.4g of Anhydrous Sodium Phosphate Monobasic (NaH_2PO_4), (Product Code 0751) were dissolved in approximately 700 ml of water. The volume was diluted to one liter with de-ionized water. pH was adjusted with either NaOH

or H_3PO_4. Phosphate buffer was then stored in a tightly capped container at room temperature.

3. Sodium Hydroxide Solution, 0.275 N
   275 ml of 1.0 N NaOH solution (Product Code 930-65) was diluted to one liter with de-ionized water in a volumetric flask. Stored in tightly capped container at room temperature.

4. Hydrochloric Acid Solution, 0.325 M
   325 ml of 1.0 HCl solution (Product Code 920-1) was diluted to one liter with de-ionized water in a volumetric flask. Stored in tightly capped container at room temperature.

Determination

Banks along with samples were run through the entire procedure to measure any contributions to residue from reagents. Samples and blanks to be tested for dietary fiber content were run in quadruplicates in order to analyze samples for protein and ash values for improved accuracy.

1. One gram of samples was weighed into tall form beakers.
2. 50 ml of pH 6.0 phosphate buffer was added to each beaker.
3. 0.10 ml of α-Amylase (Product Code A3306) was added to each beaker and mixed well.
4. Each beaker was covered with aluminum foil and placed in a boiling water bath. Beakers were agitated gently at 5 minute intervals and incubated for 15 minutes after the internal temperature of the beakers reached 95 ^{0}C.
5. Solutions were allowed to cool to room temperature.
6. The pH of the solutions was adjusted to 7.5 ± 0.2 by adding 10 ml of 0.275 N NaOH to each beaker. pH was checked and adjusted whenever required with either NaOH or HCl.
7. Immediately before use, 50mg/ml solution of Protease (Product Code P3910) was prepared in phosphate buffer and pipetted 0.1 ml (5 mg Protease) into each beaker.
8. Each beaker was covered with aluminum foil and placed in 60 ^{0}C water bath. With continuous agitation, the beakers were incubated for 30 minutes after the internal temperature of the beakers reached 60 ^{0}C.
9. Solutions were then allowed to cool to room temperature.

10. The pH of the solutions was adjusted between pH 4.0 and 4.6 by adding 10 ml of 0.325 M HCl to each beaker. pH was checked and adjusted whenever required with either NaOH or HCl.
11. 0.1 ml of Amyloglucosidase (Product Code A9913) was added to each beaker.
12. Each beaker was covered with aluminum foil and placed in 60 ^{0}C water bath. With continuous agitation, the beakers were incubated for 30 minutes after the internal temperature of the beakers reached 60 ^{0}C.
13. To each beaker 4 volumes of 95% ethanol was added.
14. The solutions were left to set overnight at room temperature to allow complete precipitation.
15. Filteration
    - The bed of Celite in each crucible was damped and redistributed using 78% ethanol. Gentle suction was applied to draw Celite onto frit as an even mat. Gentle suction was maintained and the precipitate and suspension from each beaker was quantitatively transferred to its respective crucible.
    - The residue was washed with three 20 ml portions of 78% ethanol, two 10 ml portions of 95% ethanol and two 10 ml portions of acetone.
    - Sometimes, a gum was formed with some samples that trapped the liquid and therefore interfered with the filtration. To overcome it, the surface film was usually broken down with a spatula that improved the filtration rate. The time of filtration and washing varied from 0.1 to 6 hours per crucible, averaging about 0.5 hour per crucible.
16. Crucibles containing residues were dried overnight in a 105 ^{0}C air oven or 70 ^{0}C vacuum oven.
17. All crucibles were cooled in desiccators, weighed to nearest 0.1 mg and this weight was recorded as “Residue + Celite+ Crucible Weight” or W_2.
18. The residues from two samples and two blanks were analyzed for protein by Kjeldahl nitrogen analysis as specified in the AOAC procedure. Factor 6.25 was used to convert ammonia determined in the analysis to protein except where nitrogen content in the protein sample was known.
19. The residue in the crucibles were kept for ashing from two samples and two blanks for 5 hours at 525 ^{0}C. Cooled in desiccators, weighed to nearest 0.1 mg and this weight was recorded as “Ash+Celite+Crucible Weight” or $W_{3.}$

Calculations

Residue weight= $W_2 - W_1$

Ash Weight=W_3-W_1

%TDF =[R_{SAMPLE}- P_{SAMPLE}-A_{SAMPLE}-B)/SW] ×100

Where,

TDF= Total Dietary Fiber

R=Average Residue Weight (mg)

P=Average Protein Weight (mg)

A= Average Ash Weight (mg)

SW= Average Sample Weight (mg)

Quality Control

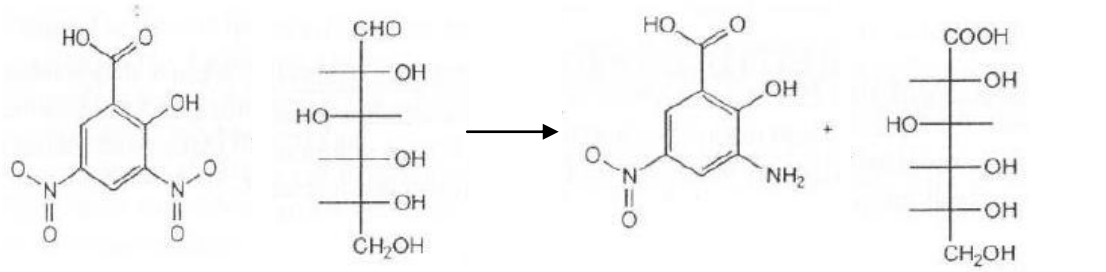
- The crucibles were properly washed, dried and stored in desiccators until use.
- The digital weighing balance was calibrated each time before weighing the samples and reagents.
- pH meter was calibrated each time before reading pH of the reagents and samples.

3. Estimation of Added Sugar

(Spectrophotometric method using Di-nitro salicylic acid, DNSA)

Principle

The method is based upon the color which forms when sugars reduce 3, 5-dinitrosalicylic acid (DNSA) to 3-amino-5-nitrosalicylic acid, as shown in the equation.



3, 5-dinitrosalicylic acid (yellow) glucose 3-amino-5-nitrosalicylic acid (red-brown) gluconic acid

As sucrose is a non-reducing sugar, it is first broken down into simple sugars like glucose by boiling the sample with hydrochloric acid. The pH is then adjusted to give a basic solution, under which condition simple sugars are good reducing agents. Therefore, when reducing sugars react with 3,5 dinitrosalicylic acid under alkaline conditions they produce red-brown color that can be measured spectrophotometrically.

Materials Required

Equipment: Spectrophotometer, weighing balance, rotary shaker, centrifuge, vortex.

Glassware: 5 ml test tubes, automatic pipettes, graduated beakers (10 ml), stirring glass rods, centrifuge tubes.

Reagents

1. DNSA

   1 g of 3,5- dinitrosalicylic acid in 20 ml of 2 M NaOH solution and 50 ml water were dissolved at room temperature, 30 g Rochelle Salt (Na-K tartrate) was added and volume was made upto 100 ml with de-ionized water. The reagent was stored at room temperature in an amber bottle (Edson and Poe, 1948).
2. Carrez I Solution
   3.60 g of potassium hexacynoferrate $\{K_4[Fe(CN)_6].3H_2O\}$ was dissolved in 100 ml of de-ionized water and stored at room temperature.
3. Carrez II Solution
   7.20 g of zinc sulphate (ZnSO4.7H2O) was dissolved in 100 ml of de-ionized water and stored at room temperature.
4. Hydrochloric Acid (5 M)
   43 ml of 11.8 M HCl was dissolved in 100 ml of de-ionized water and stored at room temperature.
5. Sodium Hydroxide
   2 g of sodium hydroxide was dissolved in 500 ml of de-ionized water and store at room temperature.

Standardization

- A blank solution containing all components of the solution except sucrose standard was used to set the spectrophotometer to a reading of zero.

- Standard series of different concentrations from 100 µl to 1000 µl was prepared using sucrose solution of known concentration.
- A volume of 100 µl to 1000 µl was pipette in quardruplicate in 5 ml test tubes.
- Volume in test tube was made up to 1 ml with de-ionized water.
- In order to hydrolyze sucrose into simple sugars, 5 M HCl was added to two test tubes of each dilution, vortexed and heated for 10 minutes in boiling water bath.
- Test-tubes were then cooled at room temperature and 5 N NaOH was added to make the solution alkaline.
- 1 ml DNSA solution was added to each hydrolyzed and unhydrolyzed sucrose dilutions, heated for 5 minutes in water bath and cooled at room temperature.
- Readings were taken on spectrophometer at 540 nm.
- A standard graph was plotted for spectrophotometer readings against different sucrose concentrations as given in Annexure XI

Sample preparation

- 1 g of ground sample was weighed in a beaker.
- 50 ml of de-ionized water was added to the beaker and kept in rotary shaker for 10 minutes.
- The diluted sample was then clarified using Carrez I and Carrez II (1 ml each) solutions and kept for 10 minutes undisturbed.
- The clear supernatant was carefully transferred in centrifuge tubes and subsequently centrifuged for 10 minutes at 30 rpm to further clear the solution if any suspended solute particles were left.

Determination

- Prepared samples (as given in sample preparation) were diluted to suitable concentrations in quardruplicates in 5 ml test tubes.
- Volume in test tube was made up to 1 ml with de-ionized water.
- In order to hydrolyze sucrose present in the samples into simple sugars, 5 M HCl was added to two test tubes of each dilution, vortexed and heated for 10 minutes in boiling water bath.
- Test-tubes were then cooled at room temperature and 5 N NaOH was added to make the solution alkaline.

- 1 ml DNSA solution was added to each hydrolyzed and unhydrolyzed samples, heated for 5 minutes in water bath and cooled at room temperature.
- Readings of blank and samples were taken on spectrophotometer at 540 nm.
- Sucrose concentration in samples was calculated from the standard sucrose graph.

Calculations

$$\text{Total Sugar (sucrose concentration)} = \frac{\text{Optical Density} \times \text{Dilution} \times 100}{1000 \times \text{Sample weight}}$$

Note: In case of further dilution, the appropriate dilution factor was applied.

Quality Control

- The spectrophotometer was kept for warming up for atleast 15 minutes prior to taking readings.
- A blank was always run to set zero before sample analysis.
- Cuvette were thoroughly rinsed and wiped before each sample reading.
- All the samples (hydrolyzed and unhydrolyzed) were analyzed in duplicates for precision.

4. DETERMINATION OF FATTY ACID PROFILE

(AOAC 996.06- Gas Chromatography Method)

A. Principle

Fat and fatty acids are extracted from food by hydrolytic methods (acidic hydrolysis for most products, alkaline hydrolysis for dairy products, and combination for cheese). Pyrogallic acid is added to minimize oxidative degradation of fatty acids during analysis. Triglyceride, triundecanoin ($C_{11:0}$), is added as internal standard. Fat is extracted into ether, then methylated to fatty acid methyl esters (FAMEs) using Boron Triflouride (BF_3) in methanol. FAMEs are quantitatively measured by capillary gas chromatography (GC) against $C_{11:0}$ internal standard. Total fat is calculated as sum of individual fatty acids expressed as triglyceride equivalents. Saturated and monosaturated fats are calculated as sum of respective fatty acids. Monounsaturated fat includes only *cis* form.

B. Apparatus

(a) Gas chromatograph (GC) – Equipped with hydrogen flame ionization detector, capillary column, split mode injector, oven temperature programming sufficient to implement a hold-ramp-hold sequence. Operating conditions: temperature (^{O}C): injector, 225; detector, 285; initial temp, 100 (hold 4 min); ramp, 3^{O}C/min; final temp 240; hold 15 min; carrier gas, helium; flow rate, 0.75mL/min; linear velocity, 18 cm/s; split ratio, 200:1.

(b) Capillary Column – Separating the FAME pair of adjacent peaks of $C_{18:3}$ and $C_{20:1}$ and the FAME trio of adjacent peaks of $C_{22:1}$, $C_{20:3}$, and $C_{20:4}$ with a resolution of 1.0 or greater. SP2560 100 m X 0.25 mm with 0.20 m film is suitable.

(c) Mojonnier flasks.

(d) Stoppers – Synthetic rubber or cork.

(e) Mojonnier centrifuge basket.

(f) Hengar micro boiling granules.

(g) Baskets – Aluminum and plastic.

(h) Shaker water bath – Maintaining 70-80^{O}C.

(i) Steam bath – Supporting common glassware.

(j) Water bath – With nitrogen stream supply, maintaining 40$\pm$ 5^{O}C.

(k) Wrist action shaker – Designed for Mojonnier centrifuge baskets.

(l) Mojonnier motor driven centrifuge – Optional; maintaining 600 X g.

(m) Gravity convection oven – Maintaining 100 $\pm$ 2^{O}C.

(n) Vortex mixer.

(o) Gas dispersion tubes – 25 mm, porosity “A”, extra coarse 175 m.

(p) Three dram vials – About 11 mL.

(q) Phenolic closed top caps – With polyvinyl liner, to fit vials.

(r) Teflon/silicone septa – To fit vials.

C. Reagents

(a) Pyrogallic acid.

(b) Hydrochloric acid – 12M and 8.3M. To make 8.3M HCl, add 250 mL 12M HCl to 110 mL H_2O. Mix well. Store at room temperature (20-25^{O}C).

(c) Ammonium hydroxide – 58% (w/w).

(d) Diethyl ether – Purity appropriate for fat extraction.

(e) Petroleum ether – Anhydrous.

(f) Ethanol – 95% (v/v).

(g) Toluene – Nanograde.

(h) Chloroform.

(i) Sodium sulfate – Anhydrous.

(j) Boron trifluoride reagent – 7% BF_3 (w/w) in methanol, made from commercially available 14% BF3 solution. Prepared in the hood.

(k) Diethyl ether – petroleum ether mixture – 1 + 1 (v/v).

(l) Triglyceride internal standard solution – $C_{11:0}$-triundecanoin; 5.00 mg/mL in $CHCl_3$. Accurately weighed 2.50 g $C_{11:0}$-triundecanoin into 500 mL volumetric flask. 400 mL $CHCl_3$ was added and mixed until dissolved. The volume was diluted with $CHCl_3$. The flask was inverted at least 10 additional times. Triglyceride internal standard solution is stable up to 1 month when stored in refrigerator (2-8^{O}C).

(m) Fatty acid methyl esters (FAMEs) standard solutions-

(1) Mixed FAMEs standard solution – Reference mixture containing series of FAMEs, including $C_{18:1}$ *cis* and *trans* (available as GLC-85 from Nu Chek Prep, Elysian, MN 56028, USA, or equivalent). To prepare mixed FAMEs standard solution, top of glass vial was broken, opened and the contents were carefully transferred to 3-dram glass vial. Original vial was washed with hexane to ensure complete transfer and washings were added to 3-dram glass vial. Diluted to ca 3 mL with hexane.

(2) C11:0 FAME standard solution – C11:0-Undecanoic methyl ester in hexane. Used only in preparation of individual FAME standard solutions. To prepare $C_{11:0}$ FAME standard solution, top of glass vial was broken, opened and the contents were carefully transferred to 50mL volumetric flask. Original vial was washed with hexane to ensure complete transfer and washings were added to 50 mL volumetric flask. The volume was diluted with hexane. $C_{11:0}$ FAME standard solution is stable up to 1 week when stored at 0^{O}C.

(3) Individual FAME standard solutions – 37-Component FAME Mix contains methyl esters of fatty acids ranging from C4 to C24, including key monounsaturated and polyunsaturated fatty acids (PUFA). The PUFA methyl ester mixes are complex qualitative standard mixtures, which can be used to verify the presence of omega 3 and omega 6 FAMEs.

D. Extraction of Fat

Food samples were finely grounded and homogenized prior to extraction of fat.

(a) Foods excluding dairy products and cheese- The grounded and homogenized test portion was weighed accurately (containing approximately 100-200 mg fat) into labeled Mojonnier flask. The material was forced into flask as far as possible. Approximately 100 mg pyrogallic acid, **C(a)**, and 2.00 mL triglyceride internal standard solution, **C(l)** was added. Few boiling granules were added to the flask. 2.0 mL ethanol was added and mixed until entire test portion was in the solution. 10.0 mL 8.3M HCl was added and mixed well. The flask was placed into basket in shaking water bath at 70-80^{o}C set at a moderate agitation speed. Maintained for 40 min. Contents of flask were mixed on Vortex mixer every 10 min to incorporate particulates adhering to sides of flask into the solution. After digestion, the flask was removed from bath and allowed to cool to room temperature (20-25^{o}C). Enough ethanol was added to fill the bottom reservoir of the flask and mixed gently.

(b) Dairy products- The grounded and homogenized test portion was weighed accurately (containing approximately 100-200 mg fat) into labeled Mojonnier flask. The material was forced into flask as far as possible. Approximately 100 mg pyrogallic acid, **C(a)**, and 2.00 mL triglyceride internal standard solution, **C(l)** was added. Few boiling granules were added to the flask. 2.0 mL ethanol was added and mixed until entire test portion was in the solution. 4.0 mL H_2O was added and mixed well. 2.0 mL NH_4OH, **C(c)** was added and mixed well. The flask was placed into the basket in shaking water bath at 70-80^{o}C set at a moderate agitation speed. Maintained for 10 min. The contents of flask were mixed on Vortex mixer every 5 min to incorporate particulates adhering to sides of flask into solution. After digestion, the flask was removed from bath and a few drops of phenolphthalein was added. The solution was kept basic (pink) with addition of ammonium hydroxide. Enough ethanol was added to fill the bottom reservoir of flask and mixed gently.

(c) Cheese- The grounded and homogenized test portion was weighed accurately (containing approximately 100-200 mg fat) into labeled Mojonnier flask. The material was forced into the flask as far as possible.100 mg pyrogallic acid, **C(a)** and 2.00 mL triglyceride internal standard solution, **C(l)** was added. Few boiling granules were added to the flask. 2.0 mL ethanol was added and mixed well until entire test portion was in solution. 4.0 mL H_2O was

added and mixed well. 2.0 mL NH_4OH, **C(c)** was added and mixed well. The flask was placed into the basket in shaking water bath at 70-80^oC set at a moderate agitation speed. Maintained for 20 min. The contents of flask were mixed on Vortex mixer every 10 min to incorporate particulates adhering to sides of flask into the solution. 10.0 mL 12M HCl was added and the flask was placed into the boiling steam bath and maintained for 20 min. Flask contents were mixed every 10 min using Vortex mixer. The flask was removed from the steam bath and allowed to cool to room temperature (20-25^oC). Enough ethanol was added to fill bottom reservoir of the flask and mixed gently.

25 mL diethyl ether was added to Mojonnier flask from (a), (b) or (c). The flask was stopperred and placed in centrifuge basket. The basket was placed in wrist action shaker, securing flask in shaker with rubber tubing. The flask was shaken for 5 min. The stopper was rinsed into the flask with diethyl ether-petroleum ether mixture, C(k). 25 mL petroleum ether was added, the flask was stopperred, and shaken for 5 min. The flask was centrifuged (in basket) for 5 min at 600 X g. (Note: If centrifuge is not available, allow contents to set at least 1 h until upper layer is clear). The stopper was rinsed into the flask with diethyl ether-petroleum ether mixture. The ether (top) layer was decanted into 150mL beaker and lip of flask was carefully rinsed into the beaker with diethyl ether-petroleum ether mixture. Ether was slowly evaporated on steam bath, using nitrogen stream to aid in evaporation. The remaining residue in beaker contained extracted fat.

E. Methylation

The extracted fat residue was dissolved in 2-3 mL chloroform and 2-3 mL diethyl ether. The mixture was transferred to 3 dram glass vial and then evaporated to dryness in 40 ^oC water bath under nitrogen stream. 2.0 mL 7% BF3 reagent, **C(j)**, and 1.0 mL toluene, **C(g)** was added. The vial was sealed with screwcap top containing Teflon/silicone septum. The vial was heated in oven for 45 min at 100^oC. The vial ca was shaken every 10 min. (Note: Evaporation of liquid from vials indicate inadequate seals; if this occurs, discard solution and repeat the entire procedure). The vial was cooled to room temperature (20-25^oC). 5.0 mL H_2O, 1.0 mL hexane and ca 1.0 g Na_2SO_4, **C(i)** was added. The vial was capped and shaken for 1 min. The layers were allowed to separate and the top layer was transferred to another vial

containing ca 1.0 g Na_2SO_4. (Note: Top layer contains FAMEs including FAME of triglyceride internal standard solution.)

FAMEs were then injected into GC column or transferred to auto sampler vial for GC analysis.

F. Determination

Relative retention times (versus FAME of triglyceride internal standard solution) and response factors of individual FAME was obtained by GC analysis of individual FAME standard solutions and mixed FAME standard solution. 2 µL each of individual FAME standard solutions and 2 µL of mixed FAMEs standard solution were injected. Mixed FAMEs standard solution was used to optimize chromatographic response before injecting and test solutions. After all chromatographic conditions have been optimized, test solutions were injected from **E**.

G. Calculations

Total fat is the sum of fatty acids from all sources, expressed as triglycerides. Expressing measured fatty acids as triglycerides requires mathematical equivalent of condensing each fatty acid with glycerol. For every 3 fatty acid molecules, 1 glycerol ($HOCH_2CHOHCH_2OH$) is required. Essentially, 2 methylene groups and 1 methine group are added to every 3 fatty acids.

Retention times for each FAME in individual FAMEs standard solutions, **C(m)**(3) was calculated by subtracting retention time of $C_{11:0}$ peak from retention time of fatty acid peak. These retention times were used to identify FAMEs in mixed FAMEs standard solution. Additional FAME solutions (from the same supplier) were used whenever required for complete FAME identity verification. The 37-FAME mix standard graph (chromatogram) has been presented in Annexure XII.

H. Quality Control

For each batch of analysis of fatty acids (15 samples), one blank, one spike and one control sample was analyzed for quality assurance purpose. For fatty acids, a control food sample (ghee sample, Annexure XIII) was repeatedly run during the analysis. The maximum precision of duplicate analysis and average recovery spiked triglycerides were 9.5 and 96%, respectively.