

PROTOCOL FOR SAMPLING AND METHODS OF ANALYSIS FOR

MALAYSIAN FOOD
COMPOSITION
DATABASE



National Technical
Working Group of
Malaysian Food
Composition
Database



MINISTRY OF HEALTH

PROTOCOL FOR SAMPLING AND METHODS OF ANALYSIS FOR MALAYSIAN FOOD COMPOSITION DATABASE

**National Technical Working Group of
Malaysian Food Composition Database**

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We are pleased that the National Technical Committee of Malaysian FCD has successfully initiated the first phase of the MyFCD update, establishing the standard protocol or methodology for creating MyFCD. This protocol is an essential guideline for participating institutions to ensure a standard methodology is used for all food analyses.

It is hoped that this document will be useful to all stakeholders, especially the participating agencies, in ensuring a harmonized application of the methods. This new revision of FCD will benefit those in the field of food and nutrition from various institutions and industries. This strong collaboration should always be maintained for our present and future benefits.

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LIST OF ABBREVIATIONS

Abbreviations	Definition/Meaning
MEASUREMENT	
mg	Milligram
g	Gram
µL	Microliter
mL	Milliliter
L	Liter
nm	Nanometer
µm	Micrometer
mm	Millimeter
cm	Centimeter
in.	Inch
TIME	
sec	Second
min	Minute
METHODS	
AOAC	Association of Official Analytical Chemists
HPLC	High-Performance Liquid Chromatography
UPLC-MS	Ultra-Performance Liquid Chromatography Mass Spectrometry
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
GC	Gas Chromatography
IDF	Insoluble Dietary Fibre
SDF	Soluble Dietary Fibre
PT	Proficiency Testing
RM	Reference Material
OTHERS	
i.e.	In other words (id est)
e.g.	For example (exempli gratia)
etc.	And the rest (et cetera)
approx. / ca.	Approximately
std.	Standard



PART I

SAMPLING OF FOODS

1.1 INTRODUCTION

Appropriate sampling is essential in the process of updating and improving the quality of food composition data. A sampling plan is designed to sample food items in the local supply to obtain robust and nationally representative estimates of mean nutrient content. It is important to update and expand the quantity and quality of food composition data in the Malaysian Food Composition Database (MyFCD).

The primary sampling method for the current Food Composition Database (FCD) is stratified sampling, whereby units are selected from defined strata (subparts) of the population. Within each stratum, the food items are selected randomly. Strata may be based on regional, seasonal, retail sales points, or others as defined by knowledge of the food being studied [1][2]. The collected foods are categorised into raw, processed, and prepared (food as consumed) foods (Figures 1.2.1 and 1.2.2).

1.2 DEFINITIONS

Raw food refers to food in its natural, unprocessed form that has not undergone the final stages of processing or has not been cooked[3].

Processed food is food that has been altered from its natural, fresh, or whole state [2]. Categories of processed food include:

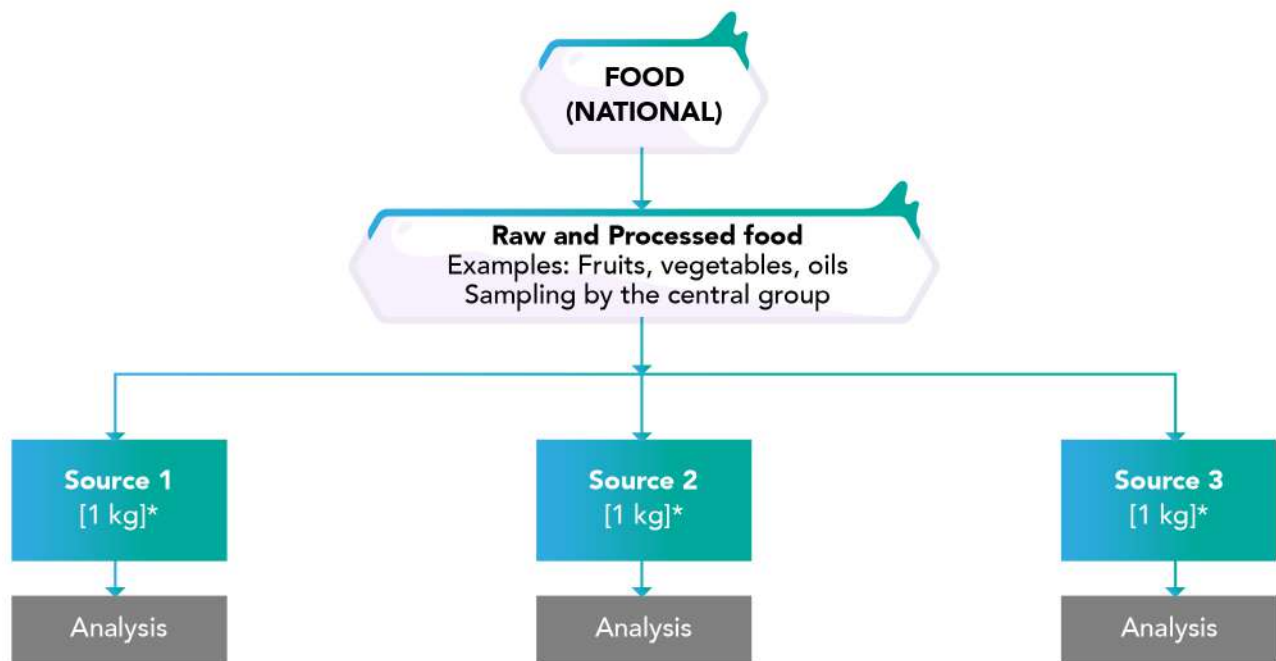
1. **Minimally processed foods** (e.g. washed and/or packaged fruits and vegetables)
2. **Preserved foods** (e.g. canned or frozen fruits and vegetables, salted fish)
3. **Mixtures of ingredients** (e.g. cake mixes, salad dressings)
4. **Ready-to-eat foods** (e.g. bread, cakes and cookies, sausage rolls, pies, cheese, breakfast cereals, lunch meats, milk, drinks, and carbonated beverages)
5. **Convenience foods** (e.g. frozen meals and pizzas, instant hotpots, and noodles)
6. **Savoury salted snacks** (e.g. chips, salted or flavoured nuts)
7. **Meat products** (e.g. bacon, sausage, ham, salami, fish balls, meatballs, nuggets, keropok lekor) [4][5][6].

Prepared food refers to food sold in a heated state or food that is heated by the seller; food in which two or more food ingredients are mixed or combined by the seller and sold as a single item. It may also include food that is packaged or prepared in advance in a container, ready for offer to consumers, or for catering purposes[7] or food sold with eating utensils provided by the seller such as plates, knives, forks, spoons, glasses, cups, napkins, or straws.

Restaurant refers to a food premise which means any place either permanently or temporarily where food or drink, intended for human consumption is prepared, stored, offered for sale, or sold, either for immediate consumption on or off the premises. Today's restaurants include traditional dine-in outlets, self-service, or takeaway restaurants with or without seating facilities.

Stall refers to any locked stalls or open setup with tables, storage, benches, baskets, chairs, wall display compartments or signboards of any kind, whether mobile or non-mobile and whether wheeled or non-wheeled, on which or in which food, drink or goods of any kind are sold or exposed for sale in any private or public place. Sellers, known as vendors or hawkers, may sell raw food or prepared food is either prepared in advance or lightly cooked at the stall.

The food truck refers to a vehicle equipped to cook and sell food that moves from place to place.



Notes:

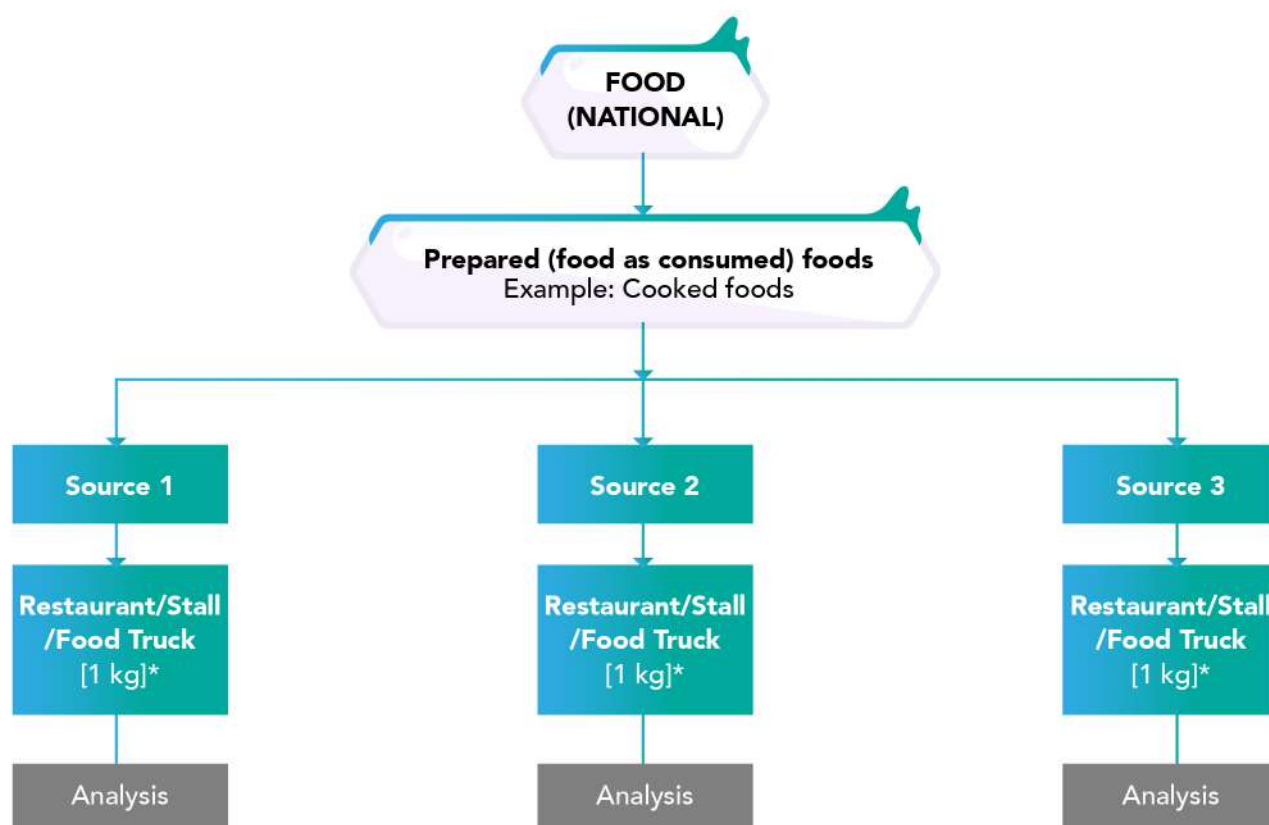
For example, for bread, three **(3) brands** such as A, B and C will be collected by the central group

For example, for sardine, three **(3) place** such as X, Y and Z will be collected by the central group

For example, for durian, three **(3) districts** (Kajang, Klang, Kuang) will be collected by the central group

*Weight of food to be collected for analysis. Refer to form 2.6.1 (raw food) and 2.6.2 (processed food)

Figure 1.2.1: Sampling of raw and processed foods



Notes:

Source refers to region states such as Northern peninsular states (Kedah, Perlis, Penang), Central peninsular states (Selangor, Perak, KL or Putrajaya, Southern peninsular states (Johor, Melaka, Negeri Sembilan), East peninsular states (Kelantan, Terengganu, Pahang) and East Malaysia states (Sabah, Sarawak, or Labuan). A minimum of one source or more can be selected to represent the region, and **a minimum of THREE sample must be collected** from stalls/restaurants.

*Weight of food to be collected for analysis. Refer to form 2.6.3 (prepared food)

Figure 1.2.2: Sampling of prepared (as consumed) foods

1.3 CLASSIFICATION

Foods collected are classified into raw, processed, and prepared foods.

Raw and processed foods include cereals and grain products (rice, white/wholemeal/flavoured breads, biscuits, flours, and processed cereal products); starchy roots, tubers and products (chips and flours); legumes and legume products (flours and sauces); nuts, seeds and products; fruits and fruits products; vegetables (starchy roots, tubers, bulbs, leaves, stems and shoots, flowers); and vegetables products; sugars and syrups; meat (beef, chicken, duck, lamb and mutton, pork) and meat products (burgers, patties, hotdogs/sausages, bologna etc); eggs (whole eggs, pasteurised eggs, frozen eggs, dried eggs, high selenium/omega-3 eggs); fish (freshwater and saltwater fish); shellfish (crustacean- crab, lobster, shrimp; mollusc- bivalve (clam, mussel, oyster, scallop), univalve; (abalone, conch, snail), cephalopod (octopus, squid); and fisheries products (crackers, snacks, sauces); milk & milk products (packed liquid/powdered milks, yogurts, cheeses, butter, creams, ghee), beverages (coffee/tea/cocoa mixtures other flavoured packed/ bottled beverages); and oils and fats.

As for prepared (food as consumed) foods, they include traditional Malaysian kuih (rice and rice flour-based, wheat flour-based, legume-based, glutinous rice-based, porridge and pengat and miscellaneous) cooked dishes and meals (cereal-based, meat and egg dishes, fish and seafood dishes, vegetable dishes and miscellaneous) and franchised fast foods (chicken, burger, pizza, spaghetti, sandwiches, satay and miscellaneous). Apart from the traditional local dishes, an emerging style of food known as food fusion refers to prepared food that involves a combination of various forms of cookery [8]. The food often combines elements of different culinary traditions such as a combination of ingredients and techniques, often from different parts of the world, such as spaghetti masak lemak cili padi, sushi rendang, pizza sambal petai, banana fritter cheese, to name a few.

1.4 SAMPLING PROCEDURES

For raw and processed foods, a total number of three sources are required as primary samples (the collection of units initially taken from the total population of food) (Figure 1.4.1). For food products in the same category, a total number of three brands/places/districts are to be included as the primary samples. Each brand shall be analysed individually, with each nutrient analysed in duplicates.

For prepared (food as consumed) foods, the collection of samples shall follow the specifically- defined stratum (regional, seasonal, retail sale point, landing areas, etc) of the individual food (Figure 1.4.2). For prepared or consumed foods such as nasi goreng, a minimum of one or more sources are to be considered, and the source refers to the region. As recommended, one or more regions are to be determined based on the importance of food items from North (Perlis, Kedah, and Penang), Central (Kuala Lumpur, Perak, Putrajaya, and Selangor), South (Johor, Melaka and Negeri Sembilan), East (Pahang, Terengganu, and Kelantan), and East Malaysia (Sabah/Labuan and Sarawak). In each region, one state will be chosen based on its comparatively highest population, with its capital city as the focus of the sample collection. One primary sample will be collected from the capital city's selected restaurant or stall which will be analysed individually for each nutrient. Therefore, the total number of samples of prepared foods is three.

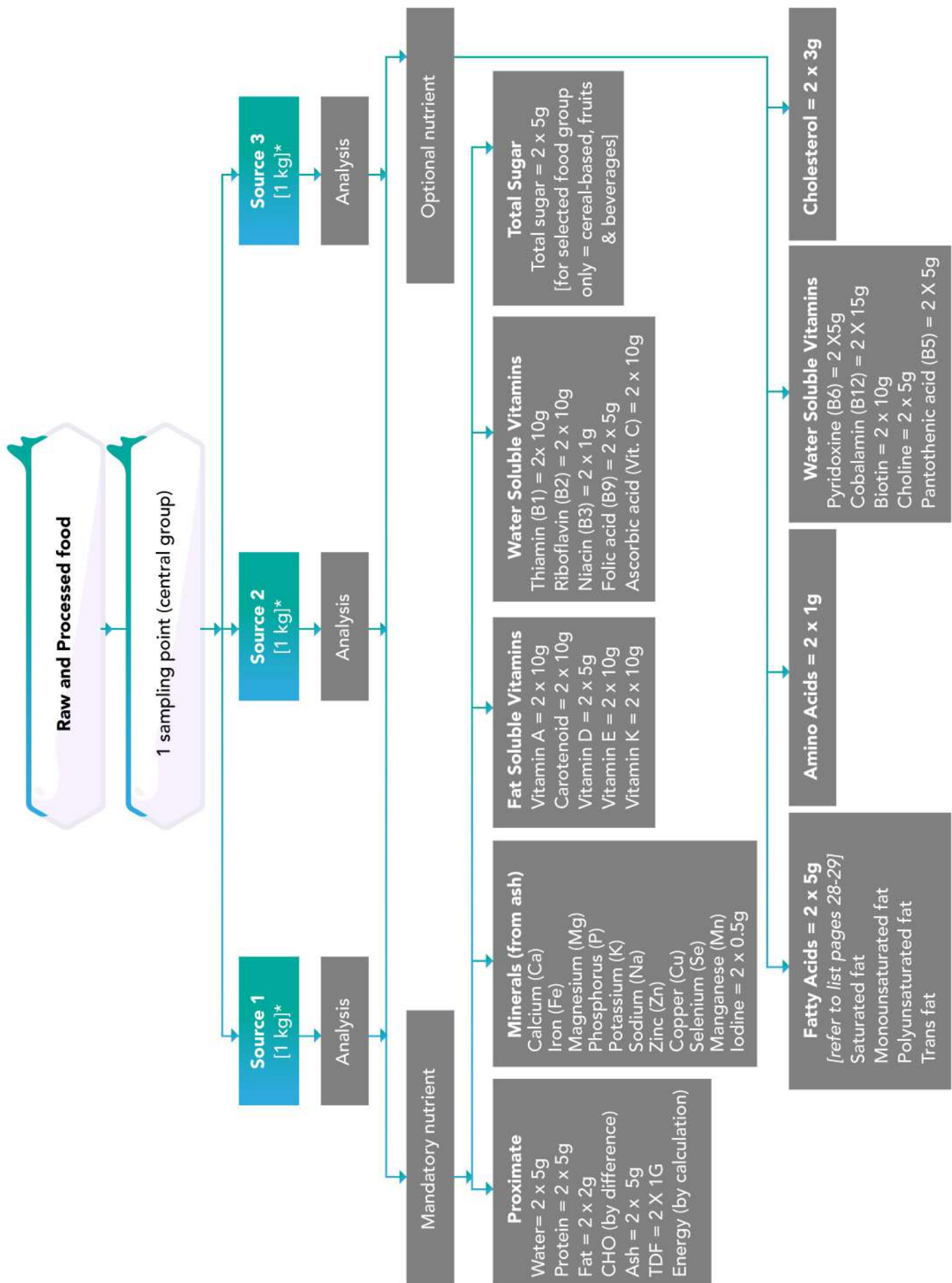


Figure 1.4.1: Nutrients analysed in raw and processed foods.

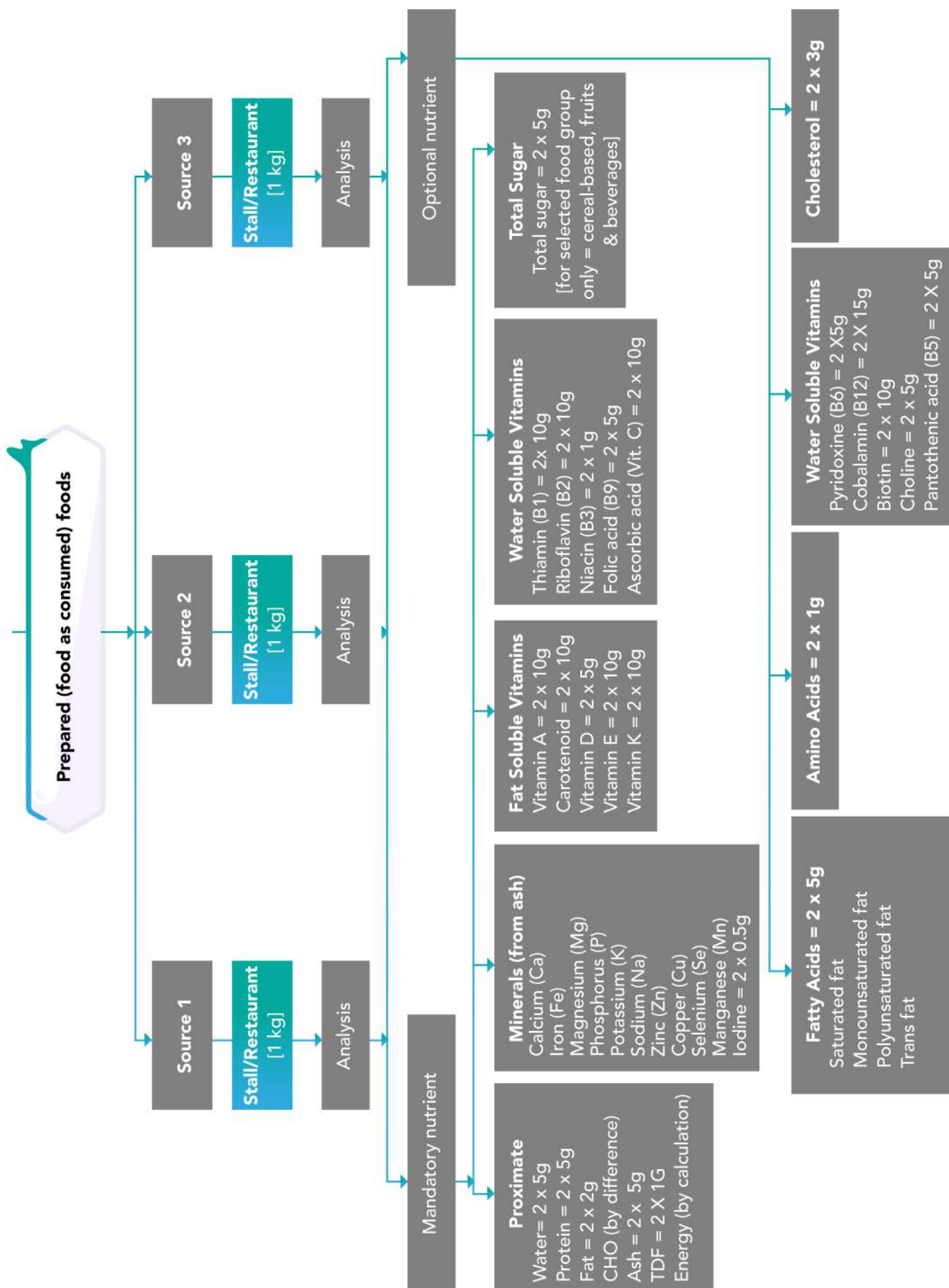


Figure 1.4.2: Nutrients analysed in prepared (as consumed) foods. A minimum of 3 samples should be collected and analysed.

1.5 PORTIONS OF FOOD

Most food composition databases/tables use analytical values obtained from the analysis of the edible portion of food. Therefore, when selecting foods for inclusion in the database, it is necessary to identify the edible portions of the food to be analysed. This will often be influenced profoundly by the cultural norms of the population for whom the database is being prepared. The non-edible portion, or refuse, should also be measured and recorded in the database since many users, particularly those in food service management, calculate nutrient content based on foods as purchased. Table 1.5 provides examples of edible and inedible portions of some foods.

Table 1.5: Examples of edible and non-edible portions of foods¹

Food	Edible	Non-edible
Cereals and grain products • Barley	All	Nil
Starchy roots, tubers, and products • Potato	Flesh	Peel
Legumes and legume products • Chickpea	All	Nil
Nuts, seeds, and products • Almond	All or Flesh	Nil or Peel
Vegetables and vegetable products • Cabbage	Remaining leaves and stalk	External yellow or wilted leaves, thick stalk
Fruits and fruit products • Banana • Orange • Passion fruit • Pineapple	Flesh Segments, residual albedo Flesh (seeds) Flesh	Peel Peel, albedo, central pith Peel (seeds) Peel, tuft, base, core
Sugars and syrups • Honey	All	Nil
Meat and meat products • Meat • Liver • Chicken	Muscle (fat), connective tissue Remaining tissue Muscle, skin from the chest and some fat pads	Bone, gristle, (fat) Blood vessels, connective tissue Bones, (skin from back), leg, subcutaneous fat connective tissue
Eggs • Duck egg	Yolk and white egg	Shell
Fish, shellfish and products • Fish • fresh • canned in brine or (oil) • dried, small	Muscle, roe, (head), (skin) Flesh/bones All	Bone, viscera, (head), fins, (skin) Oil Bones, brine, (oil), (nil) Nil
Milk and milk products • Butter • Cheese	All (Rind), inner part	Nil (Rind)
Oils and fats • Margarine	All	Nil

Food	Edible	Non-edible
Beverages • Sugar cane	Juice	Woody layers, pith
Prepared foods • Nasi Ayam (Chicken Rice)	Rice, salad, cucumber, muscle, skin from chest and some fat pads from chicken	Bones (skin from the back)

Note: The non-edible portions usually include co-product/spoiled material. The decision whether the part is edible or not depends on cultural norms and individual preferences. The portions in parentheses may or may not be discarded.

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PART II

GUIDELINE PROTOCOLS FOR
COLLECTION AND HANDLING OF RAW,
PROCESSED AND PREPARED
(FOOD AS CONSUMED) FOODS

2.1 INTRODUCTION

The collection, handling, and preparation of foods in the laboratory are vital aspects of the analytical protocols. Precautionary care should be taken in the processes of separating edible and non-edible portions (refuse, waste) and in recording the descriptions and weights of all parts.

2.2 OBJECTIVE

To ensure the process of collection, handling, preparation, storage, and disposal of food items received for nutrient analysis are conducted according to the proper protocols.

2.3 SCOPE

The guideline's scope covers sample collection, preparation, and analysis for the MyFCD.

2.4 SAMPLE CODING SYSTEM

The categories of samples are identified based on Table 2.4.1 – 2.4.7, as stated below, based on the coding flow:

1. Category sample code (Table 2.4.1)
2. Source code:
 - a. Raw, processed food, and prepared food (Table 2.4.2):
 - i. Source 1: brand/market/district A/region states.
 - ii. Source 2: brand/market/district B/region states.
 - iii. Source 3: brand/market/district C/region states.
3. State code (Table 2.4.3)
4. Food group code (Table 2.4.4 & 2.4.5)
5. Sample number code (Table 2.4.6)
6. Example of finalised identification of sample coding system (Table 2.4.7)

Example of recording sample coding system:

Category sample	Source	State	Food Group	Sample number

Figure 2.4: Sample code number

Sample: Tepung Atta (flour making Chapati)

Type: Raw

Location: Selangor

1. Category sample code: **1**

Table 2.4.1: Category sample

Category sample	Code
Raw	1
Processed	2
Prepared	3

2. Source code: **1**

Table 2.4.2: Raw, processed and prepared food

Sources	Code
Source 1	1
Source 2	2
Source 3	3

*Prepared food - Source refers to region states such as Northern peninsular states (Kedah, Perlis, Penang), Central peninsular states (Selangor, Perak, KL or Putrajaya), Southern peninsular states (Johor, Melaka, Negeri Sembilan), East peninsular states (Kelantan, Terengganu, Pahang) and East Malaysia states (Sabah, Sarawak, or Labuan).

3. State code: **B**

Table 2.4.3: State Code

State	Code	State	Code	State	Code
Perlis	R	Negeri Sembilan	N	Terengganu	T
Kedah	K	Melaka	M	Kelantan	D
Penang	P	Johor	J	Sabah	S
Perak	A	Putrajaya	PJ	Labuan	LA
Selangor	B	Pahang	C	Sarawak	Q
Kuala Lumpur	KL				

4. Food group code: **1.01**

Table 2.4.4: Raw and Processed Food

Raw and Processed Food	Code
Cereals and grain products	1.01
Starchy roots, tubers and products	1.02
Legumes and legume products	1.03
Nuts, seeds and products	1.04
Vegetables and vegetable products	1.05
Fruits and fruit products	1.06
Sugars and syrups	1.07
Meat and meat products	1.08
Eggs	1.09
Fish, shellfish and products	1.10
Milk and milk products	1.11
Oils and fats	1.12
Beverages	1.13
Miscellaneous	1.14

Table 2.4.5: Prepared Food

Prepared Food		Code
2.1 Traditional Malaysian "Kuih"	Rice and rice flour-based	2.1.1
	Wheat flour-based	2.1.2
	Legume-based	2.1.3
	Glutinous rice-based	2.1.4
	Tuber-based	2.1.5
	Bubur and pengat	2.1.6
	Miscellaneous	2.1.7
2.2 Cooked dishes and meals	Cereal-based	2.2.1
	Meat dishes	2.2.2
	Fish and seafood dishes	2.2.3
	Vegetable dishes	2.2.4
	Miscellaneous	2.2.5
2.3 Franchised "fast food"	Chicken	2.3.1
	Burger	2.3.2
	Pizza	2.3.3
	Spaghetti	2.3.4
	Sandwiches	2.3.5
	Satay	2.3.6
	Miscellaneous	2.3.7

5. Sample number code:1

Table 2.4.6: Raw and Processed Food

Raw and Processed Food	Code
Brand 1	1
Brand 2	2
Brand 3	3
Prepared Food	Code
Restaurant	1
Stall	2
Food Truck	3

6. Example of finalised identification of sample coding system

Table 2.4.7: Sample code number for Tepung Atta (Flour making chapati)

Category sample	Source	State	Food Group	Sample number
1	1	B	1.01	1

Sample code number: **1/1/B/1.01/1**

2.5 GENERAL PRECAUTIONARY MEASURES

Table 2.5.1 shows the effects of sample storage and preparation on nutrient content and the precautions required to minimise them.

Table 2.5.1: General Precaution Measures

Effects	Potential changes	Nutrients affected	Precaution
Drying out	Loss of water	All Nutrients	Keep samples in sealed or covered containers. Record food weight at initial sampling and during preparation
Absorption	Gain of water	All nutrients, especially in low-moisture and hygroscopic foods	Keep samples in sealed containers
Microbial activity	Degradation/ autolysis synthesis	Losses of carbohydrates and proteins. Gains in thiamin, vitamin B6, niacin and vitamin B12	Store at low temperature. Pasteurisation or the addition of inhibitors may be necessary
Oxidation	Destruction of unsaturated fatty acids	Alterations in the profile of fats	Store at –30°C in sealed containers with added nitrogen. Addition of antioxidants or bacteriostatic agents
	Loss of vitamins	Losses of vitamin C, riboflavin, and folates	
Acid	Hydrolysis	Losses of sucrose and higher oligosaccharides	Store at low temperature. Neutralise acid
Alkaline	Destruction	Loss of thiamin	Avoid alkaline conditions and SO ₂
Light	Photodegradation	Loss of riboflavin	Protect from light
Contamination during sampling	From cooking vessels, soil, dust, etc.	Increase in inorganic nutrients	Minimise contamination by gently rinsing it with distilled water
Contamination (from metallic blades, milling equipment, glassware, etc.)	Increase in inorganic nutrients	Increase in major trace elements	Select apparatus with care. Clean all utensils thoroughly before use and store them in plastic bags
Separation	Separation of fats. Fractionation of particles	Changes in composition overall, alteration in fibre content	Avoid over-vigorous mixing and thaw/freezing cycles
Enzymatic and metabolic activity	Change in organic nutrients	Losses of sugars, vitamin C, and folate deconjugation	Store at low temperatures. Protect the sample with folates with ascorbic acid

Sources:

- 1) Greenfield H, Southgate DAT. Food Composition Data: Production, Management, and Use. Food and Agriculture Organization of the United Nations; 2003.
- 2) Fairulnizal M, Gunasegavan RDN, Khalid NM, Balasubramaniam V, Mustar S, Rashed AA. Recent techniques in nutrient analysis for food composition database. MDPI Molecules. 2020;25(19). doi:10.3390/molecules25194567

2.6 IDENTIFICATION OF FOOD (PRIMARY SAMPLE)

FORM 2.6.1: RECORDING OF RAW SAMPLES

Category sample	Source	State	Food Group	Sample number

Common name of food			
Other Names (if any)	Alternative: Scientific:		
Date and time of collection	Date:	Time:	
Date and time of receipt in laboratory	Date:	Time:	
Plant-based food	☐ Entire plant ☐ Leaves ☐ Fruits ☐ Root/tuber ☐ Flowers ☐ Shoot ☐ Stem ☐ Seeds ☐ Others: _____		
Animal-based food	☐ Entire animal ☐ Internal organ ☐ Wings ☐ Leg ☐ Chest ☐ Others: _____ ☐ head ☐ Drumstick		
Sampling location	☐ Supermarket ☐ Pasar Tani ☐ Wet Market ☐ Stall ☐ Others: _____		
Purchase price (RM)			
Graphical record	☐ Visual record with scales		

Food Group	☐ Cereals and grain products ☐ Starchy roots, tubers, and products ☐ Legumes and legume products ☐ Nuts, seeds, and products ☐ Vegetables and vegetable products ☐ Fruits and fruit products ☐ Sugars and syrups ☐ Meat and meat products ☐ Eggs ☐ Fish, shellfish, and products ☐ Milk and milk products ☐ Oils and fats ☐ Beverages ☐ Miscellaneous
State of Maturity	☐ Immature ☐ Mature ☐ Others: _____
Physical state	☐ Liquid ☐ Solid ☐ Whole ☐ Divide ☐ Pieces
Container or wrapping type	☐ Can ☐ Foil ☐ Polystyrene ☐ Glass ☐ Plastic ☐ Others: _____ ☐ Paper
Number of items (pieces)	
Weight of food collected (gram)	
Weight of individual food (gram)	
Weight of household measurement (gram)	☐ Cup: _____ ☐ Tablespoon: _____ ☐ Scoop: _____ ☐ Bowl: _____ ☐ Teaspoon: _____ ☐ Others: _____ ☐ Plate: _____
Other details	☐ Pre-processing sample ☐ Freeze dry

Name and sign of Collector:

 (Name: _____)
 Date:

FORM 2.6.2: RECORDING OF PROCESSED FOOD SAMPLES

Category sample	Source	State	Food Group	Sample number

Common name of food			
Other Names (if any)	Alternative: Scientific:		
Date and time of collection	Date:	Time:	
Date and time of receipt in laboratory	Date:	Time:	
Plant-based food	☐ Entire plant ☐ Leaves ☐ Fruits ☐ Root/tuber ☐ Flowers ☐ Shoot ☐ Stem ☐ Seeds ☐ Others: _____		
Animal-based food	☐ Entire animal ☐ Internal organ ☐ Wings ☐ Leg ☐ Chest ☐ Others: _____ ☐ head ☐ Drumstick		
Sampling location	☐ Supermarket ☐ Pasar Tani ☐ Wet Market ☐ Stall ☐ Others: _____		
Purchase price (RM)			
Graphical record	☐ Visual record with scales		
Physical dimensions (H x W x L)			

Food Group	☐ Cereals and grain products ☐ Starchy roots, tubers, and products ☐ Legumes and legume products ☐ Nuts, seeds, and products ☐ Vegetables and vegetable products ☐ Fruits and fruit products ☐ Sugars and syrups ☐ Meat and meat products ☐ Eggs ☐ Fish, shellfish, and products ☐ Milk and milk products ☐ Oils and fats ☐ Beverages ☐ Miscellaneous
State of Maturity	☐ Immature ☐ Mature ☐ Others: _____
Physical state	☐ Liquid ☐ Solid ☐ Whole ☐ Divide ☐ Pieces
Container or wrapping type	☐ Can ☐ Foil ☐ Polystyrene ☐ Glass ☐ Plastic ☐ Others: _____ ☐ Paper
Batch number and expiry date	
Number of items (pieces)	
Weight of food collected (gram)	
Weight of individual food (gram)	
Weight of household measurement (gram)	☐ Cup: _____ ☐ Tablespoon: _____ ☐ Scoop: _____ ☐ Bowl: _____ ☐ Teaspoon: _____ ☐ Others: _____ ☐ Plate: _____
Other details	

Name and sign of Collector:

(Name: _____)

Date:

FORM 2.6.3: RECORDING OF PREPARED (FOOD AS CONSUMED) FOOD SAMPLES

Category sample	Source	State	Food Group	Sample number

Common name of food			
Other Names (if any)	Alternative: Scientific:		
Date and time of collection	Date:	Time:	
Date and time of receipt in laboratory	Date:	Time:	
Plant-based food	☐ Entire plant ☐ Flowers ☐ Fruits ☐ Root/tuber ☐ Seeds ☐ Shoot ☐ Stem ☐ Leaves ☐ Others: _____		
Animal-based food	☐ Entire animal ☐ Internal organ ☐ Wings ☐ Leg ☐ Chest ☐ Others: _____ ☐ Head ☐ Drumstick		
Purchase price (RM)			
Graphical record	☐ Visual record with scales		
Physical dimensions (H x W x L)	Traditional Malaysian Kuih ☐ Rice and rice flour-based ☐ Wheat flour based Cooked Dishes and Meals ☐ Cereal based ☐ Meat and egg dishes		

	☐ Legume based ☐ Glutinous rice based ☐ Tuber based ☐ Bubur and pengat ☐ Miscellaneous: _____ ☐ Fish and seafood dishes ☐ Vegetable dishes ☐ Miscellaneous: _____ Franchised fast foods ☐ Chicken ☐ Burger ☐ Pizza ☐ Spaghetti ☐ Sandwiches ☐ Satay ☐ Miscellaneous: _____
Physical dimension (H x W x L)	
Physical state	☐ Liquid ☐ Solid ☐ Whole ☐ Divide ☐ Pieces
Process and preservation method	☐ Canned ☐ Smoked ☐ Sun-dried ☐ Freeze-dry ☐ Other: _____
Preparation method for consumption (cooking method)	☐ Sauteing ☐ Steaming ☐ Broiling ☐ Stir-frying ☐ Baking ☐ Grilling ☐ Braising ☐ Roasting ☐ Other: _____
Extend of preparation	☐ Raw ☐ Fully Cooked ☐ Frozen ☐ Uncooked ☐ Thawed ☐ Dehydrating ☐ Partially Cooked ☐ Reheated ☐ Other: _____
Container or wrapping type	☐ Can ☐ Glass ☐ Paper ☐ Foil ☐ Plastic ☐ Polystyrene ☐ Others: _____
Number of items (pieces)	
Weight of food collected (gram)	
Weight of individual food (gram)	
Weight of household measurement (gram)	☐ Cup: _____ ☐ Bowl: _____ ☐ Plate: _____ ☐ Tablespoon: _____ ☐ Teaspoon: _____ ☐ Scoop: _____ ☐ Others: _____
Other details (recipes, ingredients, etc.)	☐ Pre-processing sample ☐ Freeze dry

Name and sign of Collector:

(Name: _____)
Date: _____

2.7 SAMPLE HANDLING IN THE FIELD

Table 2.7.1: Frozen Food

Description	Examples of record
Quantity	1 kg minimum
Packaging	Sealed plastic bag for the sample, wrapped with dark plastic
Logistic: Timing	Within 24 hours, the sample should reach the lab.
Condition for storage during transportation	Cooled box with ice pack, cooler or ice cube (0-4°C).
Transport conditions	Mode and conditions of transport
Note	Samples from various small food packages must be collected from the same production batch number.
Other details	Any relevant details as deemed important by the recorder (e.g., vacuum sealing of fresh samples immediately after collection)

Table 2.7.2: Highly Perishable And Refrigerated Food

Description	Examples of record
Quantity	1 kg minimum
Packaging	Sealed plastic bag for the sample, wrapped with dark plastic
Logistic: Timing	Within 24 hours, the sample should reach the lab.
Condition for storage during transportation	Cooled box with ice pack, cooler or ice cube (0-4°C).
Transport conditions	Mode and conditions of transport
Note	• Samples from various small food packages must be collected from the same production batch number. • Fresh eggs and outer cartoons are wrapped in several layers of bubble wrap, and thick layers of newspaper are placed between each carton.
Other details	Any relevant details as deemed important by the recorder (e.g., vacuum sealing of fresh samples immediately after collection)

Table 2.7.3: Shelf-stable Food

Description	Examples of record
Quantity	1 kg minimum
Packaging	Sealed plastic bag for the sample, wrapped with dark plastic
Logistic: Timing	Within 24 hours, the sample should reach the lab. If delayed, keep it at room temperature.
Condition for storage during transportation	Cardboard boxes
Transport conditions	Mode and conditions of transport
Note	• Samples from various small food packages must be collected from the same production batch number. • Items packed in glass bottles are wrapped in bubble wrap or newspaper to offer cushioning during transport. • Marked fragile for items like eggs.
Other details	Any relevant details as deemed important by the recorder (e.g., vacuum sealing of fresh samples immediately after collection)

Table 2.7.4: Prepared (Food As Consumed) And Fast Food

Description	Examples of record
Quantity	1 kg minimum
Packaging	Sealed plastic bag for the sample, wrapped with dark plastic
Logistic: Timing	Within 24 hours, the sample should reach the lab. If delayed, kept in the freeze.
Condition for storage during transportation	Cooled box with dry ice (<10°C).
Transport conditions	Mode and conditions of transport
Note	• Samples from various small food packages must be collected from the same production batch number. • Sealed plastic bags should be left unsealed for ten to fifteen minutes after purchase to allow the food to cool slightly before sealing. • Sodas and coffee are transferred to specially cleaned or prepared plastic bottles with leak-proof screw-type lids.
Other details	Any relevant details as deemed important by the recorder (e.g., vacuum sealing of fresh samples immediately after collection)

FORM 2.8: RECORD OF SAMPLE HANDLING IN LABORATORY

Sample code number			
Common name of food			
Date of receipt in laboratory			
Total sample weight received (gram)			
Plant-based food	☐ Head: _____ ☐ Fins: _____ ☐ Tail: _____ ☐ Wilted leaves: _____ ☐ Stem: _____ ☐ Skin/peel: _____ ☐ Shells: _____	☐ Bone: _____ ☐ Feet: _____ ☐ Internal organs: _____ ☐ Rotten parts: _____ ☐ Roots: _____ ☐ Seed: _____ ☐ Other: _____	
Weight of edible matter (gram)			
Animal-based food	☐ Entire animal ☐ Leg ☐ head	☐ Internal organ ☐ Chest ☐ Drumstick	☐ Wings ☐ Others: _____
Method of mixing and reduction	☐ Grinding	☐ Homogenising in blender	☐ Others: _____
Type of storage	☐ Sealed, room temperature	☐ Sealed, chiller temperature	☐ Others: _____
Completed the result sheet (Refer to form in section 2.5)			
Name and signature of the person completing the record			
Date of record			
Other details:	Any details that the Collector think may be relevant		

2.9 SAMPLE PREPARATION IN THE LABORATORY

The food items received from the sampling points may require simple preparations (e.g., wash and peel or use directly), while some require detailed cooking instructions (e.g., chicken). All food items are prepared individually.

1. Receiving of Samples

All received samples will be sent to the sample preparation area for collection by the respective laboratory analyst. Analysts are responsible for handling the samples as requested.

2. Identifying Sample

A sample is to be divided into 40 analyses. Each analyst must identify the sample type before analysis to determine the sample preparation procedure.

3. Preparation of Sample

Sample preparation must be carried out based on the type of sample. The preparation of analytical samples for particular food types is as in Table 2.9.1.

The portion of laboratory samples to be analysed should be identified according to the type of analysis. For samples requested for different types of analyses, the portions should be kept separately for each analysis. Each sample portion should be placed in a clean, chemically inert container and sealed to avoid contamination and ensure its integrity. Before storage, each sample should be correctly identified by a record and labelled accordingly by the respective analyst.

Table 2.9.1: Preparation of analytical samples for particular food types

Type of Sample	Method of Sample Preparation	Storage conditions
Dry food/solid food (separate) except for milk powder	Homogenise with a grinder/miller, then freeze-dried. For vitamin C analysis, keep the analytical portion in cold metaphosphoric acid.	Chilled at 4°C or Room Temperature
Liquid samples	Shake the samples in the original bottle/ can and pour the samples into a dedicated label bottle. e.g., syrup, juices, fresh milk, canned drinks.	Chilled at 4°C
Liquid samples with solids	Use a food processor or blender to homogenise the whole sample, e.g., mushroom soup, canned fruit, or vegetables, and then freeze-dried.	Chilled at 4°C
Semi-solid samples	Use a miller or mixer to homogenise the whole sample. e.g., marmalade, jam, etc.	Chilled at 4°C
Solid with liquid sample	Homogenise the solid part first, then add the liquid part, using a spatula to mix, and finally, homogenise all with a mixer. For example, instant noodles with seasoning and paste can be freeze-dried for a longer shelf life.	Chilled at 4°C
Milk Powder	Homogenize and put the samples into a dedicated label bottle.	Room Temperature

2.10 FLOW CHART OF SAMPLE PREPARATION IN THE LABORATORY

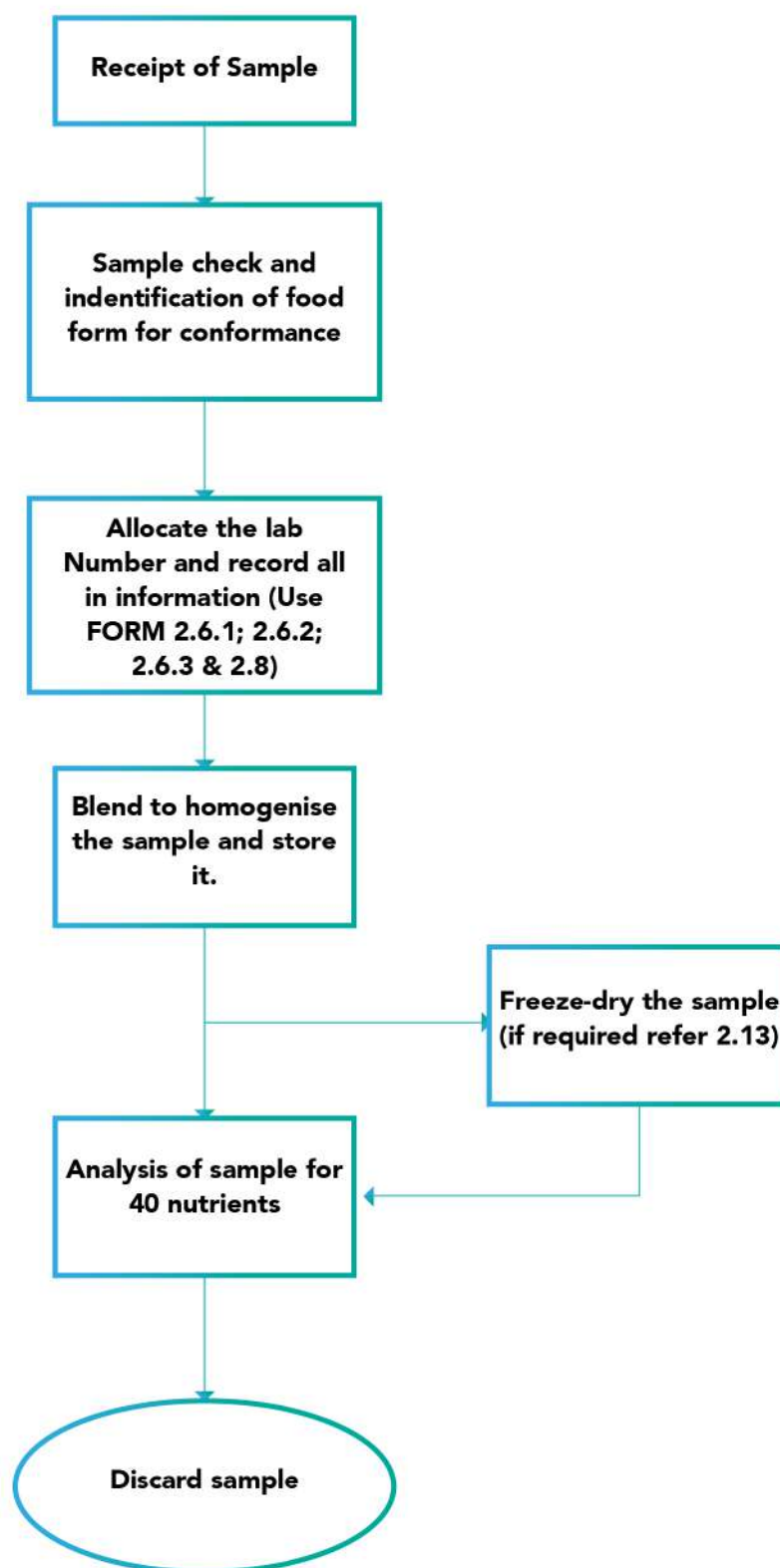


Figure 2.10: Flow chart of sample preparation in the laboratory

2.11 EQUIPMENT FOR HANDLING AND PREPARATION OF LABORATORY AND ANALYTICAL SAMPLES

A. General:

1. Trays (for carrying foods)
2. Bowls (0.5 Litre to 4 Litre capacity)
3. Spatulas
4. Chopping boards (polythene, wood)
5. Kitchen knives, knife-sharpener
6. Can-opener
7. Spoons (various sizes)
8. Plastic sieves, colanders
9. Electric heat-sealer (for freezer bags)
10. Large sheets of strong plastic (to cover benches, mix particulate foods)
11. Kitchen cutlery and tableware
12. PPE (Personal protective equipment)

B. Homogenisers:

Common domestic equipment:

1. Domestic food processor (can be equipped with titanium or other special blades)
2. Food blender

C. Freeze-dry samples (if required):

Referring to Table 2.9, freeze-drying is only done for these types of samples:

1. Dry food/solid food (separate) except for milk powder
2. Liquid samples with solids
3. Solid with liquid sample

2.12 RECORDING OF LABORATORY RESULTS

A. Form 2.12.1: Mandatory Nutrients

Sample code number:
Common name of food:

No.	Method of Sample Preparation	Unit	Results / 100 g sample	Methods of analysis	Known value results (QC)	Coefficient Variation (CV)
1	Energy	Kcal				
2	Water	g				
3	Protein	g				
4	Fat	g				
5	Available carbohydrates, by the difference	g				
6	Total Dietary Fibre, (TDF)	g				
7	Ash	g				
8	Calcium (Ca)	mg				
9	Iron (Fe)	mg				
10	Magnesium (Mg)	mg				
11	Phosphorus (P)	mg				
12	Potassium (K)	mg				
13	Sodium (Na)	mg				
14	Zinc (Zn)	mg				
15	Copper (Cu)	mg				
16	Selenium (Se)	µg				
17	Manganese (Mn)	µg				
18	Iodine	µg				
19	Ascorbic Acid (Vitamin C)	mg				
20	Thiamin (B1)	mg				
21	Riboflavin (B2)	mg				
22	Niacin (B3)	mg				
23	Folic Acid (B9)	µg				
24	Vitamin A (Retinol)	µg				
25	Carotenoid	µg				
	β-carotene	µ				
	Lycopene	µg				
	Lutein	µg				
26	Vitamin D	µg				
27	Vitamin E	mg				
28	Vitamin K	µg]				
29	Total sugar (Mandatory for cereal-based foods, fruit and beverages)	g				
	Sucrose	g				
	Glucose	g				
	Fructose	g				
	Lactose	g				
	Maltose	g				

B. Form 2.12.2: Optional Nutrients

Common name of food:
Sample code number:

No.	Method of Sample Preparation	Unit	Results / 100 g sample	Methods of analysis	Known value results (QC)	Coefficient Variation (CV)
1	Pantothenic Acid (B5)	mg				
2	Pyridoxine (B6)	mg				
3	Cobalamin (B12)	µg				
4	Choline	mg				
5	Biotin (B7)	mg				
6	Fatty acid, total saturated fat	g				
	4:0	g				
	6:0	g				
	8:0	g				
	10:0	g				
	12:0	g				
	14:0	g				
	16:0	g				
	18:0	g				
7	Fatty acids, total					
	monounsaturated fat	g				
	16:1	g				
	18:1	g				
	20:1	g				
	22:1	g				
8	Fatty acids, total					
	polyunsaturated fat	g				
	18:2	g				
	18:3	g				
	18:4	g				
	20:4	g				
	20:5	g				
9	Trans fatty acids					
10	Cholesterol	mg				
11	Amino Acid:	g				
	Tryptophan	g				
	Threonine	g				
	Isoleucine	g				
	Leucine	g				
	Lysine	g				
	Methionine	g				
	Phenylalanine	g				
	Valine	g				

2.13 SPECIFIC SAMPLE HANDLING METHOD (FREEZE-DRYING)

Drying the sample is important prior to food analysis. Freeze drying is a method of drying food by reducing the moisture content, which helps maintain important nutrients and phytochemicals at stable levels. It can also help extend the shelf-life of food and minimise food spoilage.

Principle

A freeze dryer executes a water removal process typically used to preserve perishable materials, extend shelf life, or make the material more convenient for transport. The freeze dryer works by freezing the material, then reducing the pressure and adding heat to allow the frozen water in the material to sublime. A freeze dryer functions in three phases: the Freezing Phase, the Primary Drying (Sublimation) Phase, and the Secondary Drying (Absorption) Phase.

20 – 50g of homogenous fresh samples should be set aside for moisture content determination.

Apparatus/Equipment

1. Freeze dryer machine
2. Metal tray

Procedures

1. The pressure, time, and temperature for the freeze-drying process are set based on the sample type.
2. The tray containing samples is placed on the shelves inside the chamber. A sample thickness of not more than 2 cm must be ensured to allow for optimum drying.
3. Probes are inserted into the samples to monitor the sample temperature.
4. The chamber door is closed by turning the knob clockwise. It must be ensured that the door is gripped tightly to prevent leakage during the process.).
5. The start button is pressed. The vacuum system is energised and the system is evacuated to a pre-seal value of approximately 500 Torr, 500 mbar, or 50 kPa.
6. The stop button is pressed after the drying process is completed.
7. When the system is shut down, the vacuum is automatically released to the Backfill Pressure setpoint or to atmospheric pressure.
8. When vacuum release is complete (approximately 5 minutes), the condensate is attached to the condenser host at the bottom of the condenser and placed into the basin.
9. The Defrost button is pressed to defrost the condenser.
10. When defrosting is complete, the condensate is removed and the chamber and condenser surfaces are dried using a soft cloth or tissue.

Note: The above procedure was based on Virtis Wizard 2.0 Lyophilizer, USA. Other models should follow the specific manufacturer's instructions provided to them. However, not all food is suitable for freeze-drying and needs to be frozen-dried prior to analysis. Butter, syrup, honey, jam, and spreads are examples of food types not suitable for freeze-drying.

Table 2.13: Conditions of drying using the freeze dry method

THERMAL TREATMENT STEPS				
Step	Temperature (°C)	Time (min)	Ramp/Hold	
1	-40	360	Hold	
FREEZE, CONDENSER, VACUUM PHASES				
Freeze		-40 °C		
Additional Time		60 minutes		
Condenser		-55 °C		
Vacuum		0500		
DRYING CYCLE STEPS: SAMPLE NON-GRAVY				
Step	Temperature (°C)	Time (min)	Ramp/Hold	Vacuum
1	-20	60	Hold	0400
2	-10	120	Hold	0300
3	+00	240	Hold	0100
4	+10	360	Hold	0100
5	+20	360	Hold	0100
DRYING CYCLE STEPS: SAMPLE GRAVY				
Step	Temperature (°C)	Time (min)	Ramp/Hold	Vacuum
1	-20	60	Hold	0400
2	-10	120	Hold	0300
3	+00	240	Hold	0100
4	+10	360	Hold	0100
5	+20	720	Hold	0100
DRYING CYCLE STEPS: SAMPLE HIGH SUGAR CONTENT				
Step	Temperature (°C)	Time (min)	Ramp/Hold	Vacuum
1	-20	60	Hold	0400
2	-10	120	Hold	0300
3	+00	240	Hold	0100
4	+10	360	Hold	0100
5	+20	480	Hold	0100
SECONDARY DRYING				
Secondary Set Point		+25°C		
POST HEAT SETTINGS				
Temperature		+25°C		
Time		60 minutes		
Vacuum		0100		

References

- 1) Greenfield H, Southgate DAT. Food Composition Data: Production, Management, and Use. Food and Agriculture Organization of the United Nations; 2003.
- 2) Trainer D, Pehrsson PR, Haytowitz DB, et al. Development of sample handling procedures for foods under USDA's National Food and Nutrient Analysis Program. Journal of Food Composition and Analysis. 2010;23(8):843-851. doi:10.1016/j.jfca.2010.03.020
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PART III
LABORATORY METHODS
OF ANALYSIS

3.1 INTRODUCTION

The composite laboratory method (in which multiple laboratories contribute data, following standardized protocols) is used to update the Malaysian Food Composition Database, ensuring the standardization and reliability of the data.

Reliable data on the nutrient composition of foods should only be obtained by accurate analytical methods in the hands of trained analysts. Choosing the appropriate method under a quality assurance scheme is crucial in ensuring the quality of the values in a food composition database. Several alternative analytical methods should be available for many nutrients to obtain comparable results suitable for a given analysis and differences in food matrices.

The composition of laboratory methods is divided into proximate minerals, fat-soluble vitamins, water-soluble vitamins, amino acids, fatty acids, and cholesterol analyses. Several alternative methods may be selected by considering the matrix of the food samples and the institution's laboratory capacity involved in a project.

3.2 SUMMARY OF METHOD

The tables below summarise the methods used for the nutritional analysis of food for the Malaysian Food Composition Database.

Table 3.2.1: Proximate

No.	Type of nutrient	Method of analysis	Method Reference	Nature of sample
1.	Temperature	Air oven (convection)	AOAC, Method 925.10, 2019	Most samples
		Vacuum oven	AOAC, Method 927.05; 924.45; 925.09; 926.08; 935.29; 950.46; 969.38(A), 2019	Food samples containing high proteins, sugars, and fats
		Infra-red	Bradley, 2017 Modified method by UKM	Cereal and flour-based products
		Refractometric Method	AOAC, Method 969.38 (B), 2019 CAC, Method CXS 12-1981, 2022	Honey
2.	Protein	Kjeldahl	AOAC, Method 920.152; 930.29; 950.48; 979.09; 991.22, 2019 Doc. No. J04-004 Modified method by NPHL	Refer to Table 3.3.1.2. A1
		DUMAS	AOAC, Method 992.23, 2019	All samples
3.	Fat	Soxhlet	AOAC, Method 991.36, 2019	Wet and dry samples
4.	Available carbohydrate	By difference (calculation)	Doc. No. J04-013 Modified method by NPHL	All samples

No.	Type of nutrient	Method of analysis	Method Reference	Nature of sample
5.	Total Ash	Dry ashing	AOAC, Method 900.02B; 920.1; 920.181; 923.03; 930.30; 935.42; 938.08; 940.26; 941.12; 945.46; 945.48E; 950.49B; 972.15, 2019 Doc. No. J04-003 Modified method by NPHL	All samples
6.	Total dietary fibre (TDF)	Enzymatic gravimetric method	AOAC Method 991.43, 2019 AOAC Method 985.29, 2019	All Total, soluble, and insoluble fibre content
7.	Total sugar	High-Performance Liquid Chromatography (HPLC) with Refractive Index (RI) detector	AOAC Method 980.13, 2019	Most samples. Sucrose, fructose, maltose, glucose, and lactose
8.	Energy	Calculation	Doc. No. J04-013 Modified method by NPHL	All samples

Table 3.2.2: Minerals

No.	Type of nutrient	Method of analysis	Method Reference	Nature of sample
1.	Digestion based on the food matrix	Dry Ashing	AOAC Method 991.11, 2019	Most samples
		Wet Digestion	AOAC Method 981.15D, 2019	Most samples
		Microwave Digestion	AOAC Method 2011.14, 2013.06, 2019	All samples
2.	Calcium (Ca) Ferum (Fe) Sodium (Na) Potassium (K) Magnesium (Mg) Copper (Cu) Zinc (Zn)	Atomic Absorption Spectrometer – Flame	AOAC Method 999.10, 999.11, 2019	All samples
		Inductively Coupled Plasma Mass Spectrometry (ICP-MS)		All samples
		Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES) Method	AOAC, Method 984.27, 2011.14, 2019	All samples
3.	Selenium (Se) Manganese (Mn)	ICP-MS	AOAC, Method 2015.06, 2019	All samples
4.	Iodine (I)	ICP-MS	AOAC, Method 2012.14, 2019	All samples
5.	Phosphorus (P)	Spectrophotometry	Tee et al., 1997	All samples

Table 3.2.3: Fat-Soluble Vitamins

No.	Type of nutrient	Method of analysis	Method Reference	Nature of sample
1.	Vitamin A (Retinol) Carotenoids	HPLC Method	AOAC, Method 992.04; 992.06; 2001.13; 2002.06; 2012.09; 2012.10, 2019	Most samples
2.	Vitamin D	HPLC Method	P. Moreno and V. Salvadó, 2000 A. Jedlička et al., 2005 J.B Christopher et al., 2007 Roman.K et al., 2013 Application Note 251. Sunnyvale, CA LPN 253	Solid and Liquid
		HPLC Method	AOAC, Method 995.05, 2019	Infant Formulas and Enteral Products
		HPLC Method	Jasinghe, V.J. and Perera, C.O. 2005. Food Chemistry, 92:541-546	All samples
3.	Vitamin E	HPLC Method	Journal AOAC, International 85(2), 2002 In-house based on JKM F 1203	All samples
		HPLC Method	Nielsen M.M and Hansen A., 2008 Cunha S.C et al., 2006	All samples
		HPLC Method	A.O.C.S Ce 8-89. 2017 A.O.C.S Ce 8-89. 1990.	Vegetable Oils and Fats
4.	Vitamin K	ICP-MS	AOAC 999.15, 2019	Milk and infant formulas

Table 3.2.4: Water-Soluble Vitamins

No.	Type of nutrient	Method of analysis	Method Reference	Nature of sample
	Thiamin (B1) Riboflavin (B2) Niacin (B3) Pantothenic acids (B5) Pyridoxine (B6) Biotin (B7) Cobalamin (B12) Ascorbic Acid (Vitamin C)	HPLC Method	O. Heudi et el., 2005 B. Klejdus et el., 2004 Application Notes 251	All Samples (Solid and liquid)
	Thiamin (B1)	HPLC Method	AOAC, Methods 974.29, 941.15, 957.17, 961.14, 1995 Kumar, S. & Aalbersberg, B., 2006	All samples
	Riboflavin (B2)	HPLC Method	Kumar, S. & Aalbersberg, B., 2006	All samples
	Niacin (B3)	HPLC Method	Juraja et al., 2003	All samples
	Pantothenic acids (B5)	HPLC Method	Pakin et al., 2004	All samples

No.	Type of nutrient	Method of analysis	Method Reference	Nature of sample
	Pyridoxine (B6)	HPLC Method	Kall, M.A., 2003	All samples
	Biotin (B7)	Microbiological Method	Murakami et al., 2008	All samples
	Cobalamin (B12)	Ultra Performance Liquid Chromatography Mass Spectrometry (UPLC-MS) Method	Luo et al., 2006	All samples
	Choline	Enzymatic Colorimetric Method	AOAC 999.14	Infant formula and milk
		UPLC-MS Method	Koc et al., 2004 & Zeisel et al., 2003	All samples
	Ascorbic Acid (Vitamin C)	HPLC Method	Phillips et al., 1992	All samples

Table 3.2.5: Amino Acids

No.	Type of nutrient	Method of analysis	Method Reference	Nature of sample
1.	Amino Acids	HPLC Method	AOAC 2019, Otter, D. E. 2012. British Journal of Nutrition, 108(S2), S230-S237. Puwastien, P. et al 2011. Asean manual of food analysis., 1-190.	All samples

Table 3.2.6: Fatty Acids

No.	Type of nutrient	Method of analysis	Method Reference	Nature of sample
1.	Fatty Acids	Gas Chromatography (GC) Method in food	AOAC Method 996.06, 2019	All samples

Table 3.2.7: Cholesterol

No.	Type of nutrient	Method of analysis	Method Reference	Nature of sample
1.	Cholesterol	Direct Saponification-Gas Chromatographic Method	AOAC Method 994.10, 2019 Cortés-Herrera et al, 2018. Foods 8(1), 1.	All samples
		HPLC Method	Albuquerque et al, . 2016 . Food Chemistry, 193, 18–25. Kolarič, L, 2020, Foods, 9(10). Ndhlala, A. R., 2023. Foods 12 (24).	All samples

3.3 DETAILS ON METHOD OF ANALYSIS

The details on the nutrient analysis method should include the analysis principles, the chemicals and reagents, apparatus and instruments to be used, procedures and calculations and references on the method. Users may use chemicals (i.e., solutions, buffers, eluent, diluent, standard, etc.) that come with the instruments. Each manufacturer may have their line of chemicals based on the suitability of their instruments while adhering to the internationally recognized standard reference. These chemicals are used to identify unknown substances, quantify the analytes, or calibrate the accuracy and precision of analytical measurements.

3.3.1 PROXIMATE

3.3.1.1 DETERMINATION OF MOISTURE CONTENT

Method A: Air Oven Drying

Principles

In the oven drying method, the amount of moisture in foods is the difference between their weight before and after drying. It is a simple and standard method for many kinds of food samples. The drying process is caused by the difference in the relative humidity between food and the atmosphere, and the higher the temperature, the faster the drying.

Apparatus/Instruments

1. Balance: Analytical balance with sensitivity ± 0.1 mg
2. Desiccator: Contains some moisture-absorbent material such as silica gel, calcium chloride, concentrated sulphuric acid, etc.)
3. Nickel dish with lid: Filled in with pre-dried sand and a glass rod
4. Oven: Set at 105°C
5. Tongs

Procedures

1. The experiment is performed in duplicates.
2. The dish containing the sand, glass rod, and lid is dried in an oven at 105°C overnight, then transferred to a desiccator to cool (approximately 30 minutes).
3. The dish (containing the sand and glass rod) and the lid are weighed.
4. About 5 g of the sample is weighed into the dish. The sample is spread using the glass rod, which is then placed in the dish.
5. The lid is replaced, and the dish's contents are weighed (W1).
6. With its lid slipped to one side, the dish is placed in the oven and dried for 16 hours or overnight at 105°C.
7. After drying, the dish and lid are transferred to the desiccator using a pair of tongs to cool (approximately 45 minutes). The dish, lid, and dried contents are then reweighed.
8. The dish, with its lid partially covered, is returned to the oven and dried for 1 hour. It is then transferred to the desiccator to cool and weighed again. If the weight obtained in this step is less than that obtained in Step 7, the sample is not sufficiently dried. In this case, the step is repeated until a constant weight is obtained (W2).

Calculation

$$\text{Moisture (\%)} = \frac{W1-W2}{W1} \times 100$$

Where:

W1 = weight (g) of sample before drying.

W2 = weight (g) of sample after drying

Reference

Official Methods of Analysis of AOAC INTERNATIONAL (2019) 21st Ed., AOAC INTERNATIONAL, Method 925.10

Method B: Vacuum Oven Drying

Principles

Food products that are unstable and decompose at high temperatures need to be dried at lower temperatures, such as 40 – 70°C under vacuum. Samples are dried to constant weight under a constant or reduced pressure at a prescribed temperature for a prescribed time. The moisture is the disparity between the weight measured before and after drying.

Chemicals/Reagents

1. Silica sand, 3-5 mm or Celite 545

Apparatus/Equipment

1. Vacuum Oven: Thermostatically controlled and connected through a drying train to a vacuum pump capable of maintaining the pressure in the oven below 25 mm of mercury. The oven should have an air inlet connected to a silica gel drying bottle and a trap for releasing the vacuum.
2. Convection Oven
3. Dishes: Glass or aluminium with covers
4. Desiccators: Contain silica gel as a drying agent

Procedures

1. The drying condition is decided based on the type of sample—whether a convection or vacuum oven is required, the temperature and time for drying, and a suitable amount to be weighed (refer to Table 3.3.1.1.B).
2. Dry samples with less than 20% moisture content are placed in an aluminium dish, while wet samples are placed in a glass dish with sea/silica sand or Celite 545 and a glass rod.
3. An aluminium dish with a cover, a glass dish with silica sand or Celite 545, and a glass rod are dried in a convection oven at 105°C for more than 4 hours until a constant weight is achieved.
4. The dried aluminium or glass dish is transferred into a desiccator with the lid partially opened for 20 minutes. The desiccator is closed completely and allowed to cool for at least 40 minutes.
5. The dish is weighed using an analytical balance to four decimal places. The weight is recorded as W1 in the worksheet.
6. A suitable amount of homogenised sample is added to the dish, and the final weight is recorded as W2.
7. The dish is transferred to the oven, and the sample is dried according to the temperature and time prescribed in Table 3.8.
8. For samples in a glass dish, the sample is thoroughly mixed with silica sand or Celite 545 using a glass rod. The sample is then placed in a steam water bath at a temperature not exceeding the prescribed drying condition (refer to Table 3.8).
9. Samples with high sugar content are mixed occasionally with a glass rod to prevent crystallisation and the trapping of moisture. The sample is then dried using the conditions prescribed in Table 3.8.
10. The operation of the oven is carried out according to the instrument operation manual. For vacuum ovens, refer to the Equipment Operation Procedure for Vacuum Ovens.
11. Once drying is completed, the samples are removed, and the lid is closed for the aluminium dish.
12. The dish is transferred into a desiccator with the lid partially opened for 20 minutes. The desiccator is then closed completely and cooled for at least 40 minutes.
13. The final weight is recorded as W3.

Calculation

$$\text{Moisture (g/100 g)} = \frac{W2-W3}{W2-W1} \times 100$$

Where:

W1 = Weight of dried dish

W2 = Weight of dried dish with sample

W3 = Weight of dried dish and dried sample

Table 3.3.1.1.B: Parameters for Drying the Samples

Food Matrix	Sample Weight (g)	Temperature, (°C)	Time (hour)	Type of Dish
Cereals	2	100	5	Al can
Grains flour	3-5	135	1	Al can
Bread	2-3	135	1	Al can
Bread with other ingredients, such as jam, cream, etc.	2-3	V 70	5	Glass dish*
Noodle	2-3	135	3	Glass dish*
Noodle with seasonings ingredients	2-3	105	5	Al can
Potatoes	2-3	100	5	Al can
Starch	2-3	135	1	Al can
Sugar powder	5	V 105	3	Al can
Sugar liquid	1.5	V 100	3	Glass dish*
Honey	1-1.5	V 90	3	Glass dish*
Cake, Pie	2-3	V 70	5	Glass dish*
Cookies, crackers, biscuits	3-5	100	5	Al can
Candy	1.5	V 100	2	Glass dish*
Chocolates	2-3	V 70	5	Glass dish*
Snack	3-5	105	3	Al can
Nuts	2	130	2	Al can
Beans	5	130	3	Al can
Boiled beans	2	V 100	5	Glass dish*
Soybean	5	130	2	Al can
Defatted soybean	2	130	1	Al can
Fish and its processed products	5-10	105	5	Glass dish*
Meat and its processed products	2-3	100	5	Glass dish*
Egg	2-3	V 100	5	Glass dish*
Milk liquid	2-3	100	3	Glass dish*
Milk powder, Condensed milk	2	100	4	Al can
Cream, Ice cream	2-3	100	3	Glass dish*
Lactic beverages, yogurt	2	V 100	4	Glass dish*
Cheese	3	100	4	Al can
Vegetables and fruits, its processed products	3-4	V 70	5	Glass dish*
Mushrooms, Seaweed	5	105	5	Al can
Coffee beans, Coffee powder	2-5	105	5	Al can
Salt	3	140	1.	Al can
Seasoning	3-5	V 70	5	Al can
Soy sauce, other sauces	5	V 70	5	Glass dish*
Processed foods	If the drying conditions differ between ingredients, choose the lower temperature and/or vacuum condition. For example, canned fish in tomato paste, oil, and seasoning shall use vegetable product conditions or seasoning drying conditions, NOT fish product drying conditions.			

*Use the sea sand method

V: Vacuum condition

Al: Aluminium

Reference

Official Methods of Analysis of AOAC INTERNATIONAL (2019) 21st Ed., AOAC INTERNATIONAL, Method 927.05; 924.45; 925.09; 926.08; 935.29; 950.46; 969.38(A)

Method C: Infrared (Rapid Method)

Principles

Infrared drying involves the penetration of heat into the food sample being dried. It is a rapid method of moisture content analysis, as the heat penetration enables water to evaporate quickly. Although simple and fast, the infrared method is not suitable for moisture content analysis of samples that harden or burn when heated.

Apparatus/Equipment

1. Infra-red balance
2. Disposable aluminium pan liner
3. Desiccator with moisture-absorbent material such as silica gel, calcium chloride, concentrated sulphuric acid, etc.

Procedures

1. The disposable aluminium pan liner will be pre-dried at 100°C overnight and subsequently cooled in a desiccator.
2. The infrared meter is to be balanced at zero level.
3. 10 g of flour must be accurately weighed and evenly spread onto the pan liner. The powder must not be compressed.
4. The dish containing the sample is to be placed on the infrared meter dish holder, and the heater in the moisture meter is to be switched on.
5. The percentage of moisture loss shown on the display panel is to be recorded every minute until no further changes in moisture content are detected.
6. The moisture balance must be allowed to cool before the experiment is repeated.
7. A graph showing "Moisture loss (g) against time (min)" is to be plotted.
8. Results can be read directly from the balance scale display panel.

Reference

Nielsen, S. S. 1994. Introduction to the Chemical Analysis of Foods. Boston: Jones and Bartlett Publishers, Inc.
Modified Method by UKM

Method D: Determination of Moisture Content in Honey (Refractometric Method)

Honey moisture is the quality criterion that determines the capability of honey to remain stable and to resist spoilage by yeast fermentation: the higher the moisture, the higher the probability that honey will ferment upon storage. Determining moisture by refractometry does not yield the true water content and yields lower values than the Karl Fischer method. However, it is a straightforward and reproducible method, successfully used up to the present time, and thus there is no need for alternative methods. Lower moisture limits (e.g., 19%), ensuring a better shelf-life of honey, which would be met by a large majority of commercial honey, have been proposed by some countries to revise the Codex Alimentarius.

Principles

The method is based on the principle that the refractive index increases with solids content. Table 3.3.1.1.D below was constructed from a plot of the logarithm of the refractive index minus unity plotted against the water content as determined by vacuum drying. This technique requires much greater manipulative skill. Table 3.3.1.1.D is derived from a formula developed by Wedmore from the data of Chataway and others:

$$W = \frac{1.73190 - \log \log (R.I. - 1)}{0.002243}$$

W: Water content in g per 100 g honey
R.I.: Refractive index

Apparatus/Equipment

Refractometer

Procedures

1. The sample selected for analysis must accurately represent the honey lot.
2. All honey samples are prepared in the following manner before analysis.
3. A stainless-steel sieve with a mesh diameter of 0.5 mm is used for straining.
4. Liquid or crystallised honey must be free from extraneous matter.
5. The honey sample is homogenised by stirring thoroughly for at least 3 minutes. Care is taken to stir in as little air as possible, mainly if the sample is used to determine hydroxymethylfurfural.
6. If the honey is crystallised in a hard and compact mass, it is softened by heating in a stove or thermostatic bath at a temperature not exceeding 40°C.
7. The honey's refractometer reading is determined at 20°C, and the corresponding percent moisture is obtained from Table 3.3.1.1.D.
8. According to the footnote, if the determination is carried out at a temperature other than 20°C, the reading is corrected to the standard temperature of 20°C.

Water content (g/100 g)	Refractive Index (20°C)
13.0	1.5044
13.2	1.5038
13.4	1.5033
13.6	1.5028
13.8	1.5023
14.0	1.5018
14.2	1.5012
14.4	1.5007
14.6	1.5002
14.8	1.4997
15.0	1.4992
15.2	1.4987
15.4	1.4982
15.6	1.4976
15.8	1.4971
16.0	1.4966
16.2	1.4961
16.4	1.4956
16.6	1.4951
16.8	1.4946
17.0	1.4940
17.2	1.4935
17.4	1.4930
17.6	1.4925
17.8	1.4920
18.0	1.4915
18.2	1.4910
18.4	1.4905
18.6	1.4900
18.8	1.4895

Water content (g/100 g)	Refractive Index (20°C)
19.0	1.4890
19.2	1.4885
19.4	1.4880
19.6	1.4875
19.8	1.4870
20.0	1.4865
20.2	1.4860
20.4	1.4855
20.6	1.4850
20.8	1.4845
21.0	1.4840
21.2	1.4835
21.4	1.4830
21.6	1.4825
21.8	1.4820
22.0	1.4815
22.2	1.4810
22.4	1.4805
22.6	1.4800
22.8	1.4795
23.0	1.4790
23.2	1.4785
23.4	1.4780
23.6	1.4775
23.8	1.4770
24.0	1.4765
24.2	1.4760
24.4	1.4755
24.6	1.4750
24.8	1.4745
25.0	1.4740

Footnote:

For temperatures above 20°C, add 0.00023 per °C before using the table.

For Temperatures below 20°C, subtract 0.00023 per °C before using the table.

References

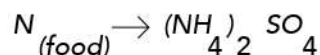
- 1) Official Methods of Analysis of AOAC INTERNATIONAL (2019) 21st Ed., AOAC INTERNATIONAL, Method 969.38 (B)
- 2) Codex Alimentarius International Food Standards, Standard for Honey, Ref. No. CXS 12-1981, Amended in 2022

3.3.1.2 DETERMINATION OF PROTEIN CONTENT

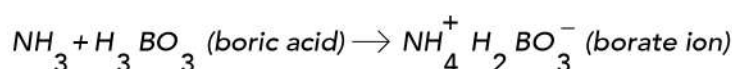
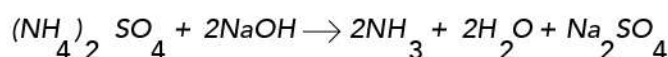
Method A: Kjeldahl Method

Principles

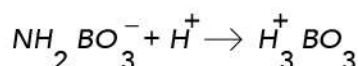
A food is digested with concentrated sulphuric acid to convert organic nitrogen to ammonium ions:



During neutralisation, alkali is added, and the liberated ammonia is distilled into an excess of boric acid solution:



The distillate is titrated with standardised hydrochloric acid to determine the ammonia absorption in the boric acid:



The amount of protein present is then calculated from the nitrogen concentration of the food.

Chemicals/Reagents

1. Catalyst tablet. (Copper sulfate: potassium sulfate = 1:9)
2. Concentrated sulphuric acid
3. Hydrogen peroxide (chilled)
4. Hydrochloric acid 0.2N:
Measure 200 mL of 1N HCl in a 1 L volumetric flask and mark up to 1 L with distilled water.
5. Methyl Red Indicator:
 - i. Weigh 100 mg of methyl red (MR) in a 10 mL volumetric flask.
 - ii. Dilute with 100 mL of methanol.
6. Bromocresol green indicator:
 - i. Weigh 100 mg of bromocresol green (BCG) in a 100 mL volumetric flask.
 - ii. Dilute with 100 mL of methanol.
7. Sodium hydroxide (NaOH), 40%:
Weigh 400 g of NaOH and dissolve in 1 L of distilled water.
8. 1% boric acid:
Weigh 10 g of boric acid and dissolve it in 1 L of distilled water.

Apparatus/Equipment

1. Automated Digestion Unit: attached with scrubber
2. Automated Protein Analyzer Machine
3. Balance: Analytical sensitivity ± 0.1 mg
4. Digestion tube
5. Volumetric Flasks: 100 mL & 1 L
6. Conical Flasks: 250 mL
7. Measuring Cylinder: 25 mL
8. Pipette: 5 mL

Procedures

a. Standardisation of hydrochloric acid 0.2N

1. Approximately 10 g of anhydrous sodium carbonate (Na_2CO_3) is weighed and dried for 1 hour at 265°C or 2 hours at 200°C. The dried sodium carbonate is transferred into a beaker with a tight lid and stored in a desiccator.
2. Approximately 0.4 g of the dried anhydrous sodium carbonate is weighed, and the weight is recorded.
3. The sodium carbonate is transferred into a receiver flask, and 40 mL of distilled water is added. Six drops of each indicator solution are added to the mixture.
4. The solution is titrated until it changes to pink, and the volume used (in mL) is noted. The solution is then boiled for a few minutes, during which it turns green. The solution is cooled to room temperature under running water.

b. Protein analysis

1. The nearest prepared sample is weighed according to its form: 0.5–2 g for dry, 3–5 g for wet or liquid on grease-proof paper (for dry/semi-solid samples). The paper is twisted and placed into a digestion tube. Liquid samples are pipetted directly into the digestion tube. (Refer to Table 3.10 for sample size guidelines.) Each sample is analysed in duplicate.
2. Tube positions No. 1 to 4 on the rack are assigned for blank samples. Positions No. 5 to 8 are designated for sample checks. Sample tubes are placed from position No. 9 onwards.
3. Two catalyst tablets are added to all tubes.
4. All sample tubes except the blank receive 15 mL of concentrated sulphuric acid, which is gently mixed by swirling.
5. 5 mL of chilled hydrogen peroxide is slowly added to each sample tube to react with the sample and acid. An additional 5 mL of hydrogen peroxide is added until the mixture turns blue, green, or slightly brown. Hydrogen peroxide must not be added to the blank sample tube.
6. The rack is placed in the digestion unit, and the samples are digested at 420°C for 1½ hours. (For operational details, refer to the Equipment Operation Procedure and Maintenance—Automated Protein Analyzer.)
7. If traces of carbon remain, the tube is allowed to cool, 3–5 mL of concentrated sulphuric acid is added, and digestion is continued.
8. Once cooled, the tube rack is transferred into the automated protein analyser machine.
9. Parameters such as sample weight, conversion factor, blank, and washing steps are set according to the Equipment Operation Procedure.
10. The machine is operated, and the results are printed.

Table 3.3.1.2.A1: Guidelines for Sample Size

Expected Protein Content (g/100 g, %)	Food Sample (example)	Sample Size (g)
80-90	Soya bean extracted powder	0.3
60	Fish powder, chlorella	0.3
30-50	Bone powder	0.4 - 0.5
10-30	Mixture of livestock feed	0.8
2-10	Others	1 - 2
1	Solid	2 - 4
	Liquid	5 - 10
Sugar, oils, and fats		1 - 4

Calculation

Nitrogen content:
$$\%N = \frac{x \div 1000 \times (V_s - V_b)}{SX14 \times 100}$$

Where x = Normality of acid

V_s and V_b = Titration volumes of the sample and blank (mL)

S = Sample weight (g)

14 = Molecular weight of nitrogen N

% Protein:
$$\%Protein = \%NXF$$

Where F = Conversion factor (referred to Table 3.11)

All the data and calculations are recorded on a worksheet.

Table 3.3.1.2.A2: Conversion factor for protein

Food Commodity	Type	N Factor
Cereals Products	Wheat, hard, medium, or soft; wholemeal or flour or bulgur	5.83
	Flour, medium or low extraction	5.70
	Macaroni, spaghetti, wheat paste	5.70
	Bran	6.31
	Rice	5.95
	Rye, barley, oats	5.83
Pulses, nuts, and seeds	Groundnuts	5.46
	Soya bean, seeds, flour, or products	6.25
Tree nuts	Almond	5.18
	Brazil nut	5.71
	Coconuts, chestnuts, tree nuts	5.30
Seeds	Sesame	5.30
	Safflower	5.30
	Sunflower	5.30
Milk and milk products	Regulation No. 82- No. 117*	6.38
Edible fats and edible oils	Regulation No. 179- No. 208*	6.25
Other foods	Other foods	6.25

*Food Act 1983 (Act 281) & Regulations 18B (8)

Reference

- 1) Official Methods of Analysis of AOAC INTERNATIONAL (2019) 21st Ed., AOAC INTERNATIONAL, Method 920.152; 930.29; 950.48; 979.09; 991.22
- 2) Doc. No. J04-004 Modified method by NPHL

Method B: Dumas Method

Principles

A sample of known mass is combusted in a high-temperature (about 950°C) chamber in the presence of oxygen for very rapid and complete combustion. This will lead to the release of carbon dioxide (CO₂), water (H₂O) and nitrogen (N₂). The CO₂ and H₂O are removed by passing the gases over a special column with a thermal conductivity detector at the end. The column helps separate the nitrogen from residual CO₂ and H₂O that may have remained in the gas stream.

The instrument is calibrated by analysing a pure material with a known nitrogen concentration, such as glycine. Thus, the thermal conductivity detector signal can be converted into nitrogen content. As the Kjeldahl method is required to convert the concentration of nitrogen content in a sample to the protein content, suitable conversion factors that depend on the precise amino sequence of the protein are necessary.

Reagents and Materials

Use only reagents of recognized analytical grade or reagents of equivalent purity:

1. Carrier gas, helium (He) as pure 99.99%
2. Oxygen (O₂) is as pure possible 99.90%
3. Pneumatic Gas-Compressed Air (oil and water free)
4. Magnesium perchlorite (Mg (ClO₄)₂), 10-16 mesh particle size saturated with carbon dioxide. Material in the reagent tube packing for moisture absorption
5. Sodium hydroxide (NaOH) on Silica Lecosorb (used for Absorption of CO₂ during combustion analysis of inorganic & organic material), size 20-30 mesh, a material in reagent tube packing
6. Glass 2 is used in the filter furnace packing to remove particulate and the reagent tube packing with the finest size.
7. Steel wool size as a material in the filter furnace packing for removing corrosive salts, scrubbing chlorine (Cl₂)
8. Particle filter to remove salt that condenses out of the flow stream
9. Cooper (stick and turning) for the reduction Heater Tube - removes excess oxygen and reduces NO_x to N₂
10. N - catalyst, grade for Carbon Hydrogen Nitrogen (CHN) Series Elemental analysers, operating temperature 500°C - 600°C (Filing material for the Reduction Heater Tube). Catalyst will absorb moisture, reduce tightly bound NO_x to N₂
11. Heated Quartz Particulate Filter size 3.2 cm
12. Quartz wool strips, filling material for combustion tube packing
13. Furnace reagent: The furnace reagent should be dry at 105°C for 2 hours before packing the combustion tube
14. Magnesium oxide as a furnace reagent for the combustion tube
15. Alumina oxide pellets as a filling material for the combustion tube

Apparatus

1. Tin capsule
2. Sample cup folder
3. Capsule storage block
4. Micropipette
5. Analytical Balance
6. Forceps
7. Beaker

Dumas apparatus

Nitrogen Analyzer consists of:

1. Autosampler or manual injector
2. Carrier gas: compressed air, O₂, and He
3. U Tube furnace
4. Thermoelectric cooler
5. Lecosorb CO₂ and anhydrous (H₂O)
6. Aliquot Solenoid
7. Thermal conductivity cell
8. Microprocessor

Preparation of Standard Solutions

1. Glycine or other suitable reference materials with a known constant certified nitrogen content.
2. Preparation of Glycine standards:
The glycine standard is prepared by appropriately weighing glycine powder in a 100 mL flask and adding distilled water until the total mass equals 100 g (where 100 g = 100 mL). The typical weight of glycine powder needed is as Table 3.12:

Table 3.3.1.2.B: Glycine Powder Needed

ID	Nitrogen Concentration, % (g/100 g)	Weight of Glycine Powder, (g)
Std 1	0.10	0.541
Std 2	0.30	1.624
Std 3	0.50	2.707
Std 4	0.75	4.060
Std 5	1.00	5.414

The following formula can be used to make a specific concentration:

$$\frac{C}{G} = PXN$$

Where:

C = desired nitrogen concentration as a percent (%)

G = grams of glycine powder

P = purity of glycine powder (e.g., 0.99 if glycine powder is 99.0%)

N = nitrogen content of glycine powder (e.g., 0.18658 if the nitrogen of glycine powder is 18.658%)

Example of 1% solution:

$$\frac{1\%}{G} = 0.99 \times 0.18658$$

$$G = 5.414 \text{ g}$$

Sample Preparation

Liquid and Semisolid Sample (Tin Capsule Method)

1. The sample cup holder is placed on the balance.
2. A tin capsule (small or medium) is placed into the sample cup holder, and the balance is tared.
3. Between 0.2 – 0.3 g of sample is weighed into the tin capsule.
4. The capsule is removed from the sample cup holder and sealed using forceps.
5. The mass is recorded in the spreadsheet.

Procedure

1. The instrument is prepared by following the procedure outlined in the operator's instruction manual (e.g., gas supplies are checked, any required maintenance is performed, and checks for leaks are conducted).
2. Five blanks (gas) are analysed until a plateau is reached. An additional 3–5 blanks are analysed, and the blank is set using these data.
3. Between 2–3 standards, each weighing 0.2–0.3 g of 0.5% glycine standard and retained sample, are logged to verify the instrument response.
4. The sample is placed into a sample carousel/autoloader and analysed.

Quality Control and Assurance

1. Retained sample for which the actual value has been previously determined for every daily batch, as for the Quality Control Sample.
2. Results within ± 3 standard deviations of the mean value are acceptable. Those outside this range will be repeated and corrective action will be taken.
3. For cases not meeting the regulations, analysis will be performed in duplicate during the first or repeat runs.

Calculation

1. Nitrogen Content:
Instrument printouts usually provide results for the total nitrogen content (TN) expressed in g per 100 g.
2. Protein content:
The sample expressed in g per 100 g is obtained by using the following equation:

$$\text{Protein content (\%w/w)} = \text{Nitrogen content (\%w/w)} \times \text{Protein factor}$$

Reference

Official Methods of Analysis of AOAC INTERNATIONAL (2019) 21st Ed., AOAC INTERNATIONAL, Method 992.23

3.3.1.3 DETERMINATION OF FAT CONTENT

Pre-Preparation of Samples (Hydrolysis) Before Fat Analysis

Pre-preparation is essential, particularly for high-carbohydrate foods. It is recommended for all types of samples.

Procedure

1. Samples are accurately weighed in a conical flask or other equivalent glassware, as recommended in Table 3.13.
2. The sample is moistened with 10 mL of deionised water.
3. Then, 10 mL of 4 N HCl is dispensed.
4. The mixture is immersed in a water bath at 80°C for 30 minutes.
5. The digestate is cooled to room temperature and filtered.
6. The filter paper with the residue is transferred to the thimble (with 2 g of Celite placed at the bottom of the thimble), and the thimble is attached to the thimble holder.
7. The thimble holder is dried in the oven at 100°C to 103°C for 3 hours.
8. It is then cooled in a desiccator and prepared for auto Soxhlet extraction.

Table 3.3.1.3: Recommended Sample Weight for Auto Soxhlet Extraction

Fat Content (%)	Sample weight as dry weight (g)
0 – 10	2 to 3
11 – 25	1 to 2
> 25	0.5 to 1

Method A: Determination of Fat Content in Dry Foods

Principles

The Soxhlet method is a semi-continuous solvent extraction method. A ground sample will be weighed in a porous thimble and placed in an extraction chamber suspended above a flask/container containing solvent. The flask is heated, the solvent is evaporated and converted into liquid at the condenser and passes through the sample, extracting the lipids and channelling them into the flask. After several hours of extraction, the flask containing the solvent is removed and evaporated. The Soxhlet method is also available in automated machines for a simpler and faster extraction technique, such as the brand FOSS Soxtec machine. Food is digested with concentrated sulphuric acid to convert organic nitrogen to ammonium ions.

Chemicals/Reagents

1. Petroleum ether
2. Diethyl ether
3. Hexane

Apparatus/Equipment

1. Automated Fat Extraction System
2. Air oven
3. Balance: Analytical sensitivity ± 0.1 mg
4. Desiccator
5. Ashless Filter paper
6. Aluminium cup/ glass cup
7. Thimble
8. Facial Cotton

Procedure

1. An aluminium or glass cup is prepared by drying it in a hot air oven at 105°C for one hour, cooling it in a desiccator, and weighing it as W1.
2. A dry sample weighing 1.0 ~ 2.0 g (S) is weighed on folded filter paper.
3. The filter paper is transferred to the thimble, and cotton is placed as a lid.
4. The extraction procedure is started (refer to the Equipment Operation Procedure for operating the Automated Fat Extraction System).
5. After the program is completed, the aluminium/glass cup is removed from the machine, dried at 105°C for 1 hour, cooled in a desiccator for 1 hour, and weighed as W2.
6. The calculation is carried out as shown below:

$$\text{Fat, g/100 g} = \frac{(W2 - W1)}{S} \times 100$$

Where W1 = Weight of dried aluminium/glass cup

W2 = Weight of dried aluminium/glass cup after extraction

S = Weight of sample

Reference

Official Methods of Analysis of AOAC INTERNATIONAL (2019) 21st Ed., AOAC INTERNATIONAL, Method 991.36

Method B: Determination of Fat Content in Wet Foods

Principles

The Soxhlet method is a semi-continuous solvent extraction method. A ground sample is weighed in a porous thimble placed in an extraction chamber suspended above a flask/container containing solvent. The flask is heated, and the solvent is evaporated and converted into a liquid at the condenser, which passes through the sample. Thus, the lipids are extracted and channelled into the flask. After several hours of extraction, the flask containing the solvent is removed and evaporated. The Soxhlet method is also available in automated machines for a simpler and less time-consuming extraction technique, such as an automated fat extraction machine. The lipid content is calculated as follows:

$$\% \text{ lipid} = \frac{M \text{ Lipid}}{M \text{ Sample}} \times 100$$

Chemicals/Reagents

1. Petroleum ether
2. Diethyl ether
3. Hexane
4. Sodium Sulfate (Na₂SO₄) anhydrous
5. Celite 545

Apparatus/Equipment

1. Automated Fat Extraction Machine (Soxhlet Method)
2. Water bath
3. Air oven
4. Balance: Analytical sensitivity $\pm 0.1\text{mg}$
5. Desiccator
6. Mortar and pestle
7. Ashless Filter paper
8. Aluminium/glass cup
9. Thimble
10. Facial cotton
11. Glass beaker: 50 mL
12. Glass rod

Procedure

1. An aluminium or glass cup is prepared by drying it in a hot air oven at 105°C for one hour, cooling it in a desiccator, and weighing it as W1.
2. A 1.0 ~ 2.0 g sample (S) is weighed into a 50 mL beaker.
3. Two to three spatulas of celite and a glass rod are added, and the mixture is dried in a water bath for over 2 hours.
4. The sample mixture is transferred qualitatively from the beaker into a clean mortar. Then, 2–3 spatulas of Na₂SO₄ are added and homogenised using a pestle.
5. The mixture is transferred into a thimble.
6. The mortar and beaker are wiped with pieces of cotton dampened with diethyl ether, and the cotton is transferred into the same thimble.
7. The thimble adapter is set with aluminium/glass cups and thimbles and placed into the automated fat extraction machine.
8. The extraction procedure is initiated (refer to the Equipment Operation Procedure for instructions on operating the Automated Fat Extraction machine).
9. After completion, the aluminium/glass cup is removed from the machine, dried at 105°C for 1 hour, cooled in a desiccator for 1 hour, and weighed as W2.

$$\text{Fat, g/100 g} = \frac{(W2 - W1)}{S} \times 100$$

Where W1 = Weight of dried aluminium/glass cup
W2 = Weight of dried aluminium/glass cup after extraction
S = Weight of sample

Reference

Official Methods of Analysis of AOAC INTERNATIONAL (2019) 21st Ed., AOAC INTERNATIONAL, Method 991.36

Method C: Determination of Fat Content in Milk

Principles

Fat is extracted with a mixture of ethers from the known weight of milk. Ether extract is decanted into a pre-weighed dry weighing dish, and ether is evaporated. The extracted fat is dried to a constant weight. The result is expressed as % fat by weight.

Chemicals/

1. Ethyl ether
2. Petroleum ether
3. Ammonium hydroxide (NH₄OH)
4. Ethyl alcohol
5. Distilled water
6. Phenolphthalein indicator

Apparatus/Equipment

1. Automated Fat Extraction System
2. Air oven
3. Balance: Analytical sensitivity ± 0.1 mg
4. Desiccator
5. Ashless Filter paper
6. Aluminium cup/ glass cup
7. Thimble
8. Facial Cotton

Procedure

1. Milk is tempered to 38°C. An empty flask with a clean, dry cork stopper is weighed. The stopper is removed, and approximately 10 g of milk is pipetted into the flask. The stopper is placed into another flask, and the flask containing milk is weighed to the nearest 0.1 mg. It is ensured that the balance reads zero between test portions.
2. 1.5 mL of NH₄OH is added to the flask and mixed thoroughly to neutralize any acid and dissolve casein. Three drops of phenolphthalein indicator are added to enhance the interface's visibility between the ether and aqueous phases. 10 mL of 95% alcohol is added, the flask is stoppered with a water-soaked cork, and shaken for 15 seconds..

First Extraction:

3. 25 mL of ethyl ether is added, the flask is stoppered, and shaken vigorously for 1 minute, releasing built-up pressure as needed by loosening the stopper. 25 mL of petroleum ether is added, the flask is restoppered, and shaken vigorously for another 1 minute. The flask is centrifuged at 600 rpm for 30 seconds to obtain a clear separation of the aqueous (bright pink) and ether phases. Decant ether solution is added to a suitable weighing dish.
4. After drying, the weighing dishes are placed in a room-temperature desiccator and cooled to room temperature. The dishes are weighed to the nearest 0.1 mg on the same day, and the weights are recorded. The ether layer is carefully decanted into a suitable weighing dish, ensuring no suspended solids or aqueous layer is transferred. The ether is evaporated from the dish at $\leq 100^\circ\text{C}$.

Third Extraction:

5. 5 mL of 95% alcohol is added to the same flask, stoppered, and shaken vigorously for 15 seconds. 15 mL of ethyl ether is added, the flask is restoppered, and shaken for 1 minute. 15 mL of petroleum ether is added, the flask is stoppered again, and shaken for another 1 minute. The flask is centrifuged at 600 rpm for 30 seconds to separate phases. If the interface lies in the lower neck of the flask, water is added down the inner surface to raise the level to mid-neck, avoiding disturbance. The ether layer is decanted into the same weighing dish used in the first extraction.

Second Extraction:

6. The procedure used for the second extraction is repeated, omitting the 95% alcohol step. Solvents are completely evaporated in a fume hood using a hot plate at $\leq 100^{\circ}\text{C}$, avoiding spattering.

Final Drying and Weighing:

7. The extracted fat in the weighing dish is dried to constant weight either in a forced air oven at $100^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for ≥ 30 minutes, or in a vacuum oven at $70^{\circ}\text{--}75^{\circ}\text{C}$ at 50.8 cm (20 in.) vacuum for ≥ 7 minutes. Weighing dishes are removed and placed in a desiccator

Reagent Blank Procedure:

8. Two reagent blanks are run daily by replacing the milk with 10 mL of H_2O and repeating the procedure. The weight of any dry residue from the blank is recorded and used in calculations. If the residue is < 0.0020 g, the blank is considered acceptable. If a negative blank occurs, the value is included in calculations as a negative number (i.e., added). (Note: To subtract the negative number [average weight blank residue] in the equation below, add it to [(weight dish + fat) – weight dish].) A negative blank indicates that dishes were not completely dry at the start of determination or that balance calibration shifted between weighing empty pans and pans plus fat. The cause of negative blanks should be identified and corrected.

$$\text{Fat, \%} = 100X[W1 - W2] - W3$$

Where W1 = Weight of dish and fat

W2 = Weight dish

W3 = Average weight of the blank residue weight test portion

Footnote:

The maximum recommended difference between duplicates is $< 0.03\%$ fat.

At 3.6% fat:

$sr = 0.015\%$; $RSDR = 0.512\%$; $sR = 0.020\%$; $r \text{ value} = 0.044\%$; $RSDr = 0.396\%$; $R \text{ value} = 0.056\%$

Reference

Official Methods of Analysis of AOAC INTERNATIONAL (2019) 21st Ed., AOAC INTERNATIONAL, Method 989.05

3.3.1.4 DETERMINATION OF TOTAL CARBOHYDRATE AND AVAILABLE CARBOHYDRATE IN FOOD (BY DIFFERENCE)

Calculation

The total carbohydrate content is calculated as follows: g/100g:

$$= 100 - \{(Asg) + (Moisture) + (Fat) + (Protein) + (Total Dietary Fiber)\}$$

*Available carbohydrate is only used for glycaemic index calculation (based on the sum of 5 different sugars)

Reference

Doc. No. J04-013 Modified method by NPHL

3.3.1.5 DETERMINATION OF ASH CONTENT

Principles

Dry ashing procedures use a high temperature capable of maintaining the temperature between 500 °C and 600°C. Water and other volatile materials are vaporised, and organic substances are burned in the presence of oxygen in the air into carbon monoxide (CO), H₂O, and N₂. Most minerals are converted to oxides, sulphates, phosphates, chlorides, or silicates.

Apparatus/Equipment

1. Silica crucible: Diameter (3.5 cm), depth (4 cm) for dry sample or bigger for liquid sample
2. Desiccators: Containing silica gel desiccant
3. Muffle Furnace: Thermostatically controlled at 550°C
4. Electric hotplate: Thermostatic controlled
5. Water Bath

Procedures

1. A clean silica crucible is placed into a muffle furnace at 550°C for at least 5 hours for pre-conditioning.
2. The crucible is cooled in a desiccator for 1 hour, then weighed using an analytical balance until a constant weight is obtained. The weight is recorded as W₁ (to four decimal places).
3. A sample is weighed into the constant-weighted crucible and recorded as W₂. For dry samples, weigh 3–5 g. For liquid samples, weigh 5–10 g.
4. The crucible containing the liquid sample is placed in a water bath or, for a dry sample, on a hot plate and heated until thoroughly dried.
5. During heating, charring will occur, indicated by the sample turning black and emitting smoke.
6. Heating is continued until the smoking subsides completely.
7. The crucible is placed into the muffle furnace and ashed overnight at 550°C.
8. The crucible is removed to check the condition of the ash. Ashing is complete when the residue is free from black particles and weighs the ash.
9. If black traces remain, the crucible is cooled, and a few drops of water are added. The residue is stirred with a glass rod to break up any remaining carbon. The crucible is dried on a hot plate, then returned to the muffle furnace to continue ashing. (The Duration depends on the type of sample.)
10. After ashing is completed, the crucible is cooled in a desiccator for 1 hour, then weighed immediately after reaching room temperature. The final weight is recorded as W₃.

Calculation

$$\text{Ash, g/100 g} = \left(\frac{W_2 - W_3}{W_2 - W_1} \right) \times 100$$

Where W₁ = Weight of dried crucible
W₂ = Weight of dried crucible with sample
W₃ = Weight of dried crucible and dried sample

Note: For seafood, if the material contains a large amount of fat, perform preliminary ashing at a low enough temperature to allow the fat to smoke off without burning.

Reference

Official Methods of Analysis of AOAC INTERNATIONAL (2019) 21st Ed., AOAC INTERNATIONAL, Method 900.02B; 920.1; 920.181; 923.03; 930.30; 935.42; 938.08; 940.26; 941.12; 945.46; 945.48E; 950.49B; 972.15

Doc. No. J04-003 Modified method by NPHL

3.3.1.6 DETERMINATION OF TOTAL, SOLUBLE AND INSOLUBLE DIETARY FIBRE CONTENTS IN FOODS

Principles

The analysis method applies to processed foods, grains, cereal products, fruits, and vegetables. Duplicate samples of dried foods, fat-extracted if containing >10% fat, undergo sequential enzymatic digestion by heat-stable amylase, protease, and amyloglucosidase to remove starch and protein. For total dietary fibre (TDF), the enzyme digestate is treated with alcohol to precipitate soluble dietary fibre (SDF) before filtering. TDF residue is washed with alcohol and acetone, dried, and weighed. For insoluble dietary fibre (IDF) and SDF, the enzyme digestate is filtered, and the residue (IDF) is washed with warm water, dried, and weighed. For SDF, combined filtrate and washes are precipitated with alcohol, filtered, dried, and weighed. TDF, IDF, and SDF residue values are corrected for protein, ash and blank.

Chemicals/Reagents

1. Use deionized water throughout.
2. Ethanol solutions:
 - a. 85% - Place 895 mL 95% ethanol into a 1 L volumetric flask and dilute to volume with H₂O.
 - b. 78% - Place 821 mL 95% ethanol into a 1 L volumetric flask, dilute to volume with H₂O.
3. Heat-stable α -amylase solution.
4. MES - 2-(N-Morpholino) ethanesulfonic acid.
5. TRIS - Tris (hydroxymethyl) aminomethane.
6. Protease - Prepare a 50 mg/mL enzyme solution daily in MES/TRIS buffer.
7. Amyloglucosidase solution - Store at 0-5°C.
8. Diatomaceous earth - Acid washed.
9. Cleaning solution - Liquid surfactant-type laboratory cleaner designed for critical cleaning. Prepare a 2% solution in H₂O.
10. MES/TRIS buffer solution - 0.05 M MES. 0.05 M TRIS, pH 8.2 at 24°C. Dissolve 19.52 g MES and 12.2 g TRIS in 1.7 L H₂O. Adjust pH to 8.2 with 6 N NaOH and dilute to 2 L with H₂O. (Note: It is important to adjust pH to 8.2 at 24°C. However, if the buffer temperature is 20°C, adjust pH to 8.3; if the temperature is 28°C, adjust pH to 8.1. For deviations between 20°C and 28°C, adjust by interpolation.)
11. Hydrochloric acid solution - 0.561N. Add 93.5 mL 6 N HCl to 700 mL H₂O in a 1 L volumetric flask. Dilute to 1 L with H₂O.

Apparatus/Instruments

1. Beakers - 400 or 600 mL tall-form.
2. Filtering crucible - With fritted disk, coarse. ASTM 40-60 μ m pore size, Pyrex 60 mL. Prepare as follows. Ash overnight at 525°C in a muffle furnace. Let the furnace temperature fall below 130°C before removing crucibles. Soak the crucible for 1 hour in a 2% cleaning solution at room temperature. Rinse crucibles with H₂O and then deionized H₂O. For the final rinse, use 15 mL of acetone and then air-dry. Add approximately 0.5 - 1.0 g Celite to dry crucibles, and dry at 130°C to constant weight. Cool the crucible for approximately 1 hour in the desiccator, and record the weight of the crucible plus Celite to the nearest 0.1 mg.
3. Vacuum system: Vacuum pump or aspirator with regulating device. 1 L heavy-walled filtering flask with side arm. Rubber ring adaptors for use with filtering flasks.
4. Shaking water bath:
 - a. Capable of maintaining $98 \pm 2^\circ\text{C}$, with an automatic on-and-off timer.
 - b. Constant temperature, adjustable to 60°C.
5. Balance - Analytical, sensitivity ± 0.1 mg.
6. Muffle furnace - Capable of maintaining $525 \pm 5^\circ\text{C}$.
7. Vacuum oven - 70°C.
8. Alternative Oven - Capable of maintaining 105°C and $130 \pm 3^\circ\text{C}$.
9. Desiccator - With silicon dioxide (SiO₂) or equivalent desiccant. Biweekly, dry desiccant overnight at 130°.
10. pH meter - Temperature compensated, standardised with pH 4.0, 7.0 and 10.0 buffer solutions.
11. Pipettes - With disposable tips, 100 - 300 μ L and 5 mL capacity.
12. Dispensers - Capable of dispensing 15 ± 0.5 mL for 78% ethanol and acetone; 40 ± 0.5 mL for buffer.
13. Magnetic stirrers and stir bars.

Procedures

A. Sample Preparation

1. The total dietary fibre in dried samples is determined.
2. The sample is homogenised and dried overnight in a vacuum oven at 70 °C. Once dried, it is cooled in a desiccator and ground to a fine powder (0.3–0.5 mm mesh) using a mortar. If the fat content is high (>10%), it is defatted using petroleum ether (three times with 25 mL portions per g of sample) before grinding.
3. The weight loss due to fat removal is recorded, and appropriate corrections are applied to the final percentage of dietary fibre.
4. The dry, ground sample is stored in a capped jar in a desiccator until analysis. For high-sugar samples, sugars are removed before analysis by extracting the sample two to three times with 85% ethanol or methanol (10 mL/g), decanting, and drying overnight at 40 °C.

B. Digestion

1. Blank assays are run alongside test samples to account for any contribution from reagents to the residue.
2. Duplicate test portions of 1.000 ± 0.005 g (labeled M1 and M2) are weighed into 400 mL or 600 mL tall-form beakers, using an analytical balance accurate to 0.1 mg.
3. Each beaker is filled with 40 mL of MES/TRIS buffer solution (pH 8.2), which is stirred on a magnetic stirrer until the sample is completely dispersed. (Note: Complete dispersion is essential to prevent lump formation, which would hinder enzyme access to the substrate.)
4. 50 μ L of heat-stable α -amylase solution is added while stirring at low speed.
5. The beakers are covered with aluminium foil and incubated in a 95–100°C water bath for 15 minutes, with continuous agitation. Timing starts when the bath temperature reaches 95°C. (Typically, a total of 35 minutes is sufficient.)
6. All beakers are removed from the water bath and cooled to 60°C. The aluminium foil is removed. Any residue forming a ring inside the beaker is scraped down using a spatula, and any gel at the bottom is dispersed. The beaker walls and spatula are rinsed with 10 mL H₂O.
7. 100 μ L of protease solution is added to each beaker. The beakers are re-covered with foil and incubated for 30 minutes at $60 \pm 1^\circ\text{C}$ with continuous agitation. Timing begins once the bath reaches 60°C.
8. The foil is removed, and 5 mL of 0.561N HCl is dispensed into each beaker while stirring.
9. The pH is adjusted to 4.0 – 4.7 at 60°C, using 1N NaOH solution as needed. (Note: pH must be adjusted while the solution is at 60°C, as pH may shift at lower temperatures.) Most cereal, grain, and vegetable products do not require pH adjustment. Once validated, routine pH adjustment may be omitted for known sample types. Blank pH should be checked routinely; sample pH must also be checked if it falls outside the target range.
10. 300 μ L of amyloglucosidase is added while stirring. The beakers are covered with foil and incubated for 30 minutes at $60 \pm 1^\circ\text{C}$ with constant agitation. Timing begins once the bath reaches 60°C.

C. Determination of Total Dietary Fibre

1. 225 mL of 95% ethanol pre-heated to 60°C is added to each digested sample. The volume is measured after heating, and the ethanol-to-sample ratio is maintained at 4:1.
2. The samples are removed from the water bath and covered with large sheets of aluminium foil. The precipitate is allowed to form at room temperature for 1 hour.
3. A pre-tared crucible B is prepared by wetting and redistributing a Celite bed using 15 mL of 78% ethanol from a wash bottle. Vacuum suction is applied to draw the Celite onto the fritted glass base, forming an even mat.
4. The alcohol-treated enzyme digestate is filtered through the prepared crucible. (Note: If a gum-like layer forms and traps liquid, it should be gently broken using a spatula to facilitate filtration.)
5. The residue is washed under vacuum using the following sequence:
 - a. Two 15 mL portions of 78% ethanol
 - b. Two 15 mL portions of 95% ethanol
 - c. Two 15 mL portions of acetone
6. The crucible containing the residue is dried overnight at 105°C and then cooled in a desiccator for 1 hour.
7. The crucible is weighed to the nearest 0.1 mg. The residue weight is calculated by subtracting the tare weight of crucible B (with Celite) from the total weight.
8. One duplicate sample is used to determine protein content, using $N \times 6.25$ as the conversion factor.
9. The second duplicate is used for ash analysis. The crucible is incinerated at 525°C for 5 hours in a muffle furnace. After cooling in a desiccator, the crucible is weighed to the nearest 0.1 mg. The ash weight is determined by subtracting the weight of the crucible and Celite B.

D. Determination of Insoluble Dietary Fibre

1. The Celite bed in the previously tared crucible B is wetted and redistributed using 3 mL of distilled water (dH_2O). Suction is applied to the crucible to draw the Celite onto the fritted glass, forming an even mat.
2. The enzyme digestate is filtered through the crucible under vacuum into a filtration flask.
3. The beaker is rinsed, and the residue is washed twice with 10 mL portions of 70°C dH_2O .
4. The filtrate and water washings are combined, transferred into a pre-tared 600 mL tall-form beaker, and reserved for the determination of soluble dietary fibre (SDF).
5. The residue is washed under vacuum twice, each with 15 mL portions of 78% ethanol and 15 mL of acetone. Note: Delay in washing IDF residues with 78% ethanol and acetone may result in inflated IDF values.
6. Duplicates are used to determine protein and ash content as described in (C).

E. Determination of Soluble Dietary Fibre

1. The procedure for IDF determination involves combining the filtrate and water washings in pre-tared 600 mL tall-form beakers.
2. The beakers containing the combined filtrate and water washings are weighed, and the volumes are estimated.
3. Four volumes of 95% ethanol preheated to 60°C are added.
4. A portion of the 60°C ethanol is used to rinse the filtering flask from the IDF determination.
5. Alternatively, the weight of the combined filtrate and water washings is adjusted to 80 g by adding distilled water, and 320 mL of 95% ethanol at 60°C is then added.
6. The mixture is left at room temperature for 1 hour to allow precipitation to form.
7. The Celite bed in a previously tared crucible B is wetted and redistributed using 15 mL of 78% ethanol from a wash bottle. Suction is applied to draw the Celite onto the fritted glass as an even mat.
8. The alcohol-treated enzyme digestate is filtered through the crucible. (Note: If a gum is formed and traps the liquid, the film is broken using a spatula.)
9. The residue is washed under vacuum twice, each with 15 mL portions of 78% ethanol, 95% ethanol, and acetone.
10. The crucible containing the residue is dried overnight in an oven at 105°C, then cooled in a desiccator for 1 hour.

11. The crucible, containing dietary fibre residue and Celite, is weighed to the nearest 0.1 mg. The residue weight is calculated by subtracting the weight of the dry crucible from Celite B.
12. One duplicate from each sample is used to determine protein, using nitrogen $\times 6.25$ as the conversion factor.
13. For ash analysis, the second duplicate is incinerated at 525°C for 5 hours. The crucible is cooled in a desiccator and weighed to the nearest 0.1 mg. The ash weight is determined by subtracting the weight of the crucible and Celite B.

Calculation

Blank, B (mg) determination:

$$B = \left\{ \frac{(BR_1 + BR_2)}{2} \right\} - P_B - A_B$$

Where, BR_1 and BR_2 = residue weights (mg) for duplicate blank determinations

P_B and A_B = weights (mg) of protein and ash, respectively, determined on the first and second blank residues.

Dietary fibre, DF (g/100 g) determination:

$$DF = \frac{\left\{ \frac{(R_1 - R_2)}{2} \right\} - P - A - B}{\left\{ \frac{(M_1 + M_2)}{2} \right\} \times 100}$$

Where R_1 and R_2 = residue weights (mg) for duplicate samples

P and A = weights (mg) of protein ash, respectively, determined on the first and second residues

B = blank weight (mg)

M_1 and M_2 = weights (mg) for samples.

TDF determination:

Determine either by independent analysis, as in I or by summing IDF and SDF, as in D and E.

Reference

- 1) Official Methods of Analysis of AOAC International, 21st Edition, 2019. Volume II, (Section 32.1.17, Method 991.43)
- 2) Official Methods of Analysis of AOAC International, 21st Edition, 2019 Volume III, Section 45.4.07, Method 985.29

3.3.1.7 DETERMINATION OF SUGAR IN FOOD (HPLC METHOD)

Principles

A simultaneous determination of sugar (sucrose, glucose, fructose, lactose, and maltose) in food samples using HPLC with Refractive Index Detector method or Evaporative Light Scattering Detector (ELSD).

Chemicals/Reagents

1. Sucrose standard
2. Glucose standard
3. Fructose standard
4. Lactose standard
5. Maltose standard
6. Acetonitrile (ACN)
7. Triethylamine (TEA)
8. Ultrapure Water, Distilled Water (dH₂O)

Apparatus/Instruments

1. Balance: analytical sensitivity ± 0.1 mg
2. Homogenizer
3. Centrifuge
4. Vortex mixer
5. Centrifuge tube: 50 mL
6. Volumetric flasks: 10 mL
7. Measuring cylinder: 1000 mL
8. Pasteur pipette
9. Micropipette
10. Syringe filter nylon 0.45 μ m
11. Ultra-filtration equipment: 0.45 μ m, cellulose acetate membrane filters
12. Syringe 10 mL
13. Vials

Chromatographic Conditions

1. Waters 1525 Binary HPLC Pump
2. Waters 2707 Autosampler
3. Waters 2414 Refractive Index (RI) Detector
4. Column: XBridge Amide Column, 3.5 μ m (4.6 x 250 mm)
5. Mobile phase: Acetonitrile/Ultrapure water/TEA (75:25:0.2%)
6. Standards: Sugar Standards (glucose, sucrose, maltose, fructose and lactose)
7. Flow rate: 1.0 mL/min
8. Column temperature: 55°C
9. Detection: RI Detector
10. Injection volume: 10 μ L

Procedures

A. Preparation of Solid Samples

1. A portion of the sample (1–3 g) is weighed into a 50 mL centrifuge tube, reaches 60°C.
2. A volume of 25 mL of 50:50 acetonitrile/water is added, and the mixture is homogenised.
3. The mixture is centrifuged at 3200 rpm for 30 minutes.
4. The supernatant is collected and filtered using a 0.45 μ m nylon syringe filter.

B. Preparation of Liquid Samples

1. A volume of 1 mL of the liquid sample is measured.
2. The sample is diluted with 50:50 acetonitrile/water to a final volume of 25 mL.
3. The diluted sample is filtered using a 0.45 µm nylon syringe filter.

C. Preparation of mobile phase (75:25:0.2%) acetonitrile/ultrapure water/TEA

1. A volume of 750 mL of acetonitrile is measured using a 1000 mL measuring cylinder.
2. Ultrapure water is added to reach a final volume of 1000 mL.
3. A volume of 2 mL of triethylamine (TEA) is added.
4. The solution is filtered using a 0.45 µm nylon membrane filter.

D. Preparation of 5% Stock Standards (Sucrose, Glucose, Fructose, Lactose, Maltose)

1. An amount of 0.5 g of each sugar standard is weighed.
2. Each standard is dissolved in 50:50 acetonitrile/deionised water (ACN/dH₂O).
3. The solutions are topped up with 50:50 ACN/dH₂O in separate 10 mL volumetric flasks.

Note: Lactose is first dissolved in dH₂O before topping up with 50:50 ACN/dH₂O.

E. Preparation of Mixed Standard

1. 1% Mixed Standard (STD 5): A volume of 1 mL from each 5% stock standard (sucrose, glucose, fructose, lactose, maltose) is combined in a 5 mL volumetric flask and mixed.
2. Working Standard (0.1%) (STD 1): 100 µL of the 1% mixed standard is transferred into a vial and topped up with 900 µL of 50:50 ACN/dH₂O. The mixture is thoroughly mixed.
3. Working Standard (0.2%) (STD 2): 200 µL of the 1% mixed standard is topped up with 800 µL of 50:50 ACN/dH₂O in a vial and mixed.
4. Working Standard (0.5%) (STD 3): 500 µL of the 1% mixed standard is combined with 500 µL of 50:50 ACN/dH₂O and mixed.
5. Working Standard (0.8%) (STD 4): 800 µL of the 1% mixed standard is topped up with 200 µL of 50:50 ACN/dH₂O and mixed.

Reference

Official Methods of Analysis of AOAC INTERNATIONAL (2019) 21st Ed., AOAC INTERNATIONAL, Method 980.13 (Adapted using LC Refractive Index Detector method)

XBridge Amide HPLC Columns Application Notebook 720003438EN September 2010 VW-PDF

3.3.1.8 DETERMINATION OF ENERGY (BY CALCULATION)

Energy level is calculated as below:

$$\text{Energy, kcal/100 g} = \{[\text{fat}] \times 9\} + \{[\text{protein}] \times 4\} + \{[\text{carbohydrate}] \times 4\}$$

$$\text{Energy, kJ/100 g} = \text{kcal} \times 4.184$$

Reference

Doc. No. J04-013 Modified method by NPHL

3.3.2 DETERMINATION OF MINERALS

The digestion method involves the separation of minerals from the food matrix by destruction of the organic matter of the sample through dry ashing, wet or microwave digestion. The mineral content is then diluted in acid and determined by atomic absorption spectrophotometer (AAS) or inductively coupled plasma (ICP).

3.3.2.1 DIGESTION METHOD

Method A: Dry Ashing

Principle

The method consists of preparing an ash using heat and hydrochloric acid to decompose the organic matter and dissolving the inorganic residue in an appropriate volume of dilute hydrochloric acid. The organic matter is first destroyed by dry ashing at 550°C overnight or until a whitish or greyish ash is obtained. The ash is dissolved in concentrated hydrochloric acid, filtered, diluted to volume, and read directly on the AAS.

Chemicals

1. Concentrated Hydrochloric Acid (HCl)
2. Deionized water

Apparatus/Instruments

1. Silica crucible: diameter 3.5 cm, depth 4 cm for dry sample or bigger for liquid sample
2. Desiccators: with silica gel desiccant
3. Muffle Furnace: thermostatically controlled at 550°C
4. Electric hotplate: thermostatically controlled
5. Water bath
6. Volumetric flask-100 mL
7. Silica basin
8. Pipettes: 2 & 5 mL
9. No. 42 filter paper
10. Glass rod
11. Glass filter funnel

Note: Use borosilicate glassware and silica basins that have been precleaned with dilute nitric acid and rinsed in distilled water immediately before use.

Procedures

1. The ashing procedure is carried out as described in the ash determination section (see page 54). A 5 mL concentrated hydrochloric acid (HCl) is added to the silica basin. The ash is broken up using a glass rod, and the mixture is dried over a water bath.
2. An additional 2 mL of concentrated HCl is added to dissolve the ash. The solution is filtered through a Whatman No. 42 filter paper into a 100 mL volumetric flask.
3. The residue is washed three times with hot water.
4. The filtrate is diluted to volume with deionised water.

Reference

Official Methods of Analysis of AOAC INTERNATIONAL (2019) 21st Ed., AOAC INTERNATIONAL, Method 999.11

Method B: Wet Digestion

Principles

The method consists of decomposing an organic material into ash by treatment with nitric acid.

Chemicals

1. Nitric acid (HNO₃), 65% Suprapur
2. Hydrogen Peroxide (H₂O₂)

Apparatus/Instruments

1. Balance: Analytical sensitivity ± 0.1 mg
2. Hotplate
3. Conical flask: 250 mL
4. Volumetric flask: 100 mL
5. Measuring cylinders: 10 mL
6. Pipette
7. Pasteur pipette
8. Watch glass

Procedures

1. A portion of 2–3 g of the food sample is weighed accurately.
2. A volume of 10 mL concentrated nitric acid (HNO₃) is added. The sample is covered with a watch glass and left to soak overnight.
3. The sample is heated on a hot plate.
4. If brown fumes are observed and the solution level is reduced, the sample is cooled, and a few mL of HNO₃ are added.
5. Heating is continued until no brown fumes are emitted.
6. Heating is stopped once white fumes begin to appear.
7. The sample is gently reheated.
8. The sample is then allowed to cool for approximately 10–15 minutes.
9. A mixture of 2 mL hydrogen peroxide (H₂O₂) and deionised water (H₂O) is prepared in a beaker. The H₂O₂:H₂O mixture is added dropwise to the sample to prevent a vigorous reaction. If brown fumes are produced during the addition, the procedure is returned to step 4. If the prepared H₂O₂:H₂O mixture is used up, concentrated H₂O₂ is added dropwise, not exceeding a total of 10 mL. Addition is continued until no further colour change is observed in the sample.
10. The digested sample is allowed to cool and then filtered.
11. The final volume is made up to 100 mL with 2% HNO₃.

Reference

Official Methods of Analysis of AOAC INTERNATIONAL (2019) 21st Ed., AOAC INTERNATIONAL, Method 981.15D

Method C: Microwave Digestion

Principles

The application of microwave power facilitates this digestion method and element volatilization is avoided using a closed digestion system. Compared to conventional digestion methods, microwave digestion methods possess advantages such as less time consumption, easy handling, reduced consumption of high-purity reagents and minimized contamination.

Chemicals

1. Concentrated nitric acid (HNO₃), 65%
2. Hydrogen peroxide (H₂O₂), 30%
3. Ultra-pure water

Apparatus/Instruments

1. Microwave oven
2. 8-position digestion rotor
3. Quartz digestion vessel
4. Grinder
5. Balance: Analytical sensitivity ± 0.1 mg
6. Pipette: 1 & 10 mL
7. Volumetric flask: 100 mL
8. PTFE bottle

Procedures

1. The sample is dried in an oven at 105 °C until a constant weight is obtained.
2. The dried sample is ground into a fine powder using a conventional grinder.
3. A portion of 0.1–0.5 g of the food sample is weighed accurately into the digestion vessel.
4. The vessel is filled with 6 mL nitric acid (HNO₃) and 1 mL hydrogen peroxide (H₂O₂).
5. The vessel's contents are ensured to be thoroughly wetted with the reagents. The vessel is then sealed securely using the vessel seal.
6. Pre-digestion is allowed to proceed for 30 minutes.
7. A reagent blank is prepared by adding the same volumes of HNO₃ and H₂O₂ into a separate blank vessel.
8. After pre-digestion, the vessels (sample and blank) are placed into the digestion rotor with protective jackets and caps. The venting screw on each vessel cap is tightened.
9. Digestion is carried out in a microwave oven according to the settings recommended by the manufacturer's application notes or instruction manual. The digestion program used is sample-dependent.
10. After digestion, the rotor is removed from the microwave oven and cooled in a fume hood.
11. Each vessel is vented by rotating the release venting screw one half revolution until complete depressurisation is achieved.
12. The vessel seal cap is removed and rinsed with deionised water.
13. The digested sample is quantitatively transferred into a 100 mL volumetric flask and made up to volume with deionised water.
14. The diluted sample is stored in a PTFE bottle and kept in a cool place before analysis.

¹Different samples might require a different ratio of HNO₃ and H₂O₂. Some samples might need the aid of other reagents, like hydrochloric acid, for better digestion results.

Reference

Official Methods of Analysis of AOAC INTERNATIONAL (2019) 21st Ed., AOAC INTERNATIONAL, Method 2011.14, 2013.06

3.3.2.2 DETERMINATION OF MAGNESIUM, COPPER, ZINC, CALCIUM, IRON, SODIUM AND POTASSIUM IN FOOD

Method A: Atomic Absorption Method

Principle

The atomic absorption spectrophotometric (AAS) method can be satisfactorily used to determine several minerals (Ca, Cu, Fe, K, Mg, Na, Zn). The digested sample is diluted to volume and read directly on the AAS. The quantity of food material taken for analysis depends on the amount available and expected mineral content. In general, a representative sample of 5 - 10 g is weighed.

Chemicals/Reagents

1. Deionized water. Use for preparing standards and dilutions.
2. Magnesium standard solutions:
 - a. Stock solution - 1000 ppm (magnesium nitrate standard solution for AAS)
 - b. Working standard - 0.1, 0.2 and 0.3 ppm.
3. Copper standard solutions:
 - a. Stock solution - 1000 ppm (copper (II) nitrate standard solution for AAS)
 - b. Working standard - 0.5, 1.0 and 1.5 ppm.
4. Zinc standard solutions:
 - a. Stock solution - 1000 ppm (zinc nitrate standard solution for AAS)
 - b. Working standard - 0.25, 0.5, 0.75, 1.0 ppm.
5. Sodium standard solutions:
 - a. Stock solution - 1000 ppm (sodium nitrate standard solution for AAS)
 - b. Working standard - 0.1, 0.2, 0.3, 0.4 and 0.5 ppm.
6. Potassium standard solutions:
 - a. Stock solution - 1000 ppm (potassium nitrate standard solution for AAS)
 - b. Working standard - 0.3, 0.6 and 0.9 ppm.
7. Calcium standard solutions:
 - a. Stock solution - 1000 ppm (calcium nitrate standard solution for AAS)
 - b. Working standard - 1, 2, 3, 4, 5 and 6 ppm.
8. Iron standard solutions:
 - a. Stock solution - 1000 ppm (ferric nitrate standard solution for AAS)
 - b. Working standard - 1, 2 and 3 ppm.

Apparatus/Instruments

1. Perkin Elmer atomic absorption spectrophotometer: model AAnalyst 400
2. Micropipette
3. Volumetric flasks: 100 mL

Procedures

A. Digestion of the sample

1. The sample is digested using the dry ashing, wet, or microwave digestion method described in Section 3.3.2.1 (Digestion Method for Mineral Determination). The digested sample is diluted to volume and analysed directly using atomic absorption spectrometry (AAS).

B. Determination

1. The instrument is set up according to the parameters specified in Table 3.14.
2. The instrument and associated computer software are prepared as the operating manual describes.
3. Calibration curves are prepared for each mineral to be determined using the appropriate standard solutions.
4. A minimum of three readings is obtained for each sample solution prepared.

Table 3.3.2.2.A: Operating Parameters Guide for Atomic Absorption Spectrophotometer (Perkin Elmer AAS model Analyst 400) *

Element	Flame type	Lamp current (mA)	Wavelength (nm)	Slit width (mm)	Working range (ppm)
Mg	Air-Acetylene	6	285.21	2.7/1.05	0.1-0.3
Cu	Air-Acetylene	15	324.75	2.7/0.8	0.5-1.5
Zn	Air-Acetylene	15	213.86	2.7/1.8	0.25-1.0
Na	Air-Acetylene	8	589.0	1.8/0.6	0.1-0.5
K	Air-Acetylene	12	766.49	2.7/0.45	0.3-0.9
Ca	Air-Acetylene	10	422.67	2.7/0.6	1-5
Fe	Air-Acetylene	30	248.33	1.8/1.35	1-3

*Parameters may differ according to the respective manufacturer's instruction manual

Calculation

Obtain from the printed report the concentration of the element in the sample, expressed in ppm. Convert results to mg per 100 g sample as follows:

$$= \text{ppm} \times \frac{1}{100} \times 100$$

Reference

Official Methods of Analysis of AOAC INTERNATIONAL (2019) 21st Ed., AOAC INTERNATIONAL, Method 999.10, 999.11

Method B: Inductively Coupled Plasma Mass Spectrometry Method

Principles

Inductively coupled plasma mass spectrometry (ICP-MS) is a can determine type of mass spectrometry that is highly sensitive and capable of determining a range of metals and several non-metals at concentrations below one part in 10¹² (parts per trillion). It is based on the coupling of an inductively coupled plasma as a method of producing ions (ionization) with a mass spectrometer as a method of separating and detecting the ions. ICP-MS is also capable of monitoring isotopic speciation for the ions of choice. As a droplet of nebulized sample enters the central channel of the ICP, it evaporates, and any solids dissolved in the liquid vaporize and then break down into atoms. At the temperatures prevailing in the plasma, a significant proportion of the atoms of many chemical elements are ionized, each atom losing its most loosely bound electron to form a singly charged ion. The ions from the plasma are extracted through a series of cones into a mass spectrometer, usually a quadrupole. The ions are separated based on their mass-to-charge ratio and a detector receives an ion signal proportional to the concentration.

Chemicals/Reagents

1. Nitric acid, 2% (HNO₃). Use for preparing standards and dilutions.
2. Sodium standard solutions:
 - a. Stock solution - 1000 ppm (sodium nitrate standard solution)
 - b. Working standard - 1 ppb- 1000 ppb
3. Magnesium standard solutions:
 - a. Stock solution - 1000 ppm (magnesium nitrate standard solution)
 - b. Working standard - 1ppb-1000ppb
4. Potassium standard solutions:
 - a. Stock solution - 1000 ppm (potassium nitrate standard solution)
 - b. Working standard - 1 ppb- 1000 ppb
5. Iron standard solutions:
 - a. Stock solution - 1000 ppm (ferric nitrate standard solution)
 - b. Working standard - 1 ppb- 1000 ppb
6. Copper standard solutions:
 - a. Stock solution - 1000 ppm (copper nitrate standard solution)
 - b. Working standard - 1 ppb- 1000 ppb
7. Zinc standard solutions:
 - a. Stock solution - 1000 ppm (Zinc nitrate standard solution)
 - b. Working standard - 1 ppb- 1000 ppb
8. Calcium standard solutions:
 - a. Stock solution - 1000 ppm (calcium nitrate standard solution)
 - b. Working standard - 1 ppb- 1000 ppb

Apparatus/Instruments

1. ICP-MS, Elan 6100 Perkin Elmer
2. Volumetric flasks: 25 & 10 mL
3. Micropipette

Procedures

a. Digestion of the sample

The sample is digested using the dry ashing, wet, or microwave digestion method described in Section 3.3.2.1 (Digestion Method for Mineral Determination). The digested sample is diluted to the required volume and analysed directly using inductively coupled plasma mass spectrometry (ICP-MS).

b. Determination

1. The instrument is set up according to the parameters provided in Table 3.15. The instrument and software are prepared for analysis as the operating manual outlines.
2. A calibration curve is prepared for each mineral using the corresponding standard solutions.
3. A minimum of three readings is obtained for each prepared sample solution.

Table 3.3.2.2.C: ICP OES Instrumental Parameters *

Measures	
Radiofrequency power	1100 W
Plasma gas flow rate	15.0 L min ⁻¹
Auxiliary gas flow rate	1.50 L min ⁻¹
Replicate read time	3 sec
Delay	15 sec
Sample Uptake delay	30 sec
Pump rate	15 rpm
Rinse Time	20 sec
Replicates	3
Torch	Quartz for axial view

**Parameters may differ according to the respective manufacturer's instruction manual*

Calculation

Obtain from the printed report the concentration of the element in the sample, expressed in ppb. Convert results to mg per 100 g sample as follows:

$$=ppb \times \frac{1}{10}$$

Reference

Official Methods of Analysis of AOAC INTERNATIONAL (2019) 21st Ed., AOAC INTERNATIONAL, Method 984.27, 2011.14

3.3.2.3 DETERMINATION OF SELENIUM AND MANGANESE USING ICP-MS

The environmental conditions and agricultural practices generally affect the selenium content of many foods, particularly plant food sources. In general, goods with higher protein content contain more selenium. Food processing and cooking can contribute to the loss of selenium from foods. Whereas manganese is mainly found in plant-based foods, with the greatest contribution from whole grains. Other food sources include nuts, fruits, leafy vegetables, legumes, and tea. Animal-based foods are generally low in manganese. For the detection of selenium and manganese, ICP-MS offers great selectivity and sensitivity.

Chemicals/Reagents

1. Nitric acid, 2% (HNO₃). Use for preparing standards and dilutions
2. Selenium standard solutions:
 - a. Stock solution - 1000 ppm (Selenium nitrate standard solution)
 - b. Working standard - 1 ppb- 1000 ppb
3. Manganese standard solutions:
 - a. Stock solution - 1000 ppm (Manganese nitrate standard solution)
 - b. Working standard - 1 ppb- 1000 ppb

Apparatus/Instruments

1. ICP-MS, Elan 6100 Perkin Elmer
2. Volumetric flasks: 25 & 10 mL
3. Micropipette

Procedures

a. Digestion of the sample

The sample is digested using the dry ashing, wet, or microwave digestion method as described in Section 3.3.2.1 (Digestion Method for Mineral Determination).

b. Determination

1. The instrument is set up according to the parameters specified in Table 3.17. The instrument and computer software are prepared for analysis as outlined in the operating manual.
2. A calibration curve is prepared for each mineral to be determined, using the corresponding standard solutions.
3. A minimum of three readings is obtained for each sample solution.

Table 3.3.2.3: ICP-MS Operating Condition (ICP-MS, Elan 6100 Perkin Elmer) *

Measures	
Spray Chamber	Scott spray chamber
Nebulizer	Cross-flow
RF power	950 watts
Argon nebulizer gas flow (Lmin-1)	0.93 (adjusted daily to maximum signal intensity)
Scan mode	Peak hopping
Resolution (amu)	0.7
Replicate time (sec)	1
Dwell time (sec)	50
Sweeps	20
Replicates	3
Isotopes	³⁴ Se, ⁵⁵ Mn

*Parameters may differ according to the respective manufacturer's instruction manual

Calculation

Obtain from the printed report the concentration of the element in the sample, expressed in ppb. Convert results to mg per 100 g sample as follows:

$$= \text{ppb} \times \frac{1}{10}$$

Reference

Official Methods of Analysis of AOAC INTERNATIONAL (2019) 21st Ed., AOAC INTERNATIONAL, Method 2015.06

3.3.2.4 DETERMINATION OF IODINE IN FOOD USING ICP-MS

Iodine is essential for producing thyroid hormones. These hormones stimulate metabolism in the body, mental growth and development. The recommended daily iodine intake is 0.1-0.2 mg, with the most common sources of iodine being fish, seafood, milk, and food supplements. Determining iodine in food has always been difficult due to the low concentrations (mg/kg), difficult sample preparation and the volatility of iodine. ICP-MS provides excellent selectivity and sensitivity for the measurement of iodine.

Chemicals/Reagents

1. Iodide standard solutions:
 - a. Stock solution -1000 ppm
 - b. Working standard- 5, 10, 15, 20 µg/L
2. Tetramethylammonium hydroxide, 25% (TMAH)
3. Tetramethylammonium hydroxide, 0.5% (TMAH)
4. Deionized water (dH₂O)

Apparatus/Instruments

1. ELAN DRC™ II ICP-MS
2. Oven
3. Centrifuge
4. Balance: Analytical sensitivity ± 0.1 mg
5. PFA tube
6. Volumetric flasks: 10, 100 & 1000 mL
7. Measuring Cylinder: 10 & 50 mL
8. Whatman No. 541 filter paper
9. Micropipettes

Procedures

a. Sample preparation

- i) Before weighing, samples are mixed or lightly crushed.
- ii) A portion of 0.25–0.5 g of the sample is placed into perfluoroalkoxy (PFA) tubes, adding 4.5 mL deionised water and 1 mL tetramethylammonium hydroxide (TMAH, 25%).
- iii) After capping, the tubes are placed in a drying oven at 90 °C for 3 hours.
- v) Once cooled, water is added to each tube to bring the total volume to 10 mL. The solutions are then centrifuged at 3000 rpm for 15 minutes.
- v) If visible particulates remain after centrifugation, the samples are filtered. In cases where high matrix concentrations are present, the resulting solutions are filtered and may be diluted further before analysis.

Note: For high-carbohydrate samples, α-amylase is added and the mixture is incubated in a water bath at 37 °C overnight before proceeding to the reagent addition step. (In-house modification)

b. Determination

- i. Set up the instrument as given in Table 3.18 below. All measurements are to be done in standard mode.
- ii. Calibration standards ranging from 5 to 20 are to be used, prepared in 0.5% (v/v) TMAH.
- iii. After each standard and sample is analysed, a 45-second rinse with 0.5% TMAH will be performed.

Table 3.3.2.4: Instrumental Conditions (ELAN DRC™ II ICP-MS)

Measures	
Spray chamber	Cyclonic
Nebulizer	Meinhard®
Sample Uptake Rate	1 mL/min
RF Power	1100 W
Plasma Gas Flow (L/min)	15
Nebulizer Gas Flow (L/min)	0.93
Auxiliary Gas Flow (L/min)	1.2
Dwell Time (ms)	50
Sweeps per reading	25
Replicates	3
Delay Time (sec)	50
Wash Time (sec)	45

**Parameters may differ according to the respective manufacturer's instruction manual*

Reference

Official Methods of Analysis of AOAC INTERNATIONAL (2019) 21st Ed., AOAC INTERNATIONAL, Method 2012.14

3.3.2.5 DETERMINATION OF PHOSPHORUS IN FOOD

Principles

The food sample is first ashed and the ash is taken up with concentrated hydrochloric acid. Phosphate in the solution is then determined based on Misson's reaction. Phosphorus present as orthophosphate reacts with a vanadate-molybdate reagent to produce a stable yellow-orange complex of vanado-molybdophosphoric acid, the optical density (OD) of which is measured at 420 nm.

Chemicals/Reagents

1. Spectrophotometer
2. Water bath
3. Balance: Analytical sensitivity ± 0.1 mg
4. Volumetric flasks: 100 & 1000 mL
5. Measuring cylinder: 25 mL
6. Glass rod
7. Whatman No.42 filter paper

Procedures

This determination uses ash from dry ash digestion (section 3.3.2.1):

1. The ash solution is prepared by adding deionized water to make up a final volume of 100 mL.
2. A suitable volume of the ash solution, containing 0.1–2 mg phosphorus (typically 1–2 mL for most foods), is transferred to a 100 mL volumetric flask.
3. Another 100 mL volumetric flask is prepared for the reagent blank.
4. A volume of 25 mL vanadomolybdate reagent is added to both flasks.
5. The flasks are immediately diluted to the mark with deionized water.
6. The solutions are allowed to stand for 10 minutes.
7. The optical density (OD) is read at 420 nm using a spectrophotometer, zeroed with the reagent blank. The colour developed remains stable for several hours.

Prepare a standard curve as follows:

1. A suitable volume of the solution, containing 0.1–2 mg phosphorus (typically 1–2 mL for most foods), is transferred to a 100 mL volumetric flask.
2. 2.5, 5, 10, 20, 30, 40, and 50 mL of the working phosphate standard solution (containing 0.125–2.5 mg phosphorus) are transferred into a series of 100 mL volumetric flasks.
3. A volume of 25 mL vanadomolybdate reagent is added to each flask.
4. All flasks are immediately diluted and made up to the mark with deionized water.
5. The solutions are allowed to stand for 10 minutes.
6. The colour formed is read at 420 nm using a spectrophotometer, zeroed with the reagent blank.

Calculation

A standard curve of OD versus mg P is plotted. From this curve, the concentration of P corresponding to the OD of the test solution is read off. The phosphorus content of the sample being analysed can then be calculated as:

mg P in 100 g sample =

$$\text{mg P corresponding to test O.D (from standard curve)} \times \frac{\text{total volume of ash solution}}{\text{volume of ash solution used}} \times \frac{100}{\text{Wt (g) of food sample}}$$

Reference

Official Methods of Analysis of AOAC INTERNATIONAL (2019) 21st Ed., AOAC INTERNATIONAL, Method 2015.06

3.3.3 DETERMINATION OF FAT-SOLUBLE VITAMIN

3.3.3.1 DETERMINATION OF VITAMIN A AND CAROTENOIDS IN FOOD USING HPLC

Principles

High-pressure liquid chromatography (HPLC) is commonly used to precisely quantify various carotenoids and retinoids in foods, primarily because it provides rapid separation, non-destructive analysis, and superior resolution.

This laboratory's method is a non-aqueous reverse-phase HPLC procedure, employing a ternary mixture of acetonitrile, methanol, and ethyl acetate (88:10:2, v/v) as the mobile phase and a μ Bondapak C18 column. A UV-visible detector capable of dual-wavelength detection will enable simultaneous detection and quantitation of retinol and carotenoids. The detector is to be set to detect retinol at 325 nm and carotenoids at 450 nm. The method is suitable for foods of animal origin and other foods in which preformed vitamin A and carotenoids are expected to be present, e.g., processed or composite foods. As described for the open column chromatography method, various sample preparation procedures must be carried out first.

Chemicals/Reagents

1. Acetonitrile, HPLC grade
2. Methanol, HPLC grade
3. Ethyl acetate, HPLC grade
4. Ethanol, analytical grade
5. Hexane, analytical grade
6. Benzene, analytical grade
7. Mixture of acetonitrile, methanol, and ethyl acetate (88:10:2, v/v) as mobile phase:
- 20 mL of ethyl acetate is pipetted into a 1 L measuring cylinder. One hundred mL of methanol is added, and the volume is made up to 1 L with acetonitrile. Using the solvent filtration kit, solvents are filtered through a 0.45 μ m nylon membrane filter and degassed using an ultrasonic bath.
8. Vitamin A standard solution:
 - a. Retinol stock solution. 70% trans-retinol is used as the standard for vitamin A determination. A 25 mg ampoule is cut and dissolved in 250 mL of absolute alcohol to give a suitable stock of 100 μ g/mL. The solution is stored in amber bottles in the freezer.
 - b. Retinol working solution for spectrophotometric reading. 200 μ L of stock solution of 100 μ g/mL is pipetted using a micropipette to make up to 10 mL with absolute alcohol, giving a working solution of 2 μ g/mL.
9. β -Carotene standard solution:
 - a. Synthetic crystalline trans-beta-carotene is used as a standard for carotene determination. Ten mg of the standard is weighed and made up to 100 mL with hexane to give a stock solution of 100 μ g/mL. The solution is stored in amber bottles in the freezer.
 - b. β -carotene working solution for spectrophotometric reading. 200 μ L of the stock solution of 100 μ g/mL is pipetted using a micropipette and made up to 10 mL volume with hexane to give a working solution of 2 μ g/mL.
10. α -Carotene standard solution:
 - a. α -carotene from carrots is used as a standard. A 10 mg ampoule is cut and made up to 100 mL volume with hexane to give a stock of 100 μ g/mL. The solution is stored in amber bottles in the freezer.
 - b. α -carotene working solution for spectrophotometric reading. 200 μ L of stock solution of 100 μ g/mL is pipetted using a micropipette and made up to 10 mL volume with hexane to give a working solution of 2 μ g/mL.
11. Lycopene standard solution:
 - a. Lycopene from tomatoes is used. A 5 mg ampoule is cut and made up to 25 mL volume with benzene to give a stock of 200 μ g/mL. The solution is stored in amber bottles in the freezer.
 - b. Lycopene working solution for spectrophotometric reading. 100 μ L of the stock solution of 200 μ g/mL is pipetted using a micropipette and made up to 10 mL with benzene to give a working solution of 2 μ g/mL.

12. Standard mixture solution for HPLC injection. The following amounts of retinol and carotenoid stock are pipetted using micropipettes into a 10 mL volumetric flask, made up to volume, and then filtered with a 0.45 μ m regenerated cellulose membrane filter before 50 μ L is injected into the HPLC:
 - a. 30 μ L of 100 μ g/mL of retinol stock to give a solution of 0.3 μ g/mL.
 - b. 20 μ L of 100 μ g/mL of carotene stock to give a solution of 0.2 μ g/mL.
 - c. 20 μ L of 100 μ g/mL of carotene stock to give a solution of 0.2 μ g/mL.
 - d. 5 μ L of 200 μ g/mL of lycopene stock to give a solution of 0.1 μ g/mL.

*Note: *Other sources of standards (β , -carotene and lycopene) can be used with known apurity information preferably more than 90%.*

**Preparation of all standards for retinol and carotenoids should be carried out in a room with subdued light and windows tinted with a light-protective film. All sample treatment and analytical procedures were also carried out in this room.*

Apparatus/Instruments

1. Balance: Analytical sensitivity $\pm$ 0.1 mg
2. Vortex mixer
3. Glass balls
4. Measuring cylinder: 50 mL
5. Round-bottom, short-neck flasks: 250 mL
6. Round-bottom, short-neck flasks: 250 mL
7. Steam bath: located in the fume cupboard
8. Pipettes: 5 mL
9. Micropipettes
10. Volumetric flasks: 10 mL
11. Separating funnel
12. Reflux apparatus
13. Electric heating mantle
14. Conical flask: 250 mL
15. Pasteur pipette
16. Ultrafiltration equipment: using 0.45 μ m, 47 mm diameter membrane filters
17. Ultrasonic bath for degassing solvent
18. Syringe filter: 0.45 μ m nylon
19. Syringe
20. Amber vials

HPLC conditions

1. 10- μ m μ Bondapak C18 stainless steel column of 30 cm x 3.9 mm I.D.
2. Guard column holder housing a disposable pre-column insert packed with the same material as that of the analytical column
3. A Gilson 234 autoinjector or a 7125 Rheodyne injector with a 500 μ L variable loop
4. A Gilson 305 piston pump to deliver the mobile phase.
5. A Gilson 805 manometric module to dampen the pump's pulsations and supply the current pressure value to the 305 piston pump.
6. A computer with a Gilson System Controller Software (i.e., the 715 controller software).
7. Attach the detector to a printer to print the results and chromatograms.
8. Gilson UV 119 detector with a minimum dual wavelength detection (i.e., 325 nm for retinol detection and 450 nm for carotenoids).

Procedures

A. Alkaline Hydrolysis

1. An accurate weight of 10 g of the foodstuff is weighed into a 250 mL boiling flask.
2. A volume of 40 mL of 95% ethanol and 40 mL of water is added to the sample, and the contents are mixed thoroughly.
3. A volume of 100% potassium hydroxide solution, equal to the sample's weight, is added slowly and mixed.
4. A few boiling chips are added.
5. The flask is attached to a water-cooled reflux apparatus and heated using a water bath or electric heating mantle.
6. The temperature is adjusted to achieve a reflux rate of approximately two drops per second.
7. The flask is agitated frequently during refluxing to disperse any aggregates formed.
8. The mixture is refluxed for 30 minutes from the point of boiling.
9. The flask is cooled rapidly to room temperature.

B. Extraction

1. The hydrolysate is extracted three times with 50 mL aliquots of hexane.
2. The hexane extracts are combined and washed with distilled water until the washings are free of alkali (confirmed by testing with phenolphthalein).
3. The hexane extract is passed through anhydrous sodium sulphate to remove moisture.
4. The extract volume is reduced by heating on a water bath at 60–65 °C under a nitrogen stream.
5. The concentrated extract is transferred to a 10 mL volumetric flask and made up to the mark with hexane (V_1).
8. The mixture is refluxed for 30 minutes from the point of boiling.
9. The flask is cooled rapidly to room temperature.

C. Instrument Set Up Procedures

1. Set up procedures following the instrument manufacturer's instruction manual.

D. Chromatography of Carotenoids and Retinol

1. The extract's hexane is first evaporated on a water bath, and the residue is immediately redissolved in a suitable volume of the mobile phase.
2. Suitable volumes are injected into the chromatograph after filtering through a 0.45 µm nylon membrane filter (using a sample filtration kit).
3. Identification and quantitation of carotenoids are carried out by comparing with reference retinol and carotenoids similarly chromatographed.
4. The appropriate extinction coefficients published are used to calculate the exact concentration of each of the carotenoids.
5. The chromatogram's total peak areas are used to determine the total carotenoid content, which is calculated using a β-carotene standard curve.

Calculation

g vitamin A alcohol (retinol) or g carotene per 100 g food =

$$\frac{\text{Peak area of sample}}{\text{peak area of standard}} \times \text{amount of standard in } \mu\text{g} \times \frac{V_1 \text{ (mL)}}{\text{inject volume in mL}} \times \frac{100}{\text{wt of food used}}$$

Reference

Official Methods of Analysis of AOAC INTERNATIONAL (2019) 21st Ed., AOAC INTERNATIONAL, Method 992.04; 992.06; 2001.13; 2002.06; 2012.09; 2012.10

3.3.3.2 DETERMINATION OF VITAMIN D IN FOOD

Method A: Determination of Fat-Soluble Vitamins (A, D2, D3, K1, K2, Lutein & B-Carotene) Level in Food Sample (Solid and Liquid)

Principles

The determination of fat-soluble vitamins (A, D2, D3, K1, K2, lutein, and β -carotene) in food samples involves extracting the vitamins from the sample using organic solvents. If the sample contains lipids, saponification breaks down fats before extraction. The extracted vitamins are separated using high-performance liquid chromatography (HPLC) with UV-Vis or fluorescence detection, as these vitamins have specific absorbance or fluorescence properties. The concentration of each vitamin is quantified by comparing the sample's chromatographic peaks with calibration curves from standard solutions. This method effectively isolates, separates, and quantifies fat-soluble vitamins in solid and liquid food samples.

Materials/Chemicals:

1. Methanol HPLC grade from Merck
2. Acetonitrile HPLC grade from Merck
3. Ethanol HPLC grade from Merck
4. Ethyl acetate analytical grade from Merck
5. 1-Chlorobutane analytical grade from Merck
6. Butylated hydroxytoluene from Sigma
7. Potassium hydroxide from Merck

Instrumentation and conditions:

1. UHPLC Dionex Ultimate 3000 series with Photo-diode Array Detector
2. Column Dionex Acclaim Polar Advantage II C18 – 5 μ m particle size, 4.6×250 mm Diameter and length
3. Flow: 1.000 mL/min
4. Injection volume: 10 μ L
5. Wavelength:
265 nm: D2, D3, K1, K2
291 nm: E
325 nm: A
450 nm: Lutein, Lycopene, β -carotene

Mobile Phase and Gradient:

1. Mobile phase: A: Deionized water
B: 100% Methanol
C: 100% Acetonitrile
2. Gradient:

Table 3.3.3.2A: Gradient of Mobile Phase

Time	Flow	Solvent A	Solvent B	Solvent C
(min)	(mL/min)	(%)	(%)	(%)
0.0	1.000	0.0	100.0	0.0
4.0	1.000	0.0	100.0	0.0
8.0	1.000	0.0	100.0	0.0
18.0	1.000	0.0	100.0	0.0
25.0	1.000	0.0	100.0	0.0
		Washing**		
0.0	0.500	30.0	0.0	70.0
4.0	0.500	30.0	0.0	70.0
16.5	0.500	30.0	0.0	70.0
60.0	0.500	30.0	0.0	70.0

**Done in the smart shutdown setting

Procedures

1. Chemical Preparation:
 - a. Ethanol with 0.5% BHT (w/v): Exactly 1.0 g of BHT is dissolved in 200 mL of ethanol.
 - b. Ethanolic potassium hydroxide (KOH) solution (7.5% w/v): 7.5 g of KOH is dissolved in 100 mL of ethanol. This solution is prepared fresh daily.
 - c. Extraction solvent: 0.25 g of BHT is dissolved in 2.5 L of 1-chlorobutane and homogenised thoroughly before use.
2. Standard Preparation:
 - a. 200 ppm Standard Preparation:
 - i. Precisely 1.0 mg of each vitamin is individually weighed, except for vitamin E, which is weighed at 5.0 mg. The exact weight of each standard is recorded. All weighed standards are subsequently dissolved in 5.0 mL of ethyl acetate.
 - ii. All standards are prepared fresh daily.
 - iii. 20 ppm mixed Fat-Soluble Vitamin (FSV) standard: 500 μ L of each stock vitamin is pipetted and combined with 500 μ L of ethyl acetate, bringing the final volume to 5.0 mL to obtain a 20 ppm mixed standard.
 - iv. Calibration standards: Calibration standards are prepared in the range of 2–10 ppm by diluting the 20 ppm mixed FSV standard accordingly to 2.0, 4.0, 6.0, 8.0, and 10.0 ppm in 1.5 mL HPLC vials.
3. Sample Preparation:
 - a. Sample Extraction:
 - i. Extraction protocol for vitamin D2, D3, K1, K2 and lutein: Weigh 1.0 g of food sample in a 50 mL centrifuge tube, and the weight was recorded. 2.0 mL of ultra-pure water, 5.0 mL each of ethanol and ethyl acetate were added. This mixture is vortexed and subjected to sonication for 20 minutes. Upon sonication, the mixture is centrifuged at 4000 rpm for 20 minutes. The resulting supernatant is then filtered using a 0.45 μ m membrane filter into HPLC vial, and finally subjected to sample analysis.
 - ii. Extraction protocol for Vitamin A and β -carotene: Weigh 5.0 g of food sample in a 100 mL amber volumetric flask, and the weight was recorded. 30.0 mL of ethanolic potassium hydroxide and 7.5 mL of BHT in ethanol solution were added to the flask. This mixture was subjected to saponification at 80°C for 45 minutes. The saponified mixture was allowed to cool down, followed by adding 30.0 mL each of ultra-pure water and extraction solvent, respectively. The separated upper layer was transferred into a 50 mL centrifuge tube and mixed with ultra-pure water in a ratio of 2:1. This step was repeated twice. The remaining supernatant was then transferred into the Eppendorf tube and subjected to evaporation using a Sample Concentrator at 2000 rpm for 1 hour and 30 minutes. The residue was then re-constituted in 0.5 mL ethyl acetate and finally subjected to HPLC analysis.

List of Fat-Soluble Vitamins

- 1) A: Retinol
- 2) D2: Ergocalciferol
- 3) D3: Cholecalciferol
- 4) E: α -Tocopherol
- 5) K1: Phyloquinone
- 6) K2: Menaquinone
- 7) Lutein
- 8) Lycopene
- 9) β -carotene

References:

- 1) A. Jedlička and J. Klimeš (2005). Determination of Water and Fat Soluble Vitamins in Different Matrices Using High Performance Liquid Chromatography. *Chemical Papers*. 59(3), 202 – 222.
 - 2) Christopher John Blake (2007). Status of Methodology for the determination of Fat Soluble Vitamins in Foods, Dietary Supplements, and Vitamin Premixes. *Journal of AOAC International*. 90(4), 897 – 910.
 - 3) P. Moreno and V. Salvadó (2000). Determination of eight water- and fat-soluble vitamins in multi-vitamin pharmaceutical formulations by high-performance liquid chromatography. *Journal of Chromatography A*. 870, 207 – 215.
 - 4) Christopher J. Bates (2006). Vitamins: Fat and Water Soluble: Analysis. MRC Human Nutrition Research, *Encyclopedia of Analytical Chemistry*; John Wiley and Sons Ltd.
 - 5) Roman Kand'ár, Pavla Novotná and Petra Drábková (2013). Determination of Retinol, α -Tocopherol, Lycopene, and β -carotene in Human Plasma Using HPLC with UV-Vis Detection: Application to a Clinical Study. *Journal of Chemistry*. 10, 1-7.
 - 6) Dionex Corporation (2010). Determination of Water- and Fat-Soluble Vitamins in Nutritional Supplements by HPLC with UV Detection. Application Note 251. Sunnyvale, CA LPN 253
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Method B: Vitamin D in Infant Formulas and Enteral Products

Principles

The analysis of vitamin D in infant formula typically involves a series of preparatory steps to isolate the nutrient. First, the test portion is saponified to break down fats and other lipids, followed by extraction to separate the vitamin D from the matrix. Afterward, the extract is evaporated to concentrate the nutrients, ensuring an accurate measurement of vitamin D content. Reversed-phase liquid chromatography (LC) is employed to determine vitamin D. This technique is coupled with a UV detector set at 265 nm, which allows for the precise quantification of vitamin D. The use of reversed-phase LC provides high resolution and specificity, ensuring reliable results in the analysis of vitamin D levels in infant formula.

Reagents/Chemicals

1. Solvents (HPLC grades)-N-hexane, dichloromethane, acetonitrile, isopropanol, methanol, ethyl Acetate
2. Ethanol-Absolute, Pharmaceutical grade
3. Phenolphthalein solution-1%- 1 g of phenolphthalein is dissolved in 100 mL of absolute ethanol.
4. Dichloromethane-isopropanol (IPA) solutions:
 - a. 99.8 + 0.2 (v/v)- 2 mL of isopropanol is transferred into a 1 L volumetric flask. It is then diluted to volume with dichloromethane and mixed.
 - b. 80 + 20 (v/v)-200 mL of isopropanol is transferred into 1 L volumetric flasks, diluted to volume with dichloromethane, and mixed.
5. Acetic acid solution-10 %: 10 mL of glacial acetic acid (AR grade) is transferred into a 100 mL volumetric flask, diluted to volume with H₂O, and mixed well.
6. Ethanolic potassium hydroxide (KOH) solution-140 g of KOH pellets (AR grade) is dissolved in 310 mL of absolute ethanol, and 50 mL of H₂O is added. This solution is prepared on the day of use.
7. Mobile phase-Gradient mixture of acetonitrile, methanol, and ethyl acetate. See Table 3.3.3.2B for the concentration of mobile phase components.
8. Vitamin D2 standard solutions:
 - a. Stock standard solution (180 µg/mL)- 45 mg of vitamin D2 is accurately weighed and quantitatively transferred to a 250 mL amber volumetric flask. It is then diluted to volume with absolute ethanol.
 - b. Working standard solution (2.88 µg/mL)- 4.0 mL of stock standard solution is transferred into a 250 mL amber volumetric flask and diluted to volume with absolute ethanol. It is stored in the refrigerator for ≤ 7 days.
 - c. Internal standard solution (46 ng/mL). Vitamin D3 is quantitated using this solution. 4 mL of stock standard solution is transferred into a 250 mL amber volumetric flask and diluted to volume with absolute ethanol. It is stored in the refrigerator for ≤ 7 days.
9. Vitamin D3 standard solutions-USP reference standard or traceable secondary standard is prepared and stored in the refrigerator ≤ 7 days.
 - a. Stock standard solution (180 µg/mL)-45 mg of vitamin D2 is accurately weighed and quantitatively transferred to a 250 mL amber volumetric flask. It is then diluted to volume with absolute ethanol.
 - b. Working standard solution (2.88 µg/mL)-4.0 mL of stock standard solution is transferred into a 250 mL amber volumetric flask and diluted to volume with absolute ethanol. It is stored in the refrigerator for ≤ 7 days.
 - c. Internal standard solution (46 ng/mL). This is used to quantitate vitamin D3.
10. System suitability standard solution-125 mg of certified vitamin D2 standard and 125 mg of certified vitamin D3 standard are dissolved in 10 mL of acetonitrile. The solution is heated for 45 min at 90°C under reflux, then cooled. One mL of refluxed solution is transferred to a 100 mL amber volumetric flask and diluted to volume with acetonitrile.

(Note: Protect vitamin D2 and D3 standard solutions from high temperature, oxygen, and light to minimize degradation.)

Apparatus/Instruments

1. Liquid chromatograph (LC)—with UV detector, meeting system suitability requirements. Operation at $27 \pm 1^\circ\text{C}$ higher temperature results in a loss of resolution
2. LC Column-250-250 x 4.6 mm id, C18, 5 μm particle size. Operating conditions: injection volume 250 μL ; column temperature, 27°C ; wavelength, 265 nm; flow rates, see Table 3.3.3.2B for flow rates; stop time, 35 min; retention times: vitamin D₂, 19.5 min; vitamin D₃, 23 min
3. Solid-phase extraction (SPE) column-Silica; 500 mg/2.8 mL
4. Vacuum manifold-For SPE column (c)
5. Evaporator-With N flow
6. Rotary Evaporator
7. Water bath shaker-Maintaining 60°C
8. Separatory funnel-250 mL
9. Amber volumetric flask-250 mL
10. Reflux apparatus

Table 3.3.3.2B: Flow rates and concentrations of mobile phase components for determination of vitamin D

Time, min	Flow rate, mL/min	Acetonitrile %	Methanol %	Ethyl acetate %
0.0	0.7	91.0	9.0	0.0
28.0	0.7	91.0	9.0	0.0
28.5	2.5	0.0	0.0	100.00
31.0	2.5	0.0	0.0	100.00
31.5	2.5	91.0	9.0	0.0
33.0	0.7	91.0	9.0	0.0
34.0	0.7	91.0	9.0	0.0

Procedures

A. Preparation of Standard Mixture and Test Portions

- a. Standard mixture-A volume of 4.0 mL of vitamin D₂ internal standard solution (8) and 4.0 mL of vitamin D₃ internal standard solution (9) is transferred into an Erlenmeyer flask. Then, 15 mL of water is added.
- b. Test portion-The formula or enteral product is prepared according to the label directions and used as the test sample. The target vitamin D concentration is set at 0.5 IU/mL. Depending on the sample's viscosity, a 15.0 mL portion of the test sample is transferred into a 125 mL Erlenmeyer flask using a pipette or syringe. Then, 4.0 mL of the internal standard solution is added to the flask.

The vitamin D₂ standard solution (8)(c) is used to quantitate vitamin D₃, while the vitamin D₃ standard solution (9)(c) is used to quantitate vitamin D₂.

B. Saponification and Extraction

- a. Aliquots of 15.0 mL ethanolic KOH solution (6) are added to the standards mixture (A1) and the test portion from A2. The flasks are closed with stoppers and placed in a water bath shaker at 60°C for 30 minutes. The flasks are then removed from the shaker and cooled to room temperature.
- b. The contents of each flask are transferred into 250 mL separatory funnels. To each emptied flask, 15 mL of water is added, the flask is closed with a stopper, and shaken vigorously. The rinse is transferred to the corresponding separatory funnel. Each flask is then rinsed again with 60 mL of hexane, and the rinse is transferred to the same funnel. Each funnel is closed with a stopper and shaken vigorously for 90 seconds. The layers are allowed to separate for approximately 10 minutes. The aqueous layer is drained and discarded. Next, 15 mL of water is added to the hexane layer remaining in each funnel. The funnel is closed with a stopper and shaken vigorously. After allowing the layers to separate, the aqueous layer is drained and discarded.
- c. Each funnel adds one drop of phenolphthalein solution (3) and 15.0 mL of water. A 10% acetic acid solution (5) is added dropwise, shaking, until the washing is neutral to phenolphthalein (colourless). The aqueous layer is drained and discarded. The hexane layer is drained through sodium sulfate

(Na₂SO₄), supported by a small cotton plug, into a 100 mL round-bottom flask. Each funnel and the Na₂SO₄ bed are rinsed with a few millilitres of hexane, and the rinses are collected in the same round-bottom flask.

C. Evaporation and solid-phase extraction

- The round-bottom flask is placed on a rotary evaporator, and the hexane is evaporated to dryness at 40 °C. Immediately after evaporation, 2.0 mL of dichloromethane-IPA solution [(4)(a)] is added to the flask.
- The SPE column is washed with 4.0 mL of dichloromethane-IPA solution [(4)(b)], followed by 5.0 mL of dichloromethane-IPA solution [(4)(a)]. The solution from the round-bottom flask is transferred to the SPE column. The empty flask is rinsed with 1.0 mL of dichloromethane-IPA solution [(4)(a)], and the rinse is transferred to the SPE column. The SPE column is washed with 2.0 mL of dichloromethane-IPA solution [(4)(a)], and this fraction is discarded. Vitamins D₂ and D₃ are eluted with 7.0 mL of dichloromethane-IPA solution [(4)(a)] into a 16 × 100 mm disposable culture tube.
- The culture tube is placed in a water bath, and the dichloromethane-IPA solution is evaporated at 40 °C using nitrogen. Once dry, 1.0 mL of acetonitrile is immediately added to the tube, and the solution is swirled to rinse down the sides of the tube. The solution is transferred to an LC vial using a disposable dropper.

D. Chromatographic Determination

- The standard mixture is injected into the LC column at the beginning, middle, and end of each run of test solutions.
- The content of vitamins D₂ and D₃ is calculated using the internal standard procedure and peak heights.

E. Calculation

Perform following calculations:

- The concentration of vitamin D₂ in the standards mixture (CSD₂) is calculated as follows:

$$CS_{D2}(IU/mL) = \frac{WX4X4X4X40000}{250X250X250} \times 1.05$$

Where:

W = weight of vitamin D₂ certified reference standard (mg)

4 = dilution factor (repeated three times for serial dilutions)

40,000 = IU of vitamin D per mg

250 = volumes of subsequent dilutions of the vitamin D₂ standard solution (mL)

1.05 = correction factor for pre-vitamin D

- The concentration of vitamin D₃ in the standards mixture (CSD₃) is calculated using the same formula above, replacing the weight with that of the vitamin D₃ certified reference standard.

- The response ratio of vitamin D₂ in the standards mixture (RSD₂) is calculated as follows:

$$RS_{D2} = \frac{PS_{D2}}{PS_{D3}}$$

Where:

PSD₂ = peak height of vitamin D₂ in the standards mixture

PSD₃ = peak height of vitamin D₃ in the standards mixture

- The response ratio of vitamin D₃ in the standards mixture (RSD₃) is calculated similarly, using the peak heights of vitamin D₃ and vitamin D₂, respectively.
- The response ratio of vitamin D₂ in the test portion (RTD₂) is calculated as follows:

$$RS_{D2} = \frac{PS_{D2}}{PS_{D3}}$$

Where:

PTD₂ = peak height of vitamin D₂ in the test portion

PTD₃ = peak height of vitamin D₃ in the test sample

- f. The response ratio of vitamin D₃ in the standards mixture (RSD₃) is calculated similarly, using the peak heights of vitamin D₃ and vitamin D₂, respectively.
- g. The concentration of vitamin D₂ (CTD₂) or vitamin D₃ (CTD₃) in the test portion is calculated using the following formulas:

$$CS_{D_2}(IU/mL) = \frac{RT_{D_2} \times CS_{D_2}}{RS_{D_2}} \times \frac{U \times D}{V_t}$$

$$CS_{D_3}(IU/mL) = \frac{RT_{D_3} \times CS_{D_3}}{RS_{D_3}} \times \frac{U \times D}{V_t}$$

Where:

V = volume of test portion (mL), usually 15

U = conversion factor to appropriate units (if necessary)

D = dilution factor for diluted powders or liquids

F. System Suitability

The standards mixture D (i.1) for routine operation assesses system suitability. The resolution factor between vitamin D₂ and vitamin D₃ is expected to be 2.0.

The resolution factor is calculated using the following formula:

$$R = \frac{2(t_2 - t_1)}{W_1 + W_2}$$

Where:

t₁ and t₂ = elution times for vitamins D₂ and D₃, respectively

W₁ and W₂ = peak widths of vitamins D₂ and D₃.

Peak width is measured as the time interval between the points where the baseline intersects the two lines produced by extending the relatively straight sides of the peak.

Separation between these peaks should be sufficient to allow additional peaks, namely pre-vitamin D₃, to be resolved between vitamin D₂ and D₃.

To verify that pre-vitamin D₃ is resolved, the system suitability standard solution, C(j), is injected. This solution contains, in order of elution, pre-vitamin D₂, vitamin D₂, pre-vitamin D₃, and vitamin D₃.

Optimize chromatography by adjusting the amount of methanol in the mobile phase, a decrease in methanol content increases retention times. Maintain column temperature at 27 ± 1°C; increased temperature decreases retention and vice versa. Six replicate injections of the standard should have <2% relative standard deviation (RSD). RSD values for standards throughout the run should be <4%.

Reference

Official Methods of Analysis of AOAC INTERNATIONAL (2019) 21st Ed., AOAC INTERNATIONAL, Method 995.05

Method C: Vitamin D in Food Using HPLC with UV Detector

Principles

The method for Vitamin D₂ and D₃ is a reverse phase HPLC procedure, employing a mixture of acetonitrile and methanol (75:25, v/v) as the mobile phase at a flow rate of 2.3 mL/min and a C18 column. The UV detection of the evaluate is usually performed at 282 nm. The vitamins D₂ and D₃ are determined by comparing the retention times of standards obtained, and quantification is done by using a calibration curve.

Chemicals/Reagents

1. Vitamin D₂ and D₃ standard
2. Cholecalciferol as the internal standard
3. Sodium ascorbate
4. Sodium hydroxide, NaOH
5. Ethanol (95% pure)
6. Potassium hydroxide (85% pure)
7. Ethanol
8. n-pentane
9. Potassium Hydroxide, KOH
10. Mixture of acetonitrile and methanol (HPLC grade, Merck Chemicals (75/25, v/v) as the mobile phase:
 - a. 750 mL of acetonitrile is added to a one-litre measuring cylinder. 250 mL of methanol is then added. Using the solvent filtration kit, solvents are filtered through a 0.45 µm nylon membrane filter and degassed using an ultrasonic bath.

Apparatus/Instruments

1. HPLC Condition:
 - a. A Waters 600E HPLC system equipped with Waters 486 tunable absorbance UV detector (Waters, Milford, MA, USA).
 - b. Eluted through a reverse phase C18 column (Maxsil 5 C18, 250 · 4.6 mm, Phenomenex, Torrance, CA, USA).
2. Balance: Analytical sensitivity ± 0.1 mg
3. Rotary evaporator
4. Round bottom flask: 250 mL
5. Measuring cylinder: 10, 25 & 50 mL
6. Reflux apparatus
7. Separating funnel
8. Conical flask: 250 mL
9. Volumetric flasks: 5 mL
10. Pasteur pipette
11. Ultrafiltration equipment: using 0.45 µm, 47 mm diameter membrane filters
12. Ultrasonic bath: for degassing solvent
13. Syringe filter: 0.45 µm non-pyrogenic filter
14. Syringe
15. Vials

Procedures

A. Preparation of Sample

1. 0.5 g of samples are to be accurately weighed into 250 mL round bottom flasks and mixed with 4 mL of sodium ascorbate solution (17.5 g of sodium ascorbate in 100 mL of 1 M NaOH), 50 mL of ethanol (95% pure), 10 mL of 50% potassium hydroxide (85% pure) and 50 µg of cholecalciferol as the internal standard.
2. The mixture will be saponified under reflux at 80°C for 1 hour, then immediately cooled to room temperature and transferred into a separating funnel.
3. The mixture is first extracted with 15 mL de-ionized water, followed by 15 mL ethanol and then with three stages of n-pentane of volumes 50, 50 and 20 mL, respectively.
4. The pooled organic layers are washed three times with 50 mL of 3% KOH in 5% ethanol and then finally with de-ionized water until neutralized.
5. The organic layer is transferred into a round-bottom flask, rotary evaporated to dryness at 40°C, and immediately re-dissolved in 5 mL of ethanol.
6. The samples are passed through a 0.45 µm non-pyrogenic filter.
7. 20 µL of filtered samples is injected into the HPLC system.

Reference

Jasinghe, V.J. and Perera, C.O. 2005. Distribution of ergosterol in different tissues of mushrooms and its effect on the conversion of ergosterol to vitamin D2 by UV irradiation. *Food Chemistry*, 92:541-546

3.3.3.3 DETERMINATION OF VITAMIN E IN FOOD

Method A: Determination of Vitamin A (Retinol) and Vitamin E and Alpha-Tocopherol by Cold Saponification in Food

Principles

The samples are saponified in basic ethanol-water solution, neutralized, and diluted, converting fats to fatty acids and tocopherol. Retinol and alpha-tocopherol are quantified on a separate LC system, using fluorescence detection for both retinol and alpha-tocopherol.

Chemicals/Reagents

All reagents were AR grade unless otherwise specified:

1. Vitamin standards: Alpha-Tocopherol, stored below 0°C in the absence of light
2. Ethanol
3. Acetic Acid-glacial
4. Tetrahydrofuran
5. Pyrogallol Acid
6. Potassium Hydroxide (KOH)
7. Methanol - HPLC Grade
8. Hexane

Apparatus/ Instruments

1. High Performance Liquid Chromatography consists of:
 - a. Solvent delivery system
 - b. Autosampler/ injector or a manual injector
 - c. Integrator or data station or equivalent for data processing
 - d. Detectors:
 - i. Vitamin A-fluorescence detection (excitation at 325 nm and emission at 475 nm)
 - ii. Vitamin E-fluorescence detection (excitation at 295 nm and emission at 330 nm)
 - e. Columns: Reverse-phase C18, 4µm, 3.9 mm x 150 mm steel column or equivalent.
2. 250 mL round-bottom flask with stopper (preferably amber)
3. Stirrer plate
4. Nitrogen flow
5. 100 mL volumetric flask (preferably amber)

Typical Operating Condition:

Mobile Phase

	Time	Flow	Methano I	Methano I	UHQ Water	Acetonitril e	Curve
1		1.00	86.0	0.0	14.0	0.0	
2	10.00	1.00	94.0	0.0	6.0	0.0	6.0
3	25.00	1.00	100.0	0.0	0.0	0.0	6.0
4	35.00	1.00	86.0	0.0	14.0	0.0	6.0
5	40.00	0.80	0.0	50.0	50.0	0.0	11.0
6	45.00	0.50	0.0	50.0	50.0	0.0	11.0
7	50.00	0.20	0.0	100.0	0.0	0.0	11.0

Table 3.3.3.3A1: Mobile phase, Gradient Mode

Flow: 1.0 mL/min, gradient mode

Volume of injection: 40 µL

Run time: 27 mins

Detector: Fluorescence - excitation at 325 nm and emission at 475 nm (Vitamin A)

Fluorescence - excitation at 295 nm and emission at 330 nm (Vitamin E)

Preparation of Standards

Individual stock vitamin standards:

1. Vitamin A-(1) Using USP standard - 70 mg of vitamin A palmitate concentrate is weighed into a 100 mL volumetric flask. The weight is recorded to the nearest 0.1 mg. Less than 3 mL of acetone is added to aid dissolution. A pea-sized amount of pyrogallol acid is added. The mixture is dissolved and diluted to volume with hexane.
2. Vitamin E-80 mg of vitamin E acetate (α -tocopherol acetate) is weighed into a 100 mL volumetric flask. The weight is recorded to the nearest 0.1 mg. Less than 3 mL of acetone is added to aid dissolution. The solution is diluted to volume with 95% ethanol.

**Note: Store at 4°C in dark. Vitamin A working solutions are stable for 2 weeks and vitamin E working solutions are stable up to 1 month.*

Intermediate stock vitamin standards

1. Vitamin A (10.0 $\mu\text{g/mL}$) - 5 mL of stock vitamin A standard is taken and diluted in a 100 mL volumetric flask with ethanol.
2. Vitamin E (40.0 $\mu\text{g/mL}$) - 5 mL of stock vitamin E standard is taken and diluted in a 100 mL volumetric flask with ethanol.

Standard Calibration

Set up the following mixed standard for vitamin A and Vitamin E. Place the appropriate volume of each standard into a 250 mL round-bottom flask, then proceed to step 2.

Time	Volume of Working Standard (mL)				Volume of 95% Ethanol (mL)
	Vitamin A (mL)	From stock	Vitamin E (mL)	From stock	
Standard 5	10	Intermediate 6.2.1	1.5	Stock 6.1.2	28.5
Standard 4	5		1.0		34
Standard 3	3		10.0	Intermediate	27
Standard 2	1	6.2.2	3.0		36
Standard 1	0.4		1.0		39

Table 3.3.3.3A2: Standard Calibration

Sample Preparation

1. To prepare low-fat samples, 5 g of sample is weighed into a 250 mL round-bottom flask; to prepare high-fat samples, 2 g of sample is weighed into a 250 mL round-bottom flask.
2. 40 mL of 95% ethanol is added to each flask.
3. A pea-sized amount of pyrogallol acid (antioxidant) is added to each standard and sample.
4. Each flask is swirled to ensure thorough dispersion of all materials in the solution.
5. 10 mL of 50% KOH solution is pipetted into each flask.
6. Each flask is swirled slowly again.
7. A white magnetic stir bar is added.
8. A light stream of nitrogen gas is passed into the flask for approximately 1 minute.
9. The flasks are stoppered and sealed with parafilm.
10. The flasks are placed on a stirrer plate and left overnight (15–16 hours) to allow saponification to occur.
11. After saponification, 10 mL of glacial acetic acid is added to each flask, and the contents are swirled slowly.
12. A light stream of nitrogen gas is passed through the solution again to accelerate cooling.
13. The contents of each flask are transferred into a 100 mL volumetric flask using a 1:1 (v/v) tetrahydrofuran (THF): 95% ethanol mixture. The solution is then made up to 100 mL with the same solvent mixture.
14. Each volumetric flask is stoppered and inverted 10 times to ensure mixing.
15. The flasks are allowed to stand at room temperature for at least 1 hour, preferably overnight in a refrigerator, or are centrifuged at 7000–9000 rpm for about 15 minutes.

Quality control and Assurance

1. A quality control sample is run for every daily batch of samples.
2. A suitable sample is spiked with Vitamin A intermediate standard and Vitamin E intermediate/stock standard as follows:
 - a. Quality control 1: 1 mL of Vitamin A intermediate standard (approx. 5 µg) and 3 mL of Vitamin E intermediate standard (approx. 50 µg)
 - b. Quality control 2: 5 mL of Vitamin A intermediate standard (approx. 25 µg) and 1 mL of Vitamin E stock standard (approx. 400 µg)
3. The spiked samples are prepared and then processed according to the procedure described in Sample Preparation Steps 1–11.
4. A blank sample is prepared using the same sample without any standard added and processed in parallel using Sample Preparation Steps 1–11.

LC Analysis

1. The HPLC system is stabilised according to the laboratory work instruction.
2. A volume of 40 µL of each working standard solution is injected.
3. Calibration curves for all vitamins are plotted separately according to the manufacturer's manual or laboratory work instructions.
4. A volume of 40 µL of control standards is injected to verify calibration accuracy.
5. A volume of 40 µL of each sample extract is injected, and an integration report is obtained for quantification.
6. LC response is monitored by injecting one of the working mixed standards used to define the calibration curve (typically the mid-level) after every 10 samples. The monitored value is expected to be within ±5% of the calibration curve value; otherwise, recalibration may be required.

Calculation

Quantitation is achieved using software-controlled linear regression analysis of a minimum of three calibration standard levels.

Amount of vitamin A,

$$\mu\text{g} / 100 \text{ g} = \frac{H \times 100\text{mL} \times 100}{W_{\text{taken}}}$$

Amount of vitamin E,

$$\text{mg} / 100 \text{ g} = \frac{H \times 100\text{mL} \times 100}{W_{\text{taken}} \times 1000}$$

Where, H= amount reading from HPLC, µg/mL

Reporting

1. Results are reported as g per 100 g, per 100 mL, or as declared on the food or beverage label.
2. The result for vitamin A is rounded to the nearest whole number, and the result for vitamin E is reported to one decimal place.

Performance Characteristic

Analysis done on creamer (non-dairy product) and cheese (milk product)

Sample preparation was done in replicates on different days.

Table 3.3.3A3: Performance Characteristic

	VITAMIN A	VITAMIN E
PRECISION (From recovery data)	5.82%	4.70%
RECOVERY	102%	90.4%
MU (95% CL)	0.22 X Cone (Vitamin A)	0.18 X Cone (Vitamin E)
LOD	50 µg/100 g	1.9 mg/100 g
LOQ	150 µg/100 g	6.0 mg/100 g
Linearity	0.04- 1.20 µg/mL	0.40-12.00 µg/mL

Reference

Determination of Vitamin A (Retinol) and E (alpha-tocopherol) in foods by liquid chromatography: collaborative study, Journal of AOAC International vol. 85, No. 2, 2002

In-house based on JKM F 1203 Determination of Vitamin A (Retinol) and Vitamin E (alpha-tocopherol) in food by HPLC

Method B: Vitamin E in Food using HPLC with Fluorescence Detector

Principles

The determination of Vitamin E in food using High-Performance Liquid Chromatography (HPLC) with a fluorescence detector involves extracting Vitamin E from the food sample with solvents, followed by injecting the extract into the HPLC system. The Vitamin E compounds, such as tocopherols and tocotrienols, are separated using a reverse-phase column and detected based on their fluorescence properties. The intensity of the fluorescence signal, which corresponds to the concentration of Vitamin E, is measured and quantified using calibration curves, making this method highly sensitive and selective for accurately determining Vitamin E levels in complex food matrices.

Chemicals/Reagents

1. Tocopherol standard solution, Davos, Singapore
2. Tocotrienol standard solution, Davos, Singapore
3. Internal standard, PMC (2,2,5,7,8-Pentamethyl-6-chromanol), Sigma-Aldrich, USA
4. Hexane, Merck, Germany
5. Isopropyl alcohol, J.T. Baker, USA
6. Ethanol, Merck, Germany
7. Dioxane, R&M, UK
8. NaCl, Merck, Germany

Apparatus/ Instruments:

1. Balance: Analytical sensitivity ± 0.1 mg
2. Centrifuge
3. Nitrogen gas blower
4. Micropipettes
5. 12 mL test tube: with screw cap
6. 7 mL Trident vial
7. Orbital laboratory shaker

Procedures

A. Extraction of food samples

1. A homogenized food sample (1–5 g) is weighed into a 12 mL test tube.
2. A volume of 1 mL 0.9% NaCl is added and shaken vigorously. Then, 1 mL of ethanol and 4 mL of hexane are added to the tube.
3. The mixture is shaken for 1 hour at 1000 rpm using a shaker.
4. The sample is centrifuged at 2000 rpm for 15 minutes. The supernatant is transferred into a 7 mL Trident vial.
5. An additional 2 mL of hexane is added into the same 12 mL test tube.
6. The extraction is repeated.
7. The resulting supernatants are combined and dried by blowing with nitrogen gas.
8. The dried sample is reconstituted with hexane.
9. The sample is ready for HPLC injection.

B. HPLC conditions

1. The determination of vitamin E tocopherol and tocotrienol in this procedure is using HPLC Agilent 1100 series.
2. The HPLC is equipped with the Agilent model FLD G1321 A fluorescence spectrophotometer and Agilent Chemstation for LC System Rev. A.06.0x.
3. The mobile phase is prepared using a mixture of 970 parts hexane, 25 parts dioxane, and five parts propyl alcohol (v/v).
4. The mobile phase is delivered and degassed with Agilent G1311A Quaternary Pump and JP73021896 Degasser at 1 mL/min flow rate.
5. The analytical column used is a Phenomenex® Luna 5 μ (250 $\times$ 4.6 mm I.D, 5 μ m).

6. The extraction is repeated.
7. The resulting supernatants are combined and dried by blowing with nitrogen gas.
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4. The mobile phase is delivered and degassed with Agilent G1311A Quaternary Pump and JP73021896 Degasser at 1 mL/min flow rate.
5. The analytical column used is a Phenomenex® Luna 5 μ (250 $\times$ 4.6 mm I.D, 5 μ m).
6. The injection system used is Agilent Auto Injector ALS G1313A and the injection volume is 40 μ L.
7. The fluorescence detector is set at an excitation wavelength of 295 nm and an emission wavelength of 325 nm.
8. The standard solution is prepared from 0.05 μ g/mL to 10 μ g/mL consists of α , β , γ and δ tocopherol and tocotrienol including PMC as internal standard.
9. The calibration curve is based on the linearity of peak area response versus standard dilution/solution.
10. The sample is injected into HPLC and analysed with Agilent Chemstation.

References

- 1) Nielsen M.M and Hansen A. (2008). Rapid high-performance liquid chromatography determination of tocopherols and tocotrienols in cereals. *Cereal Chem.* 82 (2):248-251.
- 2) Cunha S.C., Amaral J.S., Fernandes J.O. and Oliveira B.P.P. (2006). Quantification of tocopherols and tocotrienols in Portuguese olive oils using HPLC with three different detection systems. *J Agric Food Chem.* 54(9):3351-3356.

Method C: Vitamin E in Vegetable Oils and Fats using HPLC with Fluorescence Detector

Principles

The oils or fats (or unsaponifiable matter obtained from a processed product containing tocopherol esters) are dissolved in an organic solvent and subjected to direct HPLC separation of the individual tocopherols and tocotrienols. Calibration factors are determined for each tocopherol from the chromatography of solutions of standard tocopherols; calibration factors for tocotrienols are equivalent to those of the corresponding tocopherols. The tocopherol and tocotrienol content of oil or fats is the quantity of tocopherols and tocotrienols, determined in the sample by the described procedure and expressed in micrograms per gram ($\mu\text{g/g}$).

Chemicals/Reagents

1. Alpha, beta, gamma and delta tocopherol standards
2. Methanol
3. Dichloromethane
4. Hexane
5. Propan-2-ol
6. Mixture of propan-2-ol in hexane (0.5/99.5, v/v) as mobile phase
 - a. 5 mL of propan-2-ol is pipetted into a one-litre measuring cylinder. Nine hundred ninety-five mL of hexane is added. The solvents are then filtered through a 0.45 μm nylon membrane filter using the solvent filtration kit and degassed using an ultrasonic bath.

Apparatus/Instruments

1. HPLC system: consisting of a high-pressure pump, sample injection device, fluorescence detector and recording integrator. A fluorescence detector should preferably be used with the excitation wavelength set at 290 nm and the emission wavelength at 330 nm. A UV detector may be used if a fluorescence detector is not available. The wavelength of the UV detector should be set at 292 nm.
2. HPLC analytical column: (250 mm x 4 mm) packed with micro particulate silica having a mean particle size of about 5 μm .
3. HPLC Syringe (10 μm)
4. HPLC amber vial: size 3 mL
5. Rotary evaporator
6. Water bath
7. Round bottom flask: 250 mL
8. Buchner funnel
9. Whatman filter no.1

Note-All glassware should be of low actinic activity.

Procedures

A. Preparation of solutions of tocopherol standards

- a. Alpha-tocopherol standard stock solution:
 - i. A stock solution of alpha-tocopherol is prepared by accurately weighing approximately 10 mg of the standard into a 100 mL volumetric flask and making up to volume with hexane.
 - ii. A 10 mL aliquot of this solution is pipetted into an amber round-bottom flask, and all hexane is removed using a rotary evaporator at a temperature not exceeding 40 °C.
 - iii. Nitrogen restores atmospheric pressure, and the flask is removed from the evaporator immediately after the solvent is fully evaporated.
 - iv. A volume of 10 mL of methanol is pipetted into the flask, and the solution is swirled to dissolve the tocopherol residue.
 - v. The absorbance of the resulting solution is measured at 292 nm. The concentration of alpha-tocopherol (in $\mu\text{g/mL}$) is calculated by dividing the absorbance value by 0.0076.

- b. Beta, gamma and delta-tocopherol standard stock solutions:
 - i. Stock solutions and aliquots of beta-, gamma-, and delta-tocopherol standards are prepared following the same procedure described for the alpha-tocopherol standard stock solution
 - ii. The absorbance of each tocopherol solution is measured at its respective wavelength. The concentration of each tocopherol is calculated using the appropriate divisor factor specific to the tocopherol isomer and its absorbance wavelength
 - 296 nm beta-tocopherol= 0.0089
 - 298 nm gamma-tocopherol= 0.0091
 - 298 nm delta-tocopherol= 0.0087
- c. Mixed tocopherol standards working solution:
 - i. Appropriate volumes of the tocopherol stock solutions are mixed to obtain a mixed tocopherol standard working solution.
 - ii. The mixed solution is diluted with hexane to obtain a final 1–5 µg/mL concentration for each tocopherol.

Note: A more concentrated solution may have to be prepared if a UV detector is used. All standards must be protected from light and stored in the refrigerator.

B. Optimization of working parameters

- a. The column is conditioned, if necessary. The propan-2-ol/hexane mobile phase is pumped through the column at a flow rate of 1 mL/min for at least 30 minutes. Approximately 20 µL of the mixed tocopherol standards working solution is injected onto the column. If required, the propan-2-ol content of the mobile phase and the flow rate are adjusted to achieve the desired chromatographic conditions as follows:
 - i. Alpha-tocopherol retention time is not less than 5 min.
 - ii. The resolution factor (R) for the separation of beta and gamma-tocopherol must be at least 1.0, which is almost the baseline separation.
- b. The optimum detector and integrator sensitivity and chart speed are selected. Approximately 20 µL of the mixed tocopherol standards working solution is injected, and the injection is repeated to ensure reproducible chromatograms.

C. Preparation of the test sample

- a. The test sample is prepared for liquid samples by homogenisation, ensuring that filtration is avoided.
- b. For solid samples, a representative portion (not less than 10% by weight of the laboratory sample) is transferred into a glass beaker. The sample is carefully homogenised by melting with gentle mixing in a water bath at a temperature not exceeding 40°C.
- c. The test sample preparation should be done in subdued light, preferably from direct sunlight.

D. Preparation of the test solution

- a. 2 g of the prepared test sample is accurately weighed into a 25 mL volumetric flask.
- b. A sufficient quantity of hexane is added, and the flask is swirled to dissolve the sample. The solution is made up to volume with the same solvent.
- c. If a fluorescent detector is used, further dilution of the solution prior to chromatography is necessary. It is important that the test solutions are protected from light prior to analysis and analysed on the day of preparation.

E. HPLC determination of tocopherols in the test solution

- a. A volume of 20 µL of the mixed tocopherol standards working solution is injected onto the column, and the areas of the tocopherol peaks are recorded. If an integrator is unavailable, peak heights (measured in mm) are recorded.
- b. A volume of 20 µL of the test solution is injected onto the column, and the tocopherols (and tocotrienols) present are identified by referring to the chromatograms obtained from the standards. The areas of the tocopherol peaks (or peak heights) are recorded. The areas of any tocotrienol peaks are recorded if present and required for quantification. Duplicate injections of the test solution are performed to ensure reproducibility.
- c. A further 20 µL of the mixed tocopherol standards working solution is injected, and the areas of the tocopherol peaks are recorded.

F. Number of determinations

- a. Two determinations (each consisting of duplicate injections of the prepared test solutions) are carried out in rapid succession, using a fresh test portion for each determination, analysed on the day of preparation.

Calculations

1. The alpha-tocopherol content of the sample in µg/g is given by:

$$\frac{CxaxDx25}{Axm}$$

Where:

C = concentration of the alpha-tocopherol standard (µg/mL)

A = mean of the peak areas obtained for the alpha-tocopherol standard

a = mean of the peak areas obtained for the alpha-tocopherol in test sample

m = mass of test sample taken

D = dilution factor

2. The beta, gamma and delta-tocopherol contents of the test sample are calculated similarly using the data from chromatography of the corresponding tocopherol standard.
3. The tocotrienol content of a sample can be estimated using C and A values for the corresponding tocopherol.

Precision:

When analyzing processed products such as margarines and samples containing added tocopherol esters, a cold saponification procedure must be performed prior to chromatography.

The saponification procedure:

1. If no tocopherol standards are available, a blend of wheat germ and soya bean oil can be used to obtain chromatograms containing alpha-, gamma- and delta-tocopherols.
2. These can be used to assist peak identification in test sample chromatograms.
3. Palm oil can be used to identify alpha- and gamma—tocotrienols if required.
4. The following relative retention times have been found to be typical:
 - Alpha tocopherol = 1.0
 - Beta-tocopherol = 1.6
 - Delta-tocopherol = 3.0
 - Gamma-tocopherol = 1.7
5. If fluorescence detection is used and the only standard available is the alpha-tocopherol standard, this should be clearly stated when reporting results.
6. If UV detection is used, again relate all tocopherols to the alpha-tocopherol standard, but normalize the peak areas to alpha-tocopherol using the divisor values noted in the procedure for preparing standard stock solutions.
7. Cold saponification procedure for samples containing added tocopherol esters.

References

- 1) A.O.C.S Official Method Ce 8-89. Revised 2017
- 2) A.O.C.S Official Method Ce 8-89. 1990.

3.3.3.4 DETERMINATION OF VITAMIN K IN MILK AND INFANT FORMULAS USING HPLC

Principles

Following the enzymatic digestion of fat and precipitation of fatty acids, vitamin K is extracted with hexane. Vitamin K is separated by HPLC with post-column reduction and quantitated by fluorescence detection with an external standard.

Phylloquinone geometric isomers are quantitated as a single unresolved peak with a C18 or may be estimated selectively with a C30 column (K trans < K cis) under the reversed phase employed.

It is also important to note that menaquinone-4 [MK-4] will also be present in milk and infant formulas incorporating milk fat. At the same time, 2'3'-dihydrophyloquinone may be present at appreciable levels in infant formulas containing hydrogenated soya or canola oils. If required, both may be estimated against appropriate standards. The elution order is MK-4 < K1 < 2'3'-dihydrophyloK1 [relative retention times are ca 0.6, 1.0, and 1.2, respectively].

Chemicals/Reagents

Use AR grade reagents unless otherwise stated.

1. Water-purified to >18 MΩ resistivity
2. Nitrogen-Cylinder N (low O content)
3. Lipase from *Candida rugosa*, ca 1000 units/mg (Type VII, Sigma L-1754). Other sources of lipase can be used from *Pseudomonas* and *Rhizopus* species with the correct enzyme activity.
4. Monobasic potassium phosphate (KH₂PO₄)
5. Potassium hydroxide solution -40% (w/v). Dissolve 40g KOH (10) in water and dilute to 100 mL.
6. Phosphate buffer -0.8M. Dissolve 54.0 g KH₂PO₄ (4) in 350 mL of water. Adjust pH to 7.9-8.0 with 40% KOH solution and dilute to 500 mL.
7. Ethanol
8. Methanol-LC grade
9. Reagent alcohol-Mix ethanol, (7), with methanol, (8), (95 + 5, v/v)
10. Potassium hydroxide
11. Zinc chloride
12. Sodium acetate-Anhydrous
13. Acetic acid-Glacial
14. Potassium carbonate-Anhydrous
15. Zinc powder-<60 μm. Use a freshly opened bottle.
16. Hexane-HPLC grade
17. Dichloromethane-HPLC grade
18. Isopropanol- HPLC grade
19. Mobile phase- To dichloromethane (17) and methanol (8), (100 + 900, v/v), 5 mL of methanol containing ZnCl₂ (1.37 g; 11), anhydrous sodium acetate (0.41 g; 12), and glacial acetic acid (0.30 g; 13) is added. The mixture is then filtered through a 0.45 μm filter and degassed.
20. Zinc post-column reductor-The 20 x 4 mm assembly B(c) is dry packed with Zn powder (15). Voids are minimized by sequentially adding small amounts with frequent gentle tapping. With longer columns, extra care is used. The reductor column is re-prepared before each analytical schedule. The reduction efficiency of Zn powder should be monitored over time and replaced every 1-2 years as required.
21. Vitamin K1 (phylloquinone)-USP grade. All operations must be conducted under low incandescent light conditions and in low-actinic volumetric flasks.
 - a. Stock solution-ca 1.0 mg/mL. Approximately 100 mg, accurately weighed, is dissolved in 100.0 mL of isopropanol (18) with warming at approximately 30°C and standing. It is then stored under N₂ (2) at -10°C for up to 6 months.
 - b. Intermediate I-ca 50 μg/mL. 5.0 mL of stock is diluted with methanol to 100.0 mL and stored under N₂ at -10°C for up to 1 month.
 - c. Intermediate II-ca 2.5 μg/mL. 5.0 mL of Intermediate I is diluted with methanol to 100.0 mL. It is prepared daily.

- d. Working standards-ca 6.25, 12.50, 18.75, 25.00 and 31.25 ng/mL. Respectively, 0.25, 0.50, 0.75, 1.00, and 1.25 mL of Intermediate II are diluted with methanol (8) to 100.0 mL. These are prepared daily, and accurate concentrations are calculated as follows: Accurate concentrations for working standards are calculated after phylloquinone purity is estimated from absorbance (248 nm) of a solution. This solution is prepared by evaporating 5.00 mL of Intermediate I under N flow, and the residue is then re-dissolved in 25.0 mL hexane ($a_{1\%}^{1\text{cm}}=419$).

$$\text{Theoretical } A_{248} = \frac{W}{100} \times \frac{5}{100} \times 5 \times 419 \times 100$$

Where W = weight of phylloquinone in stock standard (g)

$$\text{Purity Factor (PF)} = \frac{\text{measured } A_{248}}{\text{theoretical } A_{248}}$$

$$K_1 \text{ in intermediate II } (\mu\text{g /mL}) = \text{PF} \times \frac{W}{100} \times \frac{5}{100} \times 5 \times \frac{106}{100}$$

The accurate vitamin K1 concentration in the 5 working standards (ng/mL) is then calculated by multiplying PF by 2.5, 5.0, 7.5, 10.0, and 12.5, respectively.

Apparatus/Instruments

1. HPLC system-manual or automated isocratic system: incorporating a fluorescence detector (λ 243ex nm, λ 430em nm) exhibiting a Raman spectra signal noise specification of >200:1(water). Since UV detection is insensitive to vitamin K at the working concentration levels, configure with single mode fluorescence detection only to minimize extra-column band broadening. For multi-detector LCs, configure with the fluorescence detector in the first position. Ensure the absence of leaks.
2. Column: Any C18 column (monometric or polymeric) containing 5 μm spherical silica with $\geq 10\%$ carbon loading, preceded by a guard column of similar packing.
3. Post-column redactor: 20 x 4 mm stainless steel post-column assembly placed between analytical column and fluorescence detector (part NoWAT084550, Waters or equivalent). Alternative devices such as an empty (30-50 mm) LC column can be used.
4. Spectrophotometer: UV/Vis spectrophotometer, digital readout to 0.001 A.
5. Water bath: $37 \pm 1^\circ\text{C}$
6. Centrifuge: Fixed or swing arm rotor
7. Mechanical Shaker: orbital or wrist action
8. Vortex mixer
9. pH meter: Accurate to 0.01 pH with calibration buffers
10. Test tubes: 200 x 24 mm with ground-glass stoppers
11. Vials-Amber-tinted glass vial: (1.8-8.0 mL) with Teflon-sealed caps.
12. Volumetric flasks: low-actinic volumetric flasks, 100 mL

Procedures

All steps of the assay are performed under incandescent lighting without direct sunlight.

A. Preparation of test solution

a. Digestion:

- i. A portion of 1.0 g of powder or 10.0 g of liquid sample is weighed into a test tube. A reagent blank and a quality control test portion are included in each analytical schedule. A vortex mixer dissolves the powder sample in approximately 15 mL of warm water ($<40^\circ\text{C}$). For liquid samples, 5.0 mL of warm water is added and mixed.
- ii. 5.0 mL of phosphate buffer is added to each solution and mixed thoroughly.
- iii. 1.0 g of lipase powder is added and the mixture is vortexed.
- iv. The test tube is securely stoppered and shaken until well dispersed (30–60 seconds). The mixture is incubated at $37 \pm 2^\circ\text{C}$ for 2 hours, with shaking for 15 seconds at 20-minute intervals. After incubation, the mixture is cooled to ambient temperature by standing in water.
- v. 10 mL of reagent alcohol is added and mixed.
- vi. 1.0 g of potassium carbonate (K_2CO_3) is added and mixed.

b. Extraction:

(Note: Take precautions to prevent concentration of extracts through uncontrolled solvent evaporation).

- i. 30 mL of hexane is added to each test tube. The tube is stoppered and then shaken vigorously for ≥ 10 minutes using a mechanical shaker. The tube is then allowed to stand in the dark, preferably refrigerated, until the layers separate. If separation is slow (>10 minutes) or incomplete, the tube is centrifuged at approximately 1000 rpm (≈ 200 g) for 10 minutes. If necessary, a portion of the supernatant is decanted into a smaller centrifuge tube for centrifugation.

- ii. From the upper hexane phase, 1 mL is transferred for fortified products or 5 mL for non-fortified products into a clean vial. The solvent is evaporated to dryness under the flow of nitrogen (N₂). (Note: Larger extract volumes may require evaporation using a rotary evaporator, depending on the sensitivity of the fluorescence detector.) The dried extracts may be stored in the dark for 3–5 days at <0°C under nitrogen before LC analysis.

The dried residue is re-dissolved in 1 mL of methanol. The vial is sealed and vortex-mixed thoroughly. A low-volume vial or insert is recommended to minimize evaporation into the headspace if an autosampler is used.

B. HPLC determination

a. Set-up

- i. Prior to connecting the post-column zinc reduction assembly, stable LC operating conditions are established by equilibrating the analytical column with the mobile phase at approximately 1.0 mL/min for over ca 30 minutes. The fluorescence detector is set to an excitation wavelength (λ_{exc}) of 243 nm and an emission wavelength (λ_{em}) of 430 nm. (Note: Gain and sensitivity settings depend on the detector model and should be optimized accordingly.)
- ii. The zinc reduction assembly is installed between the analytical column and the detector. The system is further equilibrated for 30–60 minutes, ensuring anhydrous conditions in the mobile phase to preserve the zinc reducing potential and prevent leaks. The mobile phase must not be recycled.
- iii. A standard solution (20–50 µL) is injected to test the system. The flow rate is adjusted to elute phyloquinone (vitamin K₁) between 8–15 minutes, typically at 1.0–1.5 mL/min. Retention time may also be modified by adjusting the dichloromethane content in the mobile phase between 8–12%. The total run time is allowed to extend of ca 5 minutes beyond the elution of phyloquinone to ensure complete separation.

b. Calibration and analyses

- i. Methanol (standard blank) is injected to confirm the absence of chromatographic activity at the retention time of phyloquinone.
- ii. The lowest-level working standard is injected to ensure a repeatable response with a signal: noise ≥ 10.
- iii. Each working standard is injected and a linear response is ensured. The reagent blank and test extracts are injected sequentially, and a working standard is included after every 4 to 6 test solutions to monitor system stability.
- iv. When the analytical schedule is complete, the system is flushed with methanol, and the analytical column is stored in methanol when not in use.
- v. The post-column assembly is dismantled and emptied. The frits are soaked in 5 M HCl to clean them and then thoroughly rinsed with water and methanol. The assembly is thoroughly dried before being repacked with Zn.

C. Calculation

Construct a 5-level calibration using either peak area or height and calculate slope (s) by linear regression with forced zero. Calculate vitamin K₁ (cis and trans) content in test samples as follows:

$$\text{Vitamin K}_1(\mu\text{g}/100\text{ g}) = \frac{A'}{S} \times \frac{30}{V} \times \frac{100}{Wt} \times \frac{1}{1000}$$

Where:

A' = peak area (or height) of phyloquinone in test extract

Wt = weight of test portion (g)

V = volume, 1 mL (fortified) or 5 mL (non-fortified)

S = slope of calibration graph

Calibration and calculations may be achieved through data processing within the instrument or off-line.

Reference

Official Methods of Analysis of AOAC INTERNATIONAL (2019) 21st Ed., AOAC INTERNATIONAL, Method 999.15

3.3.4 DETERMINATION OF WATER-SOLUBLE VITAMIN

3.3.4.1 DETERMINATION OF WATER-SOLUBLE VITAMIN (B1, B2, B3, B5, B6, B7, B12, C) LEVEL IN FOOD SAMPLE (SOLID AND LIQUID)

Principles

The simultaneous determination of water-soluble vitamins (B1, B2, B3, B5, B6, B7, B12, and C) in food samples (solid and liquid) involves extracting the vitamins from the sample matrix using a suitable solvent. The extracted vitamins are separated using high-performance liquid chromatography (HPLC), typically reversed-phase chromatography. Detection is performed using UV-Vis. Quantification is achieved by comparing the sample's chromatographic peaks with calibration curves of standard solutions.

Chemicals/Reagents

1. Trifluoroacetic Acid (TFA) LC Grade
2. Methanesulfonic Acid (MSA) LC-MS
3. Acetonitrile LC Grade
4. Potassium Hydrogen Carbonate (KHCO₃)
5. Metaphosphoric Acid (HPO₃)
6. Glacial Acetic Acid 100%
7. Sodium Acetate

Instrumentation

1. UHPLC Photo-diode Array Detector / UPLC Single-quadrupole mass detector
2. Column C18 – 5 µm particle size, 4.6×250 mm (diameter and length)
3. Flow: 1.000 mL/min
4. Injection volume: 20 µL
5. Wavelength:
 - a. 274 nm: B1, B2, B3, B6, B9, B12, C
 - b. 204 nm: B5, B7

Mobile Phase and Gradient

1. Mobile phase:
 - a. Trifluoroacetic Acid pH 3.9 (A)
 - b. 100% Acetonitrile (B)
 - c. Methanesulfonic Acid 20 Mm (C)
 - d. Deionized water (D)

2. Table 3.3.4.1A: Gradient (solid sample)

Time (min)	Flow (mL/min)	Solvent A (%)	Solvent B (%)	Solvent C (%)	Solvent D (%)
0.0	1.00	95	5	0	0
2.5	1.00	90	10	0	0
4.5	1.00	85	15	0	0
6.5	1.00	70	30	0	0
8.5	1.00	60	40	0	0
9.0	1.00	60	40	0	0
9.5	1.00	95	5	0	0
15.5	1.00	95	5	0	0
Washing**					
0.0	1.00	0	0	100	0
15.0	1.00	0	0	100	0
15.5	1.00	0	90	0	10
25.0	1.00	0	90	0	10

3. Table 3.3.4.1B: Gradient (liquid sample)

Time (min)	Flow (mL/min)	Solvent A (%)	Solvent B (%)	Solvent C (%)	Solvent D (%)
0.0	1.00	95	5	0	0
2.5	1.00	80	20	0	0
5.0	1.00	70	30	0	0
7.0	1.00	35	65	0	0
7.5	1.00	35	65	0	0
8.0	1.00	95	5	0	0
11.0	1.00	95	5	0	0
Washing**					
0.0	1.00	0	0	100	0
15.0	1.00	0	0	100	0
15.5	1.00	0	90	0	10
25.0	1.00	0	90	0	10

Procedures

A. Mobile Phase Preparation

1. [STOCK] Trifluoroacetic Acid (0.025%) pH 2.6: 250 μ L of trifluoroacetic acid is added to a 1 L volumetric flask half-filled with deionized water. The solution is stirred, and deionized water is then added to the mark.
 - a. Trifluoroacetic Acid pH 3.9: A 100 mL volume of trifluoroacetic acid, pH 2.6, is transferred to a 2 L laboratory bottle. 1900 mL of deionized water is added. The pH is measured. The pH is adjusted with deionized water until it reaches pH 3.9.
2. 20 mM Methanesulfonic acid: A volume of 1.3 mL of methanesulfonic acid is added from the bottle to a 1 L volumetric flask that is half-filled with deionized water. The solution is stirred, and deionized water is added to the mark. The solution is transferred to a 1 L laboratory bottle.

B. Chemical Preparation

1. Potassium Hydrogen Carbonate (KHCO₃) 20 mM: Exactly 2 g of KHCO₃ is weighed and added to a 1 L volumetric flask half-filled with deionized water. The solution is stirred until dissolved, and deionized water is added to the mark.
2. [STOCK] 15.6% Metaphosphoric Acid (HPO₃): Approximately 24 g of HPO₃ is weighed and added to a 100 mL volumetric flask half-filled with deionized water. The solution is stirred until dissolved, and deionized water is added to the mark.
 - a. 0.4 M HPO₃: Approximately 3.3 g of HPO₃ is weighed and added to a 100 mL volumetric flask half-filled with deionized water. The solution is stirred until dissolved, and deionized water is added to the mark.
3. Acidified water pH 4.2: In 1 L deionized water, 0.005 M hydrochloric acid is added drop by drop until pH 4.2 is reached.
4. Reconstitute Solution: In a 500 mL volumetric flask, 482 mL of deionized water (96.4%), 12.50 mL of 20 mM KHCO₃ (2.5%), and 5.50 mL of 0.4 M HPO₃ (1.1%) are added. The solution is mixed well.
5. 1 M Acetic Acid: Deionized water is added to half the volume of a 250 mL volumetric flask. Then, 14.298 mL of 100% acetic acid is added. The flask is topped up to the mark with deionized water.
6. 50 mM Acetate buffer pH 4.5: Deionized water is added to half the volume of a 1 L volumetric flask. Then, 50 mL of 1 M acetic acid is added, followed by 2.36 g of sodium acetate. The solution is mixed well and topped up with deionized water to the mark.

C. Standard Preparation

1. 150 ppm Standard Preparation

- a. Each vitamin, except vitamin C, is individually weighed precisely at 1 mg, and the weight is recorded. To each vitamin (except B9), 20 mL of deionized water is added using a pipette.
 - b. For vitamin B2, the solution is sonicated until all particles are dissolved in water.
 - c. For vitamin B9, 5 mL of 20 mM KHCO₃ is added and stirred. Then, 250 µL of HPO₃ is added, followed by 14.75 mL of deionized water. For vitamin C, 50 µL of HPO₃ is added to stabilize the vitamin, then deionized water is added up to 19.95 mL to make a total of 20 mL.
 - d. All standards are prepared daily.
2. **15 ppm Mixture Standard:** 1 mL from each stock vitamin solution is taken and mixed. Then, 1.90 mL of deionized water and 100 µL of 0.4 M HPO₃ are added.
 3. **Standard Calibration:** The 15 ppm mixture solution is diluted to prepare calibration standards of 0.5 ppm, 1.0 ppm, 1.5 ppm, 2.5 ppm, and 5 ppm, respectively, in 1.5 mL vials.

D. Sample Preparation

1. Sample Extraction

- a. Solid Samples: A 5 g portion of the food sample is weighed into a 50 mL centrifuge tube, and the weight is recorded. A 0.50 g amount of each Taka-diastase enzyme and the protease enzyme from Papain is added. Then, 20 mL of 50 mM Acetate buffer (pH 4.5) solution is added. The mixture is stirred using a vortex stirrer. The sample is incubated in an oven at 37°C for 18 hours. After incubation, the mixture is stirred again using a vortex stirrer. The tube is centrifuged at 4000 rpm for 30 minutes. Then, 5 mL of the supernatant is collected and filtered using a 13 mm, 0.45 µm Nylon syringe filter.
- b. Liquid Samples: A 5 g portion of the liquid sample is weighed into a 50 mL centrifuge tube. Then, 20 mL of deionized water is added. The mixture is stirred using a vortex stirrer. The tube is centrifuged at 4000 rpm for 30 minutes. Then, 5.00 mL of the supernatant is collected and filtered using a 13 mm, 0.45 µm Nylon syringe filter.

2. Sample Clean-Up

- a. Solid-Phase Extraction (using Smart Prep): The C18 stationary phase is flushed with 10 mL of methanol and 10 mL of acidified water (pH 4.2). Then, 5 mL of the sample is added. The sample is eluted with 5 mL of acidified water and 10 mL of methanol at a flow rate of 1 mL/min. The eluted sample is collected.

3. Sample Reconditioning

- a. The sample is evaporated to dryness using a Sample Concentrator at 2000 rpm for 2.5 hours. The residue is dissolved in the Reconstitute solution, and the sample is ready for analysis.

References

- 1) O. Heudi, T. Kilinc, P. Fontannaz (2005). Separation of water-soluble vitamins by reversed-phase high performance liquid chromatography with ultra-violet detection: Application to polyvitamated premixes. *Journal of Chromatography A*.1070, 49-56.
 - 2) B. Klejdus, J. Petrlová, D. Pořešil, V. Adam, R. Mikelová, J. Vacek, R. Kizek, V. Kubáň (2004). Simultaneous determination of water and fat-soluble vitamins in pharmaceutical preparations by high-performance liquid chromatography coupled with diode array detection. *Analytica Chimica Acta*. 520, 57–67.
 - 3) Dionex Application Notes 251.
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3.3.4.2 DETERMINATION OF THIAMINE (VITAMIN B1) AND RIBOFLAVIN (VITAMIN B2) IN FOOD USING HPLC

Principles

Thiamine and riboflavin are water-soluble vitamins that are stable in an acid environment. Therefore, thiamine is extracted simultaneously with riboflavin through acid hydrolysis followed by enzyme digestion. At the end of digestion, thiamine and its phosphate ester are converted to thiochrome prior to HPLC analysis.

Chemicals/Reagents

1. Acetonitrile, HPLC grade
2. Takadiastase enzyme
3. Mobile phase preparation. Before analysis, filter the mobile phase (Acetonitrile and deionized water) with a 0.45 µm nylon membrane filter daily using a solvent filtration apparatus.
4. 0.1 N hydrochloric acid (HCl). Pipette 8.7 mL of HCl into a volumetric flask and make up to a volume of 1 L.
5. 1 M sodium acetate. Dissolve 82 g of sodium acetate in deionized water and top up to a volume of 1 L.
6. 1 M sodium hydroxide (NaOH). Dissolve 40 g of NaOH in deionized water and top up to a volume of 1 L.
7. **Thiamine standard solution**
 - a. Thiamine hydrochloride (99% purity) is used as a standard for thiamine determination.
 - b. Thiamine standard stock solution. Ten milligrams (10 mg) of the thiamine hydrochloride standard are precisely weighed and diluted to 100 mL with deionized water to achieve a concentration of 100 µg/mL. This solution is prepared freshly before analysis.
 - c. Thiamine standard working solution. Ten milliliters (10 mL) of the standard stock solution are accurately pipetted into a 100 mL volumetric flask. The volume is then brought to the mark with deionized water, yielding a final working solution with a concentration of 10 µg/mL.
8. **Riboflavin standard solution**
 - a. Riboflavin (≥ 98% purity) is used as a standard for riboflavin determination.
 - b. Riboflavin standard stock solution. Ten milligrams (10 mg) of riboflavin standard are weighed and made up to 100 mL with deionized water to yield a concentration of 100 µg/mL. This solution is to be prepared freshly prior to analysis.
 - c. Riboflavin standard working solution. Ten milliliters (10 mL) of the standard stock solution (b) are pipetted into a 100 mL volumetric flask, and the volume is then made up to the mark with deionized water. This results in a final working solution with a concentration of 10 µg/mL.

Apparatus/Instruments

1. Balance: Analytical sensitivity ± 0.1 mg
2. Volumetric flask: 100 mL
3. Conical flask: 100 mL
4. Filter funnel
5. Homogenizer
6. Incubator at 37°C
7. Water bath
8. Ultrafiltration system: using a 0.45 µm, 47 mm diameter membrane filter
9. Vacuum pump
10. pH meter: accurate to 0.01 pH with calibration buffers
11. HPLC with DAD detector/ UV detector: (wavelength 268 nm)
12. Analytical column: reverse phase C18 column of 150 mm x 4.6 mm I.D. and particle size 5 µm.
13. Guard column: 20 mm X 4.6 mm I.D
14. HPLC sample vial

HPLC Conditions

1. HPLC with Photo-diode Array Detector thiamine and riboflavin detection at the wavelength of 268 nm
2. Reverse phase C18 column of 150 mm x 4.6 mm I.D. and particle size 5 µm
3. A computer with HPLC control software
4. HPLC consists of:
 - a. Solvent delivery system
 - b. Autosampler/injector or a manual injector
 - c. Temperature Control Module (optional)
 - d. Integrator or data station or equivalent for data processing
 - e. Detectors: Photo-diode Array Detector in series
 - f. Precolumn: Guard-Pak Holder with reverse-phase C18 insert or equivalent.

Procedures

A. Sample Extraction

1. An accurate weight of 10 g of fresh sample or 2 g of foodstuff is weighed into a 100 mL extraction flask.
2. 60 mL of 0.1 N HCl is added to the flask, and the mixture is homogenized for 10 minutes using a homogenizer.
3. The extract is autoclaved at 120°C for 30 minutes, then immediately cooled on ice.
4. The pH of the acidic extract is adjusted to pH 4.3 – 4.7 using 1 M sodium acetate.
5. Takadiastase (at a concentration of 100 mg per g of sample) is added to the extract, and the mixture is incubated at 37°C for 18 hours.
6. After incubation, 2 mL of 50% trichloroacetic acid (TCA) is added to the extract, and the mixture is heated in a water bath at 50°C for 10 minutes.
7. The extract is quantitatively transferred into a 100 mL volumetric flask, and the volume is made up to the mark with deionized water (V1).
8. The diluted extract is filtered using ashless filter paper.

B. Instruments Set-Up Procedure

1. Before the HPLC is turned on, the column, reservoir, and mobile phase are checked to ensure the correct columns and mobile phases are used.
2. The control software and HPLC system are turned on. The instrument is warmed up for at least 30 minutes before starting.
3. The purge valve on the pump module is opened by turning the knob counter-clockwise several turns so that the priming solvent is directly delivered to the waste container.
4. The pump is set to 100% at a flow rate of 5 mL/min to prime every channel in the solvent delivery system with the respective mobile phase for several minutes.
5. The pump is turned off, the purge valve is closed, the required mobile phase composition (acetonitrile: water, 70:30) is set, and the flow rate is set to 1 mL/min for thiamine and riboflavin analysis.
6. The pump is turned on again and the column is allowed to equilibrate with the mobile phase, which usually requires 1 hour.
7. The UV and visible lamps are turned on, and the DAD detector is set at a wavelength of 268 nm.
8. To check whether the column is equilibrated, the above standard mixture is injected for 2 runs, and if the elution time of the standards is the same for both runs, then the column is ready for sample injection.
9. The sample identity is keyed in, and the sequence is built in SAMPLE SEQUENCE for the autosampler.

C. Chromatography of Thiamine/ Riboflavin

1. The cleaned extract (2 mL) is filtered through a 0.45 µm PVDF syringe filter into a sample vial for riboflavin determination.
2. For thiamine analysis, 5 mL of extract is mixed with 3 mL of 1% potassium ferricyanide, followed by 0.5 mL of phosphoric acid and 1.5 mL of water, before being filtered with a 0.45 µm PVDF syringe filter.
3. Thiamine and riboflavin in the sample are identified by comparing their retention times with the retention times of standards.
4. A standard curve is obtained using the peak area given by each standard solution (10 – 50 µg/mL).
5. Both thiamine and riboflavin in the sample are quantified by comparing the integrated chromatographic peak areas from the test samples to the peak areas of known amounts of standards. The total amount of thiamine and riboflavin is expressed in mg/100 g.

Calculation

mg thiamine/ riboflavin per 100 g food =

$$\frac{\text{Peak area of sample}}{\text{Peak area of std.}} \times \text{Amount of std. in g} \times \frac{V1(\text{mL})}{\text{Inject volume in mL}} \times \frac{100}{\text{Wt. of food used}}$$

References

- 1) Kumar, S. & Aalbersberg, B. 2006. Nutrient retention in foods after earth-oven cooking compared to other forms of domestic cooking, 2. Vitamin. Journal of Food Composition and Analysis. 19(4): 311-320.
- 2) AOAC, 1995. Official methods of analysis of the Association of Official Analytical Chemists. In: Cunniff, P. (Ed.), Methods 974.29, 941.15, 957.17, 961.14, 16th ed. Association of Official Analytical Chemists, Gaithersburg, USA.

3.3.4.3 DETERMINATION OF NIACIN (VITAMIN B3) IN FOOD (HPLC METHOD)

Principles

Alkaline hydrolysis is used to extract niacin from the sample, as acid hydrolysis cannot completely liberate bound niacin from food samples. The sample extract is then purified using solid-phase extraction with both C18 and SCX cation exchange columns in series prior to HPLC analysis. PIC® A reagent is employed in the mobile phase for better chromatography separation.

Chemicals & Reagents

1. Methanol, HPLC grade
2. Methanol, analytical grade
3. Calcium hydroxide
4. Oxalic acid, 10% (w/v)
5. Oxalic acid, 1%. (w/v)
6. Ammonium hydroxide, 2% (w/w)
7. PIC® A reagent
8. Mobile phase preparation. A mobile phase consisting of 5% methanol and 95% deionized water, with the addition of 0.005 M PIC® A reagent, is prepared. This mobile phase is then filtered daily prior to analysis using a solvent filtration apparatus equipped with a 0.45 µm nylon membrane filter.
9. Niacin standard solution
 - a. Niacin, with a purity of 99.5%, is used as the standard for niacin determination.
 - b. Niacin standard stock solution. Ten milligrams (10 mg) of the standard are weighed and made up to 100 mL with deionized water, yielding a concentration of 100 µg/mL. This solution is to be prepared freshly prior to analysis.
 - c. Niacin standard working solution. One milliliter (1 mL) of the standard stock solution (b) is pipetted into a 100 mL volumetric flask, and the volume is then made up with deionized water. The final concentration of this working solution is 1 µg/mL.

Apparatus/Instruments

1. Volumetric flask: 50 mL, 100 mL
2. Conical flask: 50 mL
3. Homogenizer
4. pH meter: accurate to 0.01 pH with calibration buffers
5. Centrifuge
6. Autoclave
7. Solid phase extraction (SPE) column: C18 (500 mg) and SCX (500 mg)
8. Vacuum manifold: for SPE column
9. Syringe filter: 0.45 µm, PTFE
10. Ultrafiltration system: 0.45 µm, 47 mm diameter membrane filter
11. Vacuum pump
12. HPLC with DAD detector/ UV detector: wavelength 254 nm
13. Analytical column: reverse phase C18 column of 150 mm x 4.6 mm I.D and particle size 5 µm
14. Guard column: 20 mm X 4.6 mm I.D
15. HPLC sample vial

HPLC Conditions

1. HPLC with Photo-diode Array Detector niacin detection at the wavelength of 254 nm
2. Reverse phase C18 column: 150 mm x 4.6 mm I.D. and particle size 5 µm
3. A computer with HPLC control software
4. HPLC consists of:
 - a. Solvent delivery system
 - b. Autosampler/injector or a manual injector
 - c. Temperature Control Module (optional)
 - d. Integrator or data station or equivalent for data processing
 - e. Detectors: diode arrays multiple wavelength detector
 - f. Precolumn: Guard column holder: with a guard column I.D. of 4.6 mm and length of 20 mm, packed with the same material as the analytical column.

Procedures

A. Sample Extraction

1. 1 g of the foodstuff is weighed accurately into a 50 mL extraction flask.
2. 0.75 g of calcium hydroxide and 20 mL of deionized water are added into the flask, and the extract is homogenized for 10 minutes with a homogenizer.
3. The extracts are autoclaved at 121°C for 2 hours, then cooled immediately on ice.
4. The cooled extract is diluted with deionized water and adjusted to 50 mL using a volumetric flask.
5. The extract is centrifuged at 2500 rpm, 10°C for 20 minutes.
6. 15 mL of the supernatant is taken, and the pH is adjusted to 7 using 10% aqueous oxalic acid. Then, the volume is made up to 25 mL.
7. The resultant supernatant is centrifuged at 2500 rpm, 10°C for 10 minutes to precipitate calcium oxalate.
8. The supernatant is decanted for further purification.

B. Sample Purification

1. C18 (500 mg) and SCX (500 mg) solid phase extraction (SPE) columns are connected in series, then attached to a vacuum manifold.
2. The SPE columns are conditioned with 10 mL of methanol and 10 mL of deionized water.
3. 10 mL of the supernatant is loaded onto the C18 column.
4. The C18 column is washed with 5 mL of water.
5. The C18 column is discarded, and the SCX column is washed with 5 mL of methanol.
6. Niacin is eluted from the SCX column with 5 mL of freshly prepared 2% concentrated ammonium hydroxide in methanol.
7. The eluate is evaporated to dryness under a stream of nitrogen at room temperature.
8. The residue is dissolved in 1 mL (V1) of deionized water, and then filtered through a 0.45 µm PTFE syringe filter before HPLC analysis.

C. Instrument Set-Up Procedure

1. Before turning on the HPLC, check the column, reservoir, and mobile phase to ensure that the correct column and mobile phase are used.
2. The control software and HPLC system are turned on, and the instrument is allowed to warm up for at least 30 minutes before starting.
3. The purge valve on the pump module is opened by turning the knob counterclockwise several turns, allowing the priming solvent to be delivered directly to the waste container.
4. The pump is set to 100% at a flow rate of 5 mL/min to prime every channel in the solvent delivery system with the respective mobile phase for several minutes.
5. The pump is then turned off, the purge valve is closed, and the required isocratic mobile phase composition and flow rate of 1 mL/min for niacin analysis are set.
6. The pump is turned on again, and the column is allowed to equilibrate with the mobile phase, which usually requires 1 hour.
7. Make sure the UV and Visible lamps are turned on and set the DAD detector at a wavelength of 254 nm.
8. To check whether the column is equilibrated, inject the above standard mixture for 2 runs. If the elution time of the standards is the same for the 2 runs, then the column is ready for sample injection.
9. The sample identity is keyed in, and the sequence is built in the SAMPLE SEQUENCE for the autosampler.

D. Chromatography of Niacin

1. The cleaned extract (2 mL) is filtered through a 0.45 µm PVDF syringe filter into a sample vial.
2. Niacin in the sample is identified by comparing its retention time with the retention time of the standards.
3. A standard curve is obtained using the peak area generated by each standard solution in the range of 10–50 µg/mL.
4. The niacin content in the sample is quantified by comparing the integrated chromatographic peak areas of the test samples to the peak areas of the known standard concentrations. The total amount of niacin in the sample is expressed in mg/100 g.

Calculation

mg niacin per 100 g food =

$$\frac{\text{Peak area of sample}}{\text{Peak area of std.}} \times \text{Amount of std. in g} \times \frac{V1(\text{mL})}{\text{Inject volume in mL}} \times \frac{100}{\text{Wt. of food used}}$$

Reference

Juraja, SM, Trenerry, VC, Millar, RG, Scheelings, P & Buick, DR. 2003. Asia Pacific food analysis network (APFAN) training exercise: the determination of niacin in cereals by alkaline extraction and high-performance liquid chromatography. *Journal of Food Composition and Analysis* 16:93-106.

3.3.4.4 DETERMINATION OF PANTOTHENIC ACID (VITAMIN B5) IN FOODS (HPLC METHOD)

Principles

Pantothenic acid (vitamin B5) exists in foodstuffs in its free form, as well as bound in coenzyme A (CoA) and acyl carrier protein (ACP). Therefore, an enzymatic hydrolysis extraction method is required to release pantothenic acid from its bound forms. Microbiological assay is the most common approach for pantothenic acid determination. However, it is time-consuming, has low specificity and exhibits relatively low precision. Therefore, liquid chromatography (LC) methods are developed using post-column derivatization of pantothenic acid into a fluorescent compound, fluorescent 1-alkylthio-2-alkylisoindole. Fluorescent compound forms when pantothenic acid is hydrolyzed to β -alanine, under hot alkaline conditions and then reacted with orthophthaldialdehyde in the presence of 3-mercaptopropionic acid. This method shows a good recovery rate (96-101%) and a low detection limit (0.65 μ g/g), thus making it suitable for pantothenic acid determination in any foodstuff.

Chemicals & Reagents

1. Sodium bicarbonate, 200 mM
2. Sodium bicarbonate, 5 M
3. TRIS buffer, 200 mM, pH 8.0
4. HCl, 1 M
5. HCl, 250 mM
6. Vitamin standard, calcium D-pantothenate
7. Acetone-dried pigeon liver powder (contains pantetheinase)
 - a. Purification: One gram (1 g) of the powder is dissolved in 10 mL of 20 mM sodium bicarbonate at 0°C. The resulting solution is then centrifuged at 3500 rpm for 10 minutes. The supernatant is collected and transferred to dialysis tubing (width 25 mm, diameter 16 mm; volume/length 2.0 mL/cm, Spectra/Por 4 membrane, MWCO 12,000 – 14,000). The filled dialysis tube is placed in 3 L of 20 mM sodium bicarbonate for 15 hours at 4°C. The purified pantetheinase solution is stored in a vial and frozen until use.
8. Pepsin
9. Alkaline phosphatase
10. Methanol, HPLC grade – mobile phase
11. Phosphate buffer (33 mM, pH 2.5) – mobile phase
12. Phosphate buffer (300 mM, pH 3)
13. Reagent for post-column derivatization
 - a. A solution is prepared containing 200 mM sodium hydroxide, 1 mM orthophthaldialdehyde, and 1.6 mM 3-mercaptopropionic acid.
14. Mobile phase preparation
 - a. The mobile phase is filtered daily prior to analysis using a solvent filtration apparatus equipped with a 0.45 μ m nylon membrane filter.
15. Pantothenic acid standard solutions
16. Calcium D-pantothenate is used as a standard for pantothenic acid determination.
17. Standard stock solution. Ten milligrams (10 mg) of the standards are weighed and made up to 100 mL with deionized water, yielding a concentration of 100 μ g/mL. This solution is to be prepared freshly prior to analysis.
18. Standard working solution. Ten milliliters (10 mL) of the standard stock solution (b) are pipetted into a 100 mL volumetric flask, and the volume is then made up with deionized water. The final concentration of the working solution is 10 μ g/mL.
19. A series of standards containing 0.05 – 2.00 μ g/mL of pantothenic acid is prepared from the working solution.

Apparatus/Instruments

1. Balance: Analytical sensitivity ± 0.1 mg
2. Extraction flask: 100 mL
3. Centrifuge
4. Pipettes: 1 mL, 5 mL, 10 mL
5. Volumetric flask: 50 mL, 5 mL
6. Incubator
7. Centrifuge
8. Cellulose acetate filter: 0.45 μ m
9. HPLC sample vials
10. SPE column: strong anion exchange, 400 mg
11. Vacuum manifold: for SPE
12. Post-column derivatization kit: containing T-connector, knitted PTFE coil reactor and peristaltic pump
13. HPLC with fluorescence detector and thermostatted column compartment
14. Analytical column: reverse phase (RP) C18 endcapped, octadecylsilyl, 250 mm x 5 mm I.D; particle size 5 μ m
15. Guard column: RP 18, 4 mm X 4 mm ID; 5 μ m particle size

HPLC Conditions

1. HPLC with Fluorescence detector pantothenic acid detection at the wavelengths of 345 nm and 455 nm
2. Reverse phase C18 endcapped column of 250 mm x 5 mm I.D; particle size 5 μ m; octadecylsilyl
3. A computer with HPLC control software
4. HPLC consists of:
 - a. Solvent delivery system
 - b. Autosampler/injector or a manual injector
 - c. Temperature Control Module (optional)
 - d. Integrator or data station or equivalent for data processing
 - e. Detectors: Programmable UV (or DAD) and fluorescence detectors in series
 - f. Precolumn: Guard column holder with a guard column that RP 18 (4 mm X 4 mm ID; 5 μ m particle size; packed with the same material as that of the analytical column.
5. A computer with an HPLC control software
6. A printer to print the results and chromatograms.
7. For post-column derivatization
 - a. The derivatization kit is set between the analytical column and the fluorescence detector.
 - b. The derivatization solution is pumped in using a peristaltic pump (e.g., Miniplus 3 peristaltic pump, Gilson). The peristaltic pump is set to a flow rate of 1 mL/min.
 - c. The derivatization solution is added to the column effluent through a T-connector. This is followed by a knitted PTFE coil reactor (0.5 mm ID x 40 m), which is housed in the thermostatted column compartment at 99°C.
 - d. Another end of the PTFE reactor is connected to the detector outlet for the determination of the derivatized compound.

Procedures

A. Sample Extraction

1. An accurately weighed 5 g of the foodstuff is placed into a 100 mL extraction flask.
2. The flask is filled with 15 mL of 50 mM acetate buffer (pH 4.5) and 1 mL of pepsin solution (50 mg/mL [4500 U/mL]).
3. The mixture is homogenized and incubated at 50 °C for 3 hours.
4. After incubation, the pH is adjusted to 8 using 5 M sodium hydroxide.
5. The mixture is then diluted with 10 mL of 200 mM Tris buffer (pH 8), 0.6 mL of alkaline phosphatase solution (20 U/mL), and 2.5 mL of pantetheinase solution (80 mU/mL).
6. The mixture is incubated at 20 °C for 18 hours, then the volume is adjusted to 50 mL with deionized water.
7. The sample is centrifuged at 11,500 rpm for 10 minutes, followed by filtration through a 0.45 μ m cellulose acetate filter.

B. Extract Purification

1. A strong anion exchange (SAX) cartridge (400 mg) is washed successively with 5 mL of methanol and 5 mL of deionized water.
2. 5 mL of the extract is loaded onto the SAX cartridge, and the first 2 mL is discarded.
3. The subsequent 3 mL of the extract is collected into a 5 mL volumetric flask.
4. 0.25 mL of 250 mM HCl is added, and the volume is made up to 5 mL using 300 mM phosphate buffer (pH 3.0).
5. A strong cation exchange (SCX) cartridge (400 mg) is prepared by washing it successively with 5 mL of methanol and 5 mL of deionized water.
6. The entire solution from the previous step is loaded onto the SCX cartridge, and the first 2 mL is rejected.
7. The next 3 mL is collected for HPLC determination.

C. Instrument Set-Up Procedure

1. Before turning on the HPLC, check the column, reservoir, and mobile phase to ensure that the correct column and mobile phase are used.
2. The Agilent ChemStation control software and HPLC system are turned on, and the instrument is allowed to warm up for at least 30 minutes before use.
3. The purge valve on the pump module is opened by turning the knob counter-clockwise several turns, allowing the priming solvent to flow directly into the waste container.
4. The pump is set to 100% at a flow rate of 5 mL/min to prime every channel in the solvent delivery system with the respective mobile phase for several minutes.
5. After priming, the pump is turned off, and the purge valve is closed. The mobile phase composition is set to phosphate buffer (33 mM, pH 2.5) and methanol. The flow rate is adjusted to 1 mL/min for pantothenic acid analysis.
6. The gradient mode is used, whereby the proportion of methanol is increased linearly from 0% to 10% over 25 minutes, then maintained at 10% (phosphate buffer: methanol = 90:10, v/v) for 8 minutes.
7. After the 8-minute hold, the mobile phase composition is immediately adjusted back to the initial composition (100% phosphate buffer).
8. The pump is turned on again, and the column is equilibrated with the initial mobile phase composition, which typically requires 1 hour.
9. The fluorescence detector is set with an excitation wavelength of 345 nm and an emission wavelength of 455 nm.
10. The standard mixture is injected for 2 consecutive runs to verify column equilibration. If the retention times of the standards are consistent in both runs, the column is considered equilibrated and ready for sample injection.
11. The sample identity is entered, and the sequence is built in the SAMPLE SEQUENCE module for the autosampler.
12. 50 μ L of the extract is injected for HPLC analysis.

D. Chromatography of Pantothenic Acid

1. Pantothenic acid in the sample is identified by comparing its retention time with the retention time of known standards.
2. A standard calibration curve is constructed using the peak areas obtained from a series of standard solutions ranging from 0.5 to 2 μ g/mL.
3. The amount of pantothenic acid in the sample is quantified by comparing the integrated chromatographic peak areas from the test samples with the peak areas of the corresponding standards on the calibration curve.
4. The final concentration of pantothenic acid in the sample is expressed as mg per 100 g of sample.

Reference

Pakin, C., Bergaentzlé, M., Hubscher, V., Aoudé-Werner, D., Hasselmann, C. 2004. Fluorimetric determination of pantothenic acid in food by liquid chromatography with post column derivatization. *Journal of Chromatography A*. 1035, 97-95.

3.3.4.5 DETERMINATION OF PYRIDOXINE (VITAMIN B6) IN FOOD (HPLC METHOD)

Principles

Vitamin B6 in food is dephosphorylated by enzyme hydrolysis and detected using fluorescence, which provides a highly sensitive and specific detection mode for analysing all vitamin B6 forms commonly encountered in foods. Pyridoxine hydrochloride (PN), pyridoxal hydrochloride (PL) and pyridoxamine dihydrochloride (PM) are detected, and total vitamin B6 is calculated with the equation $PN = PN + (1.01PL) + (0.79PM)$.

Chemicals/Reagents

1. Acetonitrile, HPLC grade
2. HCl, 0.01 M
3. HCl, 0.1 M
4. HCL, 1 M
5. Sodium acetate, 2 M
6. Potassium hydroxide, 3.5 M
7. Acid phosphatase, 25 units/mL, prepare freshly prior to analysis.
8. β -glucosidase, 45 units/mL, prepare freshly prior to analysis.
9. Triethylamine, 99%, 4 mM
10. Potassium dihydrogen phosphate, 81 mM
11. Ortho-phosphoric acid, 85%, 19 mM
12. 1-octan-sulfonic acid, 2.2 mM
13. Sodium monohydrate, 99%
14. Mobile phase preparation.
 - a. HPLC eluent buffer. A buffer (1 L) is prepared, consisting of 2.2 mM 1-octanesulfonic acid in 81 mM potassium dihydrogen phosphate, 19 mM 85% phosphoric acid, and 4.0 mM triethylamine. The pH is then adjusted to 2.75 with 3.5 M potassium hydroxide.
 - b. A mobile phase is prepared that contains 93% HPLC eluent buffer and 7% acetonitrile.
 - c. Daily, the mobile phase is filtered with a 0.45 μ m nylon membrane filter using a solvent filtration apparatus before analysis.
15. Vitamin B6 standard solutions
 - a. For vitamin B6 determination, pyridoxine hydrochloride (PN), pyridoxal hydrochloride (PL), and pyridoxamine dihydrochloride (PM) are used as standards.
 - b. Standard stock solution. Ten milligrams (10 mg) of the standards are weighed and made up to 100 mL with 0.1 M HCl, yielding a concentration of 100 μ g/mL. This solution is to be prepared freshly prior to analysis.
 - c. Standard working solution. One milliliter (1 mL) of the standard stock solution (b) is pipetted into a 100 mL volumetric flask, and then diluted to volume with 0.1 M HCl. The final concentration of the working solution is 1 μ g/mL.

Apparatus/Instruments

1. Ultrafiltration equipment: 0.45 μ m, 47 mm diameter membrane filter
2. Volumetric flask: 100 mL, 1 L
3. Balance: analytical sensitivity $\pm$ 0.1 mg
4. Conical flask: 50 mL
5. Beaker: 25 mL, 50 mL
6. Mechanical shaker
7. Autoclave
8. Centrifuge
9. Incubator: at 45°C
10. Filter paper, Whatman No.1
11. HPLC sample vials
12. Syringe filter: 0.45 μ m PVDF
13. Analytical column: C18, particle size 3 μ m, 150 mm x 4.6 mm I.D
14. HPLC with fluorescence detector

HPLC Conditions

1. Reverse phase C18 column of 150 mm x 4.6 mm I.D and particle size 3 µm.
2. Guard column holder with a guard column with an I.D of 4.6 mm and length of 20 mm packed with the same material as that of the analytical column.
3. Agilent 1200 series standard and preparative autosampler, G1239. *
4. Agilent 1200 series binary pump, G1312 to deliver the mobile phase. *
5. Agilent 1200 series micro vacuum degasser, G1379. *
6. Agilent 1200 series fluorescence detector, G1321. *
7. Solvent cabinet to keep the mobile phases. *
8. A computer with an Agilent ChemStation control software.
9. A printer to print results and chromatograms.

**Brand and model may differ for other manufacturer*

Procedures

A. Sample Extraction

1. Accurately, 5 g of the foodstuff is weighed into a 100 mL extraction flask.
2. 50 mL of 0.1 M hydrochloric acid is added, and the mixture is homogenized using an orbital shaker for 10 minutes.
3. The extract is autoclaved at 121°C for 30 minutes if the sample is of animal origin, and for 5 minutes if of vegetable origin.
4. The extract is immediately cooled to room temperature on ice.
5. The pH of the cooled extract is adjusted to 4.5 using 2 M sodium acetate.
6. The extract is diluted and adjusted to 100 mL with deionized water.
7. The diluted extract is centrifuged at 8500 g for 10 minutes at 5°C.
8. The supernatant is decanted and filtered using filter paper.
9. For animal-origin samples, 15 mL of the filtered supernatant is mixed with 1 mL of acid phosphatase solution in a 30 mL volumetric flask.
10. For vegetable-origin samples, 15 mL of the filtered supernatant is mixed with 1 mL of acid phosphatase and 3 mL of β-glucosidase in a 30 mL volumetric flask.
11. The mixture is incubated at 45°C for 18 hours.
12. After incubation, the mixture is cooled to room temperature, followed by adding 5 mL of cold 1 M hydrochloric acid. The volume is brought up to 30 mL (V1) using 0.01 M hydrochloric acid.
13. The extract is filtered into an HPLC vial through a 0.45 µm PVDF syringe filter.

Note: For turbid samples, the mixture is centrifuged at 14,000 × g for 10 minutes at 5°C prior to filtration.

C. Chromatography of Vitamin B6

1. Vitamin B6 in the sample is identified by comparing its retention time with the retention time of the standards.
2. The peak area given by each standard solution (1–50 µg/mL) is used to obtain a standard curve.
3. Vitamin B6 in the sample is quantified by comparing the integrated chromatographic peak areas from the test samples to the peak areas of known amounts of standards.
4. Vitamin B6 is calculated as pyridoxine hydrochloride (PN·HCl) using the formula:
$$\text{PN} \cdot \text{HCl} = \text{PN} + (1.01 \times \text{PL}) + (0.79 \times \text{PM})$$
5. The total amount of vitamin B6 in the sample is expressed in mg/100 g of sample.

Calculation

mg PN/ PL/ PM in food =

$$\frac{\text{Peak area of sample}}{\text{Peak area of std.}} \times \text{Amount of std. in g} \times \frac{V1(\text{mL})}{\text{Inject volume in mL}} \times \frac{100}{\text{Wt. of food used}}$$

Reference

Kall, MA. 2003. Determination of total vitamin B6 in food by isocratic HPLC: a comparison with microbiological analysis. Food Chemistry 82: 315-327.

3.3.4.6 DETERMINATION OF BIOTIN (VITAMIN B7) IN FOOD (MICROBIOLOGICAL METHOD)

Principles

Biotin occurs in relatively low concentrations in most foods. Therefore, microbiological assay remains the most versatile method for determining the total biotin concentration in foods. It is a highly sensitive method detecting biotin levels up to 10^{-9} g/L. The assay is based on the growth of *Lactobacillus plantarum* (a biotin-dependent microorganism) according to the concentration of biotin in the medium.

Chemicals/Reagents

1. Methanol, analytical grade
2. Sulfuric acid, 2 N
3. Sulfuric acid, 6 N
4. Sodium hydroxide (NaOH), 20%
5. Biotin assay medium
6. Folic acid standard solution
 - a. Use biotin as a standard for biotin determination.
 - b. Folic acid standard stock solution. One mg of standard is weighed and made up to 100 mL with 25% methanol to give a concentration of 10 μ g/mL. This solution is prepared freshly prior to analysis
 - c. Biotin standard working solution.
 - i. 0.1 mL of standard stock solution (b) is pipetted into a 100 mL volumetric flask and diluted to volume with deionized water to give the final concentration of 10 ng/mL.
 - ii. One mL of diluted stock solution (i) is pipetted into a 100 mL volumetric flask and made up to volume with deionized water. The final concentration of the working solution is 0.1 ng/mL.

Apparatus/Instruments

1. Balance: analytical sensitivity ± 0.1 mg
2. Pipettes: 1 mL, 5 mL, 10 mL
3. Extraction flask: 50 mL
4. Homogenizer
5. Autoclave
6. pH meter: accurate to 0.01 pH with calibration buffers
7. Filter paper: ashless
8. Test tubes
9. Incubator

Procedures

A. Sample Extraction

1. Exactly 1 g of homogenised foodstuff is weighed into a 50 mL extraction flask.
2. 25 mL of sulfuric acid, 2 N (for plant samples) or 6 N (for animal samples), is added.
3. The mixture is homogenised with a homogeniser for 10 minutes.
4. The mixture is autoclaved at 121°C for 2 hours.
5. The sample is cooled immediately and adjusted to pH 7 with 20% NaOH.
6. The mixture is diluted to 100 mL with deionised water and filtered through ashless filter paper.
7. The extract is kept in a refrigerator prior to analysis.

B. Microbiological Assay

1. The biotin assay medium is prepared according to the product manual (e.g., Difco).
2. Inoculum preparation
 - a. 5 mL of assay medium is added into a test tube and the volume is adjusted to 10 mL with deionised water.
 - b. The medium is autoclaved at 121°C for 5 minutes.
 - c. The autoclaved medium is supplemented with 5 ng of biotin.
 - d. *Lactobacillus plantarum* is subcultured from the stock culture into the medium.
 - e. The culture is incubated for 16–24 hours at 35–37°C.
 - f. The culture is centrifuged at 3000 g for 10 minutes.
 - g. The supernatant liquid is decanted. The cell is washed three times with 10 mL of sterile 0.85% saline.
 - h. The cell is resuspended in 10 mL of sterile 0.85% saline.
 - i. The culture is diluted at a ratio of 1:100 with sterile 0.85% saline.
3. A series of standard test tubes is prepared, containing 0.0 (inoculated blank), 0.0 (uninoculated blank), 2, 4, 6, 8, and 10 mL of the final biotin standard working solution.
4. A set of assay test tubes containing 0.1 mL of sample solution is prepared.
5. Deionised water is added into each test tube (standards and samples) to bring the total volume to 5 mL.
6. 5 mL of biotin assay solution is added to each test tube.
7. The mixtures are autoclaved at 121°C for 10 minutes and cooled rapidly.
8. 1 drop of the diluted culture is added into each test tube (except the uninoculated blank).
9. The test tubes are incubated for 18–24 hours at 35–37°C until maximum turbidity is reached.
10. The turbidity of samples and standards is measured at 660 nm using a UV/Vis spectrometer, against the inoculated blank.
11. The uninoculated blank is used to set transmittance (T) at 100
12. A standard curve of %T against the concentration of biotin standard is prepared for sample quantification.

**** During all microbiological assay procedures, use clean glassware free from detergents and other chemicals. Furthermore, heat glassware at 250°C for 1 hour to burn off any organic residues that might be present. Conduct the whole assay under sterile and cool conditions.**

Reference

Murakami, T., Yamano, T., Nakama, A., Mori, Y. 2008. Estimation of dietary intake of biotin and its measurement uncertainty using total diet sample in Osaka, Japan. *Journal of AOAC International* 91:6

3.3.4.7 DETERMINATION OF FOLIC ACID (VITAMIN B9) IN FOOD (HPLC METHOD)

Principles

Folate in foods is extracted through tri-enzyme extractions, where protease, α -amylase and conjugase are employed. The tri-enzyme extractions enable the increase of total measurable folate from various food samples, as each enzyme has a different role in extraction. For example, α -amylase digestion increases folate extract from high-starch and glycogen foods. Folate content in food is identified by comparing the fluorescence and UV peak height ratio at different wavelengths.

Chemicals/Reagents

1. Acetonitrile, HPLC grade
2. Protease, 2 mg/mL
3. α -Amylase, 20 mg/mL
4. Chicken pancreas conjugase, 5 mg/mL
5. Hydrochloric acid (HCl), 0.1 M
6. Sodium hydroxide (NaOH), 0.1 M
7. Potassium phosphate buffer, 30 mM, pH 2.3
8. Elution buffer- Sodium acetate, 0.1 M (containing 10% sodium chloride, 1% ascorbic acid and 0.1% 2-mercaptoethanol)
9. Folic acid standard solution
 - a. Folic acid (from Sigma Chemical Co.) is used as a standard for folic acid determination.
 - b. Folic acid standard stock solution. Ten milligrams (10 mg) of standard is weighed and made up to 100 mL with deionized water to give a concentration of 100 μ g/mL. This solution is prepared freshly prior to analysis.
 - c. Folic acid standard working solution. One mL of standard stock solution (b) is pipetted into a 100 mL volumetric flask and made up to volume with deionized water. The final concentration of the working solution is 1 μ g/mL.

Apparatus/Instruments

1. Balance: analytical sensitivity ± 0.1 mg
2. pH meter: accurate to 0.01 pH with calibration buffers
3. Extraction flask: 50 mL
4. Volumetric flask
5. Incubator: at 37°C
6. Pipettes: 1 mL, 5 mL, 10 mL
7. Shaking water bath
8. Vortex mixer
9. Centrifuge
10. SPE column: strong anion exchange (SAX, 500 mg)
11. Vacuum manifold
12. Vacuum pump
13. Syringe filter: 0.45 μ m PTFE
14. HPLC sample vials
15. HPLC with fluorescence detector and UV detector
16. Analytical column: RP C18, 150 mm x 4.6 mm I.D and particle size 3 μ m
17. Guard column: 4.6 mm of I.D. and length of 20 mm

HPLC Conditions

1. Reverse phase C18 column of 150 mm x 4.6 mm I.D. and particle size 3 μ m (e.g., Atlantis, Waters).
2. Guard column holder with a guard column with an I.D. of 4.6 mm and a length of 20 mm packed with the same material as the analytical column.
3. Agilent 1200 series standard and preparative autosampler, G1239. *
4. Agilent 1200 series binary pump, G1312 to deliver the mobile phase. *
5. Agilent 1200 series micro vacuum degasser, G1379. *
6. Agilent 1200 series fluorescence detector, G1321. *
7. Agilent 1200 series diode arrays multiple wavelength detector (DAD), G1315 for niacin detection at the wavelength of 254 nm. *
8. Solvent cabinet to keep the mobile phases.
9. A computer with an Agilent ChemStation control software. *
10. A printer to print the results and chromatograms.

**Brand and model may differ for other manufacturers*

Procedures

A. Sample Extraction

1. A total of 5 g of homogenised foodstuff is accurately weighed into a 50 mL extraction flask.
2. The pH is adjusted to 4.5 with 0.1 M HCl.
3. 0.8 mL of protease is added, then vortexed and incubated at 37°C for 16 hours.
4. The sample is placed in a 100°C shaking water bath for 10 minutes to deactivate the protease, then cooled on ice to room temperature.
5. An amount of 0.8 mL of α -amylase is added, then vortexed and incubated at 37°C for 4 hours.
6. The sample is placed in a 100°C shaking water bath for 10 minutes to deactivate the α -amylase, then cooled on ice to room temperature.
7. The pH is adjusted to 7.2 with 0.1 M NaOH.
8. An amount of 1 mL of chicken pancreas conjugase is added, then vortexed and incubated at 37°C for 3 hours.
9. The sample is placed in a 100°C shaking water bath for 10 minutes to deactivate enzymes, then cooled on ice to room temperature.
10. The extract is centrifuged at 3000 rpm, 10°C for 20 mins.
11. The supernatant is decanted and stored at -20°C for further purification.

B. Sample Purification

1. The thawed sample is purified using solid-phase extraction (SPE) on a strong anion exchange (SAX, 500 mg) column.
2. The SAX column is connected to a vacuum manifold.
3. The column is conditioned with 5 mL of methanol followed by 5 mL of deionised water.
4. A volume of 2.5 mL (V1) of supernatant is loaded onto the SAX column.
5. The column is washed with 5 mL of water.
6. Folic acid is eluted from the SAX column with 5 mL of freshly prepared elution buffer.
7. The first 0.5 mL of eluate is discarded, and the following 3.5 mL is collected.
8. The collected eluate is filtered through a 0.45 μ m PTFE syringe filter prior to HPLC analysis.

C. Instrument Set-Up Procedures

1. Before turning on the HPLC, the column, reservoir, and mobile phase are checked to ensure the correct column and mobile phase are used.
2. The control software and HPLC system are turned on, and the instrument is warmed up for at least 30 minutes before starting.
3. The purge valve on the pump module is opened by turning the knob counterclockwise several turns, allowing the priming solvent to be delivered directly to the waste container.
4. The pump is set at 100% flow rate of 5 mL/min to prime every channel in the solvent delivery system with the respective mobile phase for several minutes.
5. The pump is turned off and the purge valve is closed. The required mobile phase composition (gradient) and a flow rate of 0.4 mL/min for folic acid analysis are set.
6. The pump is turned on again and the column is equilibrated with the mobile phase, which usually requires 1 hour.
7. The UV and visible lamps are turned on, and the DAD detector is set at wavelengths of 280, 290, and 300 nm.
8. The fluorescence detector is set with an excitation wavelength of 290 nm and an emission wavelength of 360 nm.
9. Gradient elution is performed with acetonitrile (solvent A) and potassium phosphate buffer (solvent B). The gradient starts at 6% acetonitrile, maintained isocratically for the first 5 mins, then increased linearly to 25% within 20 mins, with a total run time of 42 mins.
10. To check if the column is equilibrated, the standard mixture is injected for 2 runs. If the elution time of the standards is the same for both runs, the column is ready for sample injection.
11. The sample identity is entered and the sequence is built in SAMPLE SEQUENCE for the autosampler.

D. Chromatography of Folic Acid

1. The peak of folic acid is identified by its retention time and its identity is confirmed by comparing the ratio of fluorescence and UV peak heights at different wavelengths.
2. The peak areas from each standard solution (10–50 µg/mL) are used to obtain a standard curve.
3. Folic acid in the sample is quantified by comparing the integrated chromatographic peak areas from the test samples with the peak areas of known amounts of standards. The total amount of folic acid in the sample is expressed in mg/100 g of the sample.

Calculation

mg folic acid per 100 g food =

$$\frac{\text{Peak area of sample}}{\text{Peak area of std.}} \times \text{Amount of std. in g} \times \frac{V1(\text{mL})}{\text{Inject volume in mL}} \times \frac{100}{\text{Wt. of food used}}$$

Reference

Johansson, M., Furuhausen, C., Frølich, W., Jägerstad, M. 2008. Folate content in frozen vegetarian ready meals and folate retention after different reheating methods. LWT 41: 528-536.

3.3.4.8 DETERMINATION OF COBALAMIN (VITAMIN B12) IN FOOD (UPLC – MASS SPECTROMETRY METHOD)

Principles

Vitamin B12 in the food sample is extracted with enzyme digestion and analysed by using the combination of UPLC-MS with positive ESI ionization.

Chemicals / Reagents

1. Acetonitrile, HPLC grade
2. Methanol, HPLC grade
3. Pepsin
4. α -Amylase
5. Sodium hydroxide (NaOH), 0.1 M
6. Sodium acetate buffer, 50 mM, pH 4
7. Sodium cyanide, 1%
8. Ammonium formate, 1 M
9. Formic acid
10. Internal standard
 - a. Ginsenoside Re is used as an internal standard.
 - b. Internal standard stock solution (40 $\mu\text{g/mL}$) is prepared by dissolving 4 mg of ginsenoside Re in deionized water, and made up to a volume of 100 mL.
 - c. The working solution (0.1 $\mu\text{g/mL}$) is prepared by pipetting 0.25 mL of stock solution (b) into a 100 mL volumetric flask and filling it up to volume with deionized water.
11. Vitamin B12 standard solution
 - a. Vitamin B12 (from Sigma Chemical Co.) is used as a standard for vitamin B12 determination.
 - b. Vitamin B12 standard stock solution. One mg of standard is weighed and made up to 100 mL with deionized water to give a concentration of 10 $\mu\text{g/mL}$. This solution is prepared freshly prior to analysis.
 - c. Vitamin B12 standard working solution. 0.1 mL of standard stock solution (b) is pipetted into a 100 mL volumetric flask and made up to volume with deionized water. The final concentration of the working solution is 10 ng/mL.
 - d. A vitamin B12 standard is prepared in the concentration range of 10-150 ng/mL. The working solution is stored at 4°C before use.

Apparatus/Instruments

1. Balance: analytical sensitivity ± 0.1 mg
2. Homogenizer
3. Incubator: at 37°C
4. pH meter: accurate to 0.01 pH with calibration buffers
5. Volumetric flask: 50 mL
6. Centrifuge
7. Ultrafiltration system: with membrane filter, 0.45 μm
8. UPLC with PDA detector, column heater, MS system and Q-ToF
9. UPLC analytical column, RP C18: 50 mm x 2.1 mm I.D. and particle size 1.8 μm .
10. Guard column: with I.D of 2.1 mm, a length of 5 mm and 1.8 μm .

UPLC-ESI-MS Conditions

1. UPLC Reverse phase C18 column of 50 mm x 2.1 mm I.D. and particle size 1.8 µm (e.g., ACQUITY UPLC HSS T3, Waters)*
2. Guard column with I.D. of 2.1 mm, length of 5 mm and 1.8 µm packed with the same material as that of the analytical column (e.g., ACQUITY UPLC HSS T3 VanGuard Pre-column, Waters)*
3. Waters ACQUITY UPLC® system*
4. Waters ACQUITY UPLC® sample organizer*
5. Waters ACQUITY UPLC® binary pump to deliver the mobile phase*
6. Waters ACQUITY UPLC® column heater/ cooler*
7. Waters ACQUITY UPLC® PDA detector*
8. Waters SYNAPT™ mass spectrometry (MS) system*
9. Water Q-ToF Premier™*
10. A computer with a MassLynx™ Informatics control software*
11. A printer to print the results and chromatograms

**Brand and model may differ for other manufacturers*

Procedures

A. Sample Extraction

1. A total of 15 g of food sample is accurately weighed into an extraction flask.
2. 40 mL of sodium acetate buffer, 1 mL of sodium cyanide, 0.25 g of α-amylase, and 1 g of pepsin are added.
3. The sample is homogenised with a homogeniser for 5 minutes.
4. The mixture is incubated in a shaking incubator at 37°C for 3 hours.
5. The pH is adjusted to 4.8 with 0.1 M NaOH.
6. The extract is heated at 100°C for 35 minutes under agitation and nitrogen steam reflux.
7. The extract is immediately cooled to room temperature.
8. The extract is quantitatively transferred into a 50 mL volumetric flask, an internal standard is added, and deionised water is added to make up the volume.
9. The diluted extract is centrifuged at 8,000 g, 4°C for 10 minutes.
10. The supernatant is filtered through a 0.45 µm membrane filter before injection.

B. Instrument for Set-Up Procedure

1. Before turning on the UPLC, the column, reservoir, and mobile phase are checked to ensure the correct ones are used.
2. The control software and UPLC system are turned on, and the instrument is warmed up for at least 30 minutes before starting.
3. The Start Up System is turned on, purging is selected for 5 minutes for valves A and B, and the sample needle wash is performed for 5 cycles.
4. The pump is set to allow the column to equilibrate with the mobile phase, which usually requires 1 hour.
5. The UV and visible lamps are ensured to be turned on, and the PDA detector is set at a wavelength of 200–500 nm.
6. To check whether the column is equilibrated, the standard mixture is injected for two runs. If the elution times of the standards are consistent in both runs, the column is considered ready for sample injection.
7. The sample identity is keyed in and the sequence is built in the SAMPLE LIST.

C. Chromatography of Niacin

1. UPLC Condition
 - a. The column temperature is set at 40°C and the sample temperature is set at 4°C.
 - b. The mobile phase flow rate is set at 0.6 mL/min.
 - c. Mobile phase A is prepared by mixing 990 mL of water, 10 mL of ammonium formate, and 1 mL of formic acid.
 - d. Mobile phase B is prepared by mixing 990 mL of methanol, 10 mL of ammonium formate, and 1 mL of formic acid.
 - e. Gradient elution is programmed as follows:

Table 3.3.4.8: Gradient elution

0.0 min	99% A
2.0 min	99% A
3.0 min	45% A
3.1 min	99% A
4.0 min	99% A

- f. A sample injection volume of 20 μL is used with full-loop injection.
 - g. The needle is washed with 0.1% formic acid in water (weak wash) or 0.1% formic acid in methanol (strong wash).
 - h. The total run time for each sample is 4 minutes.
2. MS Condition
 - a. A 0.6 mL/min portion of the column eluent is directed into the MS.
 - b. ESI-MS spectra are acquired in positive ion mode.
 - c. Nitrogen is used as the desolvation gas (350 L/hour) and the cone gas (50 L/hour).
 - d. The desolvation temperature is set at 350°C
 - e. The source temperature is set at 105°C.
 - f. The capillary voltage is set at 4 kV and the cone voltage is set at 180 V.
 - g. Argon gas is used as the collision gas at 3.5×10^{-3} mBar.
 - h. m/z 930.8 and 969.9 are used as quantifier ions for vitamin B12 and ginsenoside Re, respectively.
 3. The calibration curve is obtained using the area counts from each standard solution.
 4. The total amount of vitamin B12 in the sample is expressed in ng/g of sample.

Reference

Luo, X., Chen, B., Ding, L., Tang, F., Yao, S. 2006. HPLC-ESI-MS analysis of vitamin B12 in food products and in multivitamins-multimineral tablets. *Analytica Chimica Acta* 562: 185-189.

3.3.4.9 DETERMINATION OF CHOLINE IN FOOD

Principles

Determination of choline in food samples can be performed using three primary methods: ion chromatography, enzymatic colorimetric, and UPLC-MS. Ion chromatography separates choline as a quaternary ammonium ion after acid hydrolysis, using cation-exchange columns and suppressed conductivity detection. The enzymatic colorimetric method utilizes specific enzymes to release choline, reacting with a chromogenic reagent to produce a measurable color change. UPLC-MS combines liquid chromatography for separation with mass spectrometry for highly sensitive and accurate detection of choline by ionizing and quantifying it. Each method offers distinct advantages based on sensitivity, accuracy, and the type of sample analyzed.

Method A: Determination of Choline in Food Samples using Ion Chromatography

Chemicals

1. Choline bitartrate
2. Hydrochloric acid
3. Methane sulfonic acid (MSA)

Apparatus

1. Micropipette 1 mL
2. Spatula (small)
3. 50 mL centrifuge tube
4. 15 mL centrifuge tube
5. Aluminium foil
6. Pipette tips (1 mL)
7. Pipette tips (5 mL)
8. Syringe (1 mL)
9. Syringe (10 mL)
10. Syringe (25 mL)
11. Plastic funnel
12. Whatman 40 Ashless filter paper
13. Pasteur pipette plastic
14. Disposable Nitrile gloves (powder free)
15. Parafilm
16. Test tube rack (plastic)

Procedures

A. Chemical Preparation

1. 1N HCl

- a. To prepare 1.0 L of 1 N HCl, take the ampule that contains pure HCl.
- b. Ultra-pure water is added to the 1.0 L volumetric flask until approximately half full.
- c. The ampoule is opened according to the instructions provided on the ampoule box.
- d. Ultra-pure water is added to the mark (1.0 L), and the solution is mixed until homogeneous.

2. 5.0 mM Methane Sulfonic Acid (MSA)

- a. Ultra-pure water is added to the 1.0 L volumetric flask until the water level reaches approximately half of the flask.
- b. A volume of 400 μ L of pure MSA is pipetted into the flask.
- c. Ultra-pure water is added to the mark (1.0 L), and the solution is mixed until homogeneous.
- d. The prepared solution is transferred into a labelled bottle.

3. Stock Choline Hydroxide Solution (1000ppm)

- a. Approximately 7–10 g of choline bitartrate (MW 253.25) is dried at 102°C until a constant weight is obtained.
- b. A mass of 0.1046 g of the dried choline bitartrate is accurately weighed and dissolved in 50 mL of ultra-pure water to prepare a 1000 ppm standard solution.
- c. The solution is stored at 4°C and is considered stable for one week.

4. Working Standard Solution

- The 1000 ppm stock solution is diluted to prepare 10, 25, 50, 75, and 100 ppm working standards.
- Example calculation for preparing a 10 ppm (mg/L) standard solution:

$$M_1V_1 = M_2V_2 \quad M_1 = 10 \quad V_1 = \frac{10 \text{ ppm} \cdot 50 \text{ mL}}{1000 \text{ ppm}} \quad V_1 = 0.5 \text{ mL}$$

- 0.5 mL of the 1000 ppm stock choline hydroxide solution is pipetted into a 50 mL volumetric flask, and ultra-pure water (UPW) is added to the mark (50 mL).

Table 3.3.4.9A: Working Standard Solution

No.	Standard mg/L (ppm)	UPW (mL)	Stock (mL) 1000 mg/L (ppm)	Final volume (mL)
1	100	45	5.0	50
2	75	46.25	3.75	50
3	50	47.50	2.5	50
4	25	48.75	1.25	50
5	10	49.50	0.5	50
6	0.0	50	0.0	50

Example for final volume 50 mL:

Calculation for spiking:

Volume of Spiking Solution =

$$\text{Spike Concentration Desired} \times \frac{\text{Volume of Sample to Which Spike is Added}}{\text{Concentration of the Spiking Solution}}$$

$$\text{Spike Concentration Desired} = 50 \text{ ppm} \quad \text{Volume of Sample to which Spike is Added} = 100 \text{ mL} \quad \text{Con} = \underline{5 \text{ mL}}$$

B Sample Preparation

- A mass of 1 g of powdered sample is weighed.
- The sample is placed into a 50 mL centrifuge tube.
- 2.5 mL of 1 M HCl is added to the tube.
- The tube is capped and vortexed for 10 seconds.
- The cap is hand-loosened to avoid excessive pressure build-up before placing in the water bath.
- The tube is placed in a water bath at 70°C for 50 minutes
 - The tube is removed from the water bath, the cap is tightened, and the tube is vortexed again for 10 seconds.
 - The cap is hand-loosened and the tube is placed back into the water bath, where it is shaken for 10 minutes.
 - After 10 minutes, the shaking function is stopped.
- The steps above (a-c) are repeated two more times, for a total of 3 hours of incubation in the water bath at 70°C.
- After 3 hours, the tube is cooled to room temperature.
- 22.5 mL of ultra-pure water (UPW) is added to the tube (final dilution factor: 25×).
- The tube is screw capped and vortexed for 10 seconds.
- The mixture is centrifuged at 4000 rpm for 12 minutes.
- The supernatant is filtered through ashless filter paper into a clean 50 mL centrifuge tube.
- The filtrate is diluted 1:1 by pipetting 5 mL of sample and 5 mL of UPW into a 15 mL centrifuge tube (final dilution factor: 50×).
- The 15 mL centrifuge tube is closed with aluminium foil (the solution is stable for three days at 4°C).
- The prepared sample is placed in the sample rack.

Reference

Determination of Choline in Dry Milk and Infant Formula. 2002. Dionex Application Note 124

Method B: Enzymatic Colorimetric Method (Milk and Infant Formula containing 44-175 mg solids/100g)

Principles

The product is acid-digested at 70°C to release most of the bound choline. Following pH adjustment, residual choline phospholipids are cleaved with phospholipase D and free choline is subjected to choline oxidase with liberation of H₂O₂. In the presence of peroxidase, phenol is oxidized and a quinoneimine chromophore is formed with 4-aminoantipyrine. Absorbance is measured at 505 nm and choline content is calculated by interpolation from multilevel calibration. For this procedure, choline is defined and reported as the hydroxide. See Table 3.3.4.9B for the results of the interlaboratory study supporting the acceptance of the method.

Chemicals/Reagents

If substituting enzymes come from a different source than that specified, ensure that the declared activity (units/mg) is similar. Store enzymes and 4-aminoantipyrine at 4°C or as instructed by the supplier.

1. Water – Purified to > 18 MΩ resistivity.
2. Hydrochloric acid – 1.0 M. 85 mL of concentrated HCl is measured into a 1 L volumetric flask and diluted to the mark with water. Standardization is not required.
3. Sodium hydroxide solution—40% w/v. 50 g of NaOH is dissolved in approximately 80 mL of water in a graduated cylinder with cooling, and the solution is then diluted to 100 mL.
4. Choline bitartrate—Sigma C-2654 or equivalent. Dry 0.7-1.0 g of choline bitartrate at 102°C to constant weight. Store desiccated at ambient temperature.
 - a. Stock choline standard—2500 µg/mL as choline hydroxide. 523 mg of dry choline bitartrate is added to a 100 mL volumetric flask. It is dissolved in water and diluted to the mark. The solution is stored refrigerated at 4 ± 2°C and should not be used after 1 week.
 - b. The working choline standard is 250 µg/mL as choline hydroxide. 10 mL of the stock solution (a) is pipetted and diluted to 100 mL with water. The solution is prepared fresh daily.
5. Tris (hydroxymethyl) aminomethane (Trizma) buffer. – 0.05 M (pH 8.0). 6.057 g of tris (hydroxymethyl) aminomethane (Sigma T-1503 or equivalent) is added to 500 mL of water in a 1 L volumetric flask. The pH is adjusted to 8.0 using 1M HCl (from step 2), and the solution is diluted to the mark with water. It is stored at 4°C and remains stable for 1 month.
6. Phospholipase D. – Sigma Type VI, P-8023, from *Streptomyces chromofuscus*, 150 units/mg. Unit definition: One unit will liberate 1.0 µmol choline from L-α-phosphatidyl choline (egg yolk) per hour at pH 5.0 at 30°C. This form of phospholipase D is preferred over others due to its activity for sphingomyelins and lysophospholipids.
7. Choline oxidase – Sigma, C-5896, from *Alcaligenes species*, 10 units/mg. Unit definition: One unit will form 1.0 µmol H₂O₂ with oxidation of 1.0 µmol choline to betaine aldehyde per minute at pH 8.0 at 37°C.
8. Peroxidase – Sigma Type 1, P-8125, from horseradish, 80 unit/mg. Unit definition: One unit will form 1.0 mg purpurogallin from pyrogallol in 20 sec at pH 6.0 at 20°C.
9. 4-aminoantipyrine – Sigma A4382 or equivalent.
10. Phenol
11. Chromogenic reagent – Into a 100 mL volumetric flask, sequentially weigh:
 - a. 75-100 unit phospholipase
 - b. 100-120 unit choline oxidase
 - c. 250-280 unit peroxidase
 - d. 15 mg 4-aminoantipyrine and 50 mg phenol
 - e. Dissolve and dilute to volume with 0.05 M Trizma buffer pH 8.0. Prepare fresh daily.

Apparatus/ Instruments

1. Spectrophotometer: Digital readout to 0.001 absorbance at 505 nm. Glass cuvettes (10 mm) of a flow-through cell.
2. Covered water baths: $37 \pm 2^\circ\text{C}$ and $70 \pm 4^\circ\text{C}$.
3. pH meter: Reading to 0.001 unit, with calibration buffers, 4.0 and 7.0.
4. Pipettes: Calibrated glass, or preferably auto-pipettors for 0.100 and 3.0 mL.
5. Volumetric flasks: 10, 50 and 100 mL and 1 L.
6. Digestion vessels: A conical flask, 100 and 150 mL, with stoppers. Alternatively, boiling tubes (dimensions ca 150 x 25 mm) with stoppers can be used, with a suitable rack. Either vessel should permit the insertion of a pH probe for post-digestion pH adjustment.
7. Test tubes: 10 mL with glass stoppers, with rack. Alternatively, disposable polypropylene or polystyrene plastic tubes fitted with screw-capped lids may be used.
8. Filter paper: medium speed, porosity 1.4-2.9 microns, Whatman No. 2, or equivalent.

Procedure

Note: All assay steps are performed under incandescent lighting; fluorescent lighting and direct sunlight are avoided.

A. Hydrolysis

1. A test portion of 5.00 g is weighed into a 100 mL conical flask (or boiling tube).
2. 30 mL of 1.0 M HCl, C(b), is added. The flask is stoppered and the contents are mixed by shaking until well dispersed.
3. The flask is placed into a water bath at 70°C for 3 hours, shaking occasionally.
4. Stoppers are loosened or removed occasionally to avoid excessive pressure build-up. After hydrolysis, the mixture is cooled to ambient temperature and the pH is adjusted to 3.4–4.0 using 50% NaOH, C(c). The discoloured solution is quantitatively transferred into a 50 mL volumetric flask.
5. The solution is diluted to the mark with water and filtered through filter paper. The first 5–10 mL of filtrate is discarded, and the remaining filtrate is collected.
6. The filtrate is ensured to be free of particulates; if particulates remain, the solution is refiltered. The filtrate is considered stable and may be stored in the dark at 4°C for up to three days. The filtrate is returned to ambient temperature before proceeding with the assay.
7. Two 10 mL test tubes are separately labelled as test solution (tube 1) and blank (tube 2) for each test solution. A 0.100 mL aliquot of filtrate is dispensed into each tube.

B. Preparation of Standard Curve

1. Aliquots of 2, 4, 6, and 8 mL of the working standard, C(d)(2), (250 $\mu\text{g/mL}$) are pipetted into four separate 10 mL volumetric flasks and diluted to the mark with water.
2. The fifth calibrant consists of the undiluted working standard. A five-level standard curve (50, 100, 150, 200, and 250 $\mu\text{g/mL}$) is prepared by dispensing 0.100 mL of each standard into separately labelled 10 mL test tubes.
3. A reagent blank tube (0 $\mu\text{g/mL}$) is prepared by substituting 0.100 mL of water for the choline solution.

C. Enzymatic Determination

1. Each blank (tube 2) receives 3.0 mL of water. In addition, 3.00 mL of chromogenic reagent, C (k), is added to each test solution (tube 1), the five standards, and the reagent blank.
2. The test tubes are placed in a covered water bath at $37 \pm 2^\circ\text{C}$ for 15 minutes to develop a pink/red colour. They are then removed from the water bath and cooled to ambient temperature for 15 minutes. Without delay, the contents of each tube are transferred into 10 mm optical cells or analysed using a flow-through cuvette.
3. The spectrophotometer is zeroed at 505 nm against water, and the absorbance of the standards (Astd), test solution (A'), blanks (Abl), and reagent blank (Areag) is measured.

Calculation

1. A standard calibration of net absorbance ($A_{std} - A_{reag}$) versus choline concentration ($\mu\text{g/mL}$) is created using linear least mean square regression analysis with a forced origin.
2. The absorbance of the blanks and reagent blank is subtracted from each test solution to eliminate nonspecific spectral background:

$$\text{Net absorbance of test solution, } A = A' - A_{bl} - A_{reag}$$

3. The choline concentration in the test solution is determined and expressed as choline hydroxide:

$$\text{Choline hydroxide, } \frac{\text{mg}}{100 \text{ g}} = \frac{A}{S} \times \frac{V}{1000} \times \frac{100}{W}$$

where A = net absorbance of test solution

S = slope of standard curve

V = volume in mL of the hydrolysates (50 mL)

W = weight in grams of test portion (5.00 g)

100 = conversion to 100 g basis

1000 = conversion μg to mg. Report data to one decimal place.

Table 3.3.4.9B: Interlaboratory study results for choline in milk and infant formula

Parameter ^a	Sample ^b							
	1	2	3	4	5	6	7	8
No. of labs	28	29	29	29	29	28	29	26
Average, mg/100 g	87.66	73.22	173.0	47.13	99.71	132.68	109.39	124.94
S_D , mg/100 g	3.03	2.03	5.95	1.52	2.23	3.07	2.01	3.16
RSD _D %	3.46	2.77	3.44	3.23	2.24	2.32	1.84	2.53
2.8s, mg/100 g	8.49	5.68	16.66	4.26	6.25	8.60	5.62	8.86
S_R , mg/100 g	4.55	4.19	8.04	2.99	5.26	5.85	4.66	6.54
RSD _R %	5.19	5.73	4.64	6.34	5.27	4.41	4.26	5.23
S_R , mg/100 g	12.75	11.74	22.50	8.37	14.72	16.39	13.06	18.31

^a 1, 3, 4 = Milk/whey-based infant formula; 2 = milk-based follow on; 5 = soy-based infant formula; 6 = NIST SRM 1846, 7 and 8 = whole milks. Samples are variously vitaminized, oil-filled, lecithinated, and supplemented with choline chloride.

^b All values excluding invalid data, no outliers detected.

Reference

J. AOAC Int. 83, 131 (2000)

Method C: Ultra Performance Liquid Chromatography–Mass Spectrometry (UPLC-MS) Method

Principles

Choline, β -hydroxyethyltrimethyl-ammonium hydroxide, is a water-soluble essential nutrient required for synthesizing phospholipids in cell membranes, methyl metabolism, and normal cholinergic neurotransmission in humans. Choline compounds in food samples are extracted and partitioned into organic and aqueous phases using methanol and chloroform and analysed directly by liquid chromatography-electrospray ionization-isotope dilution mass spectrometry (LC-ESI-IDMS). Samples are analysed for free choline (Cho) and choline-contributing compounds: glycerophosphocholine (GPCCho), phosphocholine (Pcho), phosphatidylcholine (Ptdcho) and sphingomyelin (SM). Total choline content in the food sample will be calculated as the sum of Cho, GPC, Pcho, Ptdcho and SM.

Chemicals/Reagents

1. Methanol, HPLC grade
2. Chloroform, HPLC grade
3. Standards for the calibration curve
 - a. Choline chloride
 - b. Glycerophosphocholine
 - c. Phosphocholine
 - d. Phosphatidylcholine
 - e. Sphingomyelin
4. Isotopes
 - a. Choline-[N,N,N-trimethyl-d₉]bromide
 - b. Phosphocholine-[N,N,N-trimethyl-d₉] chloride
 - c. 1,2-Dipalmitoyl-sn-3-glycero-phosphocholine-[N,N,N-trimethyl-d₉]
 - d. Synthesis of glycerophosphocholine-[N,N,N-trimethyl-d₉]
 - i. 10 μ mol of 1,2-dipalmitoyl-sn-3-glycerophosphocholine-[N, N, N-trimethyl-d₉] in 1 mL of chloroform/methanol (1:4) mixture is hydrolyzed with 100 μ L of 1.2 N NaOH in methanol/water (1:1) at 37°C for 10 minutes.
 - ii. After incubation, the mixture is neutralized with 150 μ L of 1N acetic acid. Then, 2 mL of chloroform/methanol (9:1), 1 mL of isobutanol and 2 mL of water are added to the mixture.
 - iii. The lower phase is re-extracted with 1 mL of methanol/water (1:2). The aqueous phase is combined, dried, and purified by HPLC.

4. Isotopes
 - a. Choline-[N,N,N-trimethyl-d9]bromide
 - b. Phosphocholine-[N,N,N-trimethyl-d9] chloride
 - c. 1,2-Dipalmitoyl-sn-3-glycero-phosphocholine-[N,N,N-trimethyl-d9]
 - d. Synthesis of glycerophosphocholine-[N,N,N,-trimethyl-d9]
 - i. 10 µmol of 1,2-dipalmitoyl-sn-3-glycerophosphocholine-[N, N, N-trimethyl-d9] in 1 mL of chloroform/methanol (1:4) mixture is hydrolyzed with 100 µL of 1.2 N NaOH in methanol/water (1:1) at 37°C for 10 minutes.
 - ii. After incubation, the mixture is neutralized with 150 µL of 1N acetic acid. Then, 2 mL of chloroform/methanol (9:1), 1 mL of isobutanol and 2 mL of water are added to the mixture.
 - iii. The lower phase is re-extracted with 1 mL of methanol/water (1:2). The aqueous phase is combined, dried, and purified by HPLC.
 - e. Synthesis of Sphingomyelin –[N-methyl-d3]
 - i. 1 mmol of sphingomyelin and 6 mmol of 1,4-diazabicyclo(2,2,2)-octane are dissolved in 50 mL of dimethyl formamide. The mixture is refluxed under a stream of nitrogen for 6 hours.
 - ii. The mixture is cooled and poured into 20 mL of ice-cold 1 N HCl. It is then extracted with 50 mL of chloroform. The chloroform extract is collected and dried with heat. 170
 - iii. 200 mg of the dried extract is mixed with 30 µL of cyclohexylamine (0.35 mmol) and 90 µL of iodomethane-d₃ in 5 mL of methanol, then sealed tightly in a Teflon-lined screw cap tube and stored at room temperature in the dark for 18 hours.
 - iv. After that, the solvent is evaporated under vacuum. The residue is dissolved in chloroform and washed twice with 5% sodium thiosulfate, 2N HCl, and water. It is then dried and purified using an SPE cartridge (aminopropyl column).

Apparatus/ Instruments

1. Pipettes: 10 mL, 1 mL
2. Micropipettes: 100 µL
3. Incubator
4. Screw cap tube, Teflon
5. SPE cartridge, aminopropyl column
6. Balance: analytical sensitivity ± 0.1 mg
7. Vortex mixer
8. Centrifuge
9. Fume hood
10. Volumetric flask: 10 mL
11. Ultrafiltration system with membrane filter, 0.45 µm
12. UPLC with PDA detector and column heater: equipped with MS system and Q-Tof
13. Analytical column, silica column: 150 mm x 2.1 mm I.D. and particle size 1.7 µm

UPLC-ESI-MS Conditions

1. LC Silica column of 150 mm x 2.1 mm I.D. and particle size 1.7 µm (e.g., UPLC HILIC, Waters)
2. Guard column with I.D of 2.1 mm, a length of 5 mm and 1.7 µm, which is packed with the same material as that of the analytical column
3. Waters ACQUITY UPLC® system*
4. Waters ACQUITY UPLC® sample organizer*
5. Waters ACQUITY UPLC® binary pump to deliver the mobile phase*
6. Waters ACQUITY UPLC® column heater/ cooler*
7. Waters ACQUITY UPLC® PDA detector*
8. Waters SYNAPT™ mass spectrometry (MS) system*
9. Water Q-Tof Premier™*
10. A computer with a MassLynx™ Informatics control software*
11. A printer to print the results and chromatograms.

**Brand and model may differ for other manufacturers*

Procedures

A. Sample Preparation

1. The sample is frozen at -80°C and pulverised prior to analysis.
2. 100 mg food sample is accurately weighed and spiked with deuterium-labelled internal standards (isotopes) of all analytes.
3. 400 μL of methanol/chloroform (2:1, v/v) is added, and the mixture is vortexed vigorously. The sample is left at -20°C overnight before being centrifuged at $1500 \times g$ for 5 minutes.
4. The supernatant is transferred to a new tube, and the residue is re-extracted with 250 μL of methanol/chloroform/water (2:1:0.8, v/v).
5. The supernatants from both extractions are combined, followed by adding 100 μL of chloroform and then 100 μL of water to form two phases. The mixture is centrifuged.
6. After centrifugation, the aqueous phase and chloroform phase are separated.
7. The aqueous phase is dried and redissolved in 20 μL of water. Subsequently, 800 μL of methanol is added, the mixture is vortexed, centrifuged, and the supernatant is collected for LC-MS analysis (10 μL).
8. The chloroform phase is dried, resuspended in chloroform, and purified using a solid-phase extraction (SPE) cartridge (aminopropyl column) prior to LC-MS analysis.

B. Instrument Set Up Procedures

1. Before turning on the UPLC, the column, reservoir, and mobile phase are checked to ensure the correct configuration.
2. The MassLynx 4.1 control software and UPLC system are turned on, and the instrument can warm up for at least 30 minutes before starting.
3. The Start Up System is activated; purging is selected for 5 minutes for valves A and B, and the sample needle is washed for 5 cycles.
4. The pump is set to allow the column to equilibrate with the mobile phase, which usually requires approximately 1 hour.
5. The UV and visible lamps are ensured to be turned on, and the PDA detector is set at a wavelength range of 200–500 nm.
6. To check whether the column is equilibrated, the standard mixture described above is injected for two runs; if the elution times of the standards remain consistent across both runs, the column is considered ready for sample injection.
7. The sample identity is entered, and the sequence is built in the SAMPLE LIST within MassLynx.

C. Chromatography of Choline

1. UPLC Condition

- a. The column temperature is set at 40°C , and the sample temperature is maintained at 4°C .
- b. Mobile phase A is prepared as a mixture of acetonitrile/water/ethanol/1 M ammonium acetate/glacial acetic acid in the 800/127/68/3/2 (v/v) ratio.
- c. Mobile phase B is prepared as a mixture of acetonitrile/water/ethanol/1 M ammonium acetate/glacial acetic acid in the ratio 500/500/85/27/18 (v/v).
- d. The gradient elution is programmed as follows for the aqueous phase:

Table 3.3.4.9Cd: Gradient elution (programmed as follows for the aqueous phase)

0.0 min	0% B	0.4 mL/min
3.0 min	0% B	0.4 mL/min
10.0 min	40% B	0.4 mL/min
14.0 min	45% B	0.3 mL/min
18.0 min	60% B	0.3 mL/min
19.0 min	100% B	0.5 mL/min
27.0 min	100% B	0.5 mL/min
29.0 min	0% B	0.4 mL/min

- e. The gradient elution is programmed as follows for the (chloroform) phase:

Table 3.3.4.9Ce: Gradient elution (programmed as follows for the chloroform phase)

0.0 min	0% B	0.4 mL/min
3.0 min	0% B	0.4 mL/min
10.0 min	20% B	0.4 mL/min
14.0 min	60% B	0.3 mL/min
16.0 min	100% B	0.3 mL/min
17.0 min	100% B	0.5 mL/min
20.0 min	100% B	0.5 mL/min
22.0 min	0% B	0.4 mL/min

- f. A sample injection volume of 10 µL is used, with full-loop injection.
g. The injection needle is washed using either 0.1% formic acid in water (weak wash) or 0.1% formic acid in methanol (strong wash).
h. LC effluent from 5–26 minutes is directed into the mass spectrometer (MS) for the aqueous phase. LC effluent from 4–14 minutes is directed into the MS for the organic phase.

2. MS Condition

- A portion of column effluent at 0.6 mL/min is introduced into the MS.
 - ESI-MS spectra are acquired in positive ion mode.
 - Nitrogen is used as both desolvation gas (350 L/hour) and cone gas (50 L/hour).
 - The desolvation temperature is set at 350°C.
 - The source temperature is set at 105°C.
 - The capillary voltage is set at 5 kV, and the cone voltage is set at 180 V.
 - Argon is used as the collision gas at a pressure of 3.5×10^{-3} mBar.
 - For the aqueous phase, the following ions are monitored:
 - m/z 104 (Cho)
 - m/z 113 (Cho-d9)
 - m/z 258 (GPCho)
 - m/z 267 (GPCho-d9)
 - m/z 184 (PCho)
 - m/z 193 (PCho-d9)
 - For the organic phase, the following ions are monitored:
 - m/z 184 (PtdCho and SM)
 - m/z 193 (PtdCho-d9)
 - m/z 187 (SM-d3)
- Calibration curve standards are prepared by mixing 20 nmol of deuterium-labelled internal standards with varying amounts of analytes.
 - Calibration curves are constructed by relating the varying amounts of each analyte to their relative response factors (RRFs), determined by the ratio of the peak area of the analyte to that of the corresponding deuterium-labelled internal standard.
 - The sample's total choline content (mg/100 g food) is calculated as the sum of Cho, GPCho, PCho, PtdCho, and SM. Individual metabolites are reported as mg choline moiety per 100 g of food.

References

- Koc, H., Mar, M-H., Ranasinghe, A., Swenberg, J.A. & Zeisel, S. H. 2002. Quantitation of choline and its metabolites in tissues and food by liquid chromatography/ electrospray ionization-isotope dilution mass spectrometry. *Analytical Chemistry*. 74: 4734-4740.
- Zeisel, S.H., Mar, M-H, Howe, J-C, Hoilden, J.M. 2003. Concentrations of choline-containing compounds and betaine in common foods. *Journal of Nutrition*. 133: 1302-1307.

3.3.4.10 DETERMINATION OF ASCORBIC ACID (VITAMIN C) IN FOOD (HPLC METHOD)

Principles

High-pressure liquid chromatography analytical methods are now replacing the traditional titrimetric and colorimetric methods for more accurate determination of total vitamin C. The HPLC method can distinguish between L-ascorbic acid and isoascorbic acid and eliminate other deficiencies of the traditional method. For example, colour interference by highly coloured extracts during the titration's endpoint, and overestimation of total vitamin C content due to dye reduction by other substances like ferrous, tannin and cuprous.

Ascorbic acid in the sample is extracted in an acidic environment and completed rapidly in subdued light. Metaphosphoric acid is chosen as the extractant because it inhibits L-ascorbic acid oxidase and metal catalysis, precipitates proteins, and is compatible with the LC system. Besides, tris (2-carboxyethyl)-phosphine (TCEP) is included in the extractant to convert dehydroascorbic into its reduced form and stabilize ascorbic acid.

This method employs a reverse-phase HPLC procedure. 0.05% of formic acid is used as the mobile phase with a Phenomenex C18 column. The diode array detector is set at 255 nm for L-ascorbic acid determination.

Chemicals/Reagents

1. Extraction buffer preparation
 - a. An extraction buffer containing 5% metaphosphoric acid (MPA), 1 mM ethylenediaminetetraacetic acid (EDTA), and 5 mM tris(2-carboxyethyl)phosphine (TCEP) is prepared.
 - b. 5% MPA is prepared by dissolving 50 g of MPA in deionized water and diluting to a final volume of 1 L.
 - c. 150 mg of EDTA and 717 mg of TCEP are dissolved in 5% MPA, then the solution is made up to a final volume of 500 mL in a volumetric flask. The extraction buffer is refrigerated until use.
2. Mobile phase preparation
 - a. 0.05% formic acid is prepared by dissolving 0.5 g of formic acid in deionized water and diluting to a final volume of 1 L in a volumetric flask. The mobile phase is filtered using a 0.45 µm nylon membrane filter with a solvent filtration apparatus and prepared freshly prior to each analysis.
3. Vitamin C standard solution
 - a. For ascorbic acid determination, crystalline L-ascorbic acid (99% purity, e.g., from Sigma Chemical Co.) is used as a standard.
 - b. Ascorbic acid standard stock solution. 10 mg of standard is weighed and made up to 100 mL with 5% MPA to obtain a concentration of 100 µg/mL. The solution is prepared freshly prior to analysis.
 - c. Ascorbic acid standard working solution. 10 mL of the standard stock solution (b) is pipetted into a 100 mL volumetric flask and made up to volume with 5% MPA. The final concentration of the working solution is 10 µg/mL.

Note: All standards for ascorbic acid should be prepared in a room with subdued light. All sample treatment and analytical procedures were also carried out in this room.

Apparatus/ Instruments

1. Analytical balance
2. Conical flask: 100 mL
3. Homogenizer
4. Centrifuge
5. Filtration system with 0.45 µm PVDF membrane filter
6. Volumetric flask: 50 mL
7. HPLC with UV detector
8. Analytical column: RP C18, 150 mm x 4.6 mm I.D and particle size 5 µm
9. Guard column: length 20 mm, I.D. 4.6 mm

HPLC Conditions

1. Reverse phase C18 column: 150 mm x 4.6 mm I.D. and particle size 5 μ m (e.g., Atlantis, Waters).
2. Guard column holder with a guard column: it has an ID of 4.6 mm and a length of 20 mm, packed with the same material as the analytical column.
3. Agilent 1200 series standard and preparative autosampler, G1239. *
4. Agilent 1200 series binary pump, G1312, to deliver the mobile phase. *
5. Agilent 1200 series micro vacuum degasser, G1379. *
6. Agilent 1200 series diode arrays multiple wavelength detector (DAD), G1315 for ascorbic acid detection at the wavelength of 254 nm. *
7. Solvent cabinet to keep the mobile phases.
8. A computer with an Agilent ChemStation control software. *
9. A printer to print the results and chromatograms.

**Brand and model may differ for other manufacturers*

Procedures

A. Sample Extraction

1. A mass of 10 g (for fresh sample) or 2 g (for dried sample) of the food item is accurately weighed into a 100 mL conical flask.
2. 15 mL of cooled extraction buffer is added to the flask, and the sample is extracted for 10 minutes using a homogeniser.
3. The extraction procedure is repeated 3–4 times, and the extracts are combined.
4. The combined extract is centrifuged at 10°C and 5000 rpm for 30 minutes.
5. The supernatant is decanted and filtered through a 0.45 μ m PVDF membrane filter.
6. The filtered extract is quantitatively transferred into a 50 mL volumetric flask and made up to volume with extraction buffer (V1).

B. Instrument Set Up Procedures

1. Before turning on the HPLC, the column, reservoir, and mobile phase are checked to ensure the correct columns and mobile phases are used.
2. The control software and HPLC system are turned on, and the instrument can warm up for at least 30 minutes before starting.
3. The purge valve on the pump module is opened by turning the knob counter-clockwise several turns so that the priming solvent is delivered directly to the waste container.
4. The pump is set to 100% flow at a rate of 5 mL/min to prime each channel of the solvent delivery system with the respective mobile phase for several minutes.
5. The pump is turned off, the purge valve is closed, and the required mobile phase composition (isocratic) and flow rate of 1 mL/min are set for ascorbic acid analysis.
6. The pump is turned on again, and the column can equilibrate with the mobile phase, which usually requires approximately 1 hour.
7. The UV and visible lamps are ensured to be turned on, and the DAD detector is set at a wavelength of 255 nm.
8. To check whether the column is equilibrated, the standard mixture described above is injected for two runs; if the elution times of the standards remain the same across both runs, the column is considered ready for sample injection.
9. The sample identity is entered, and the sequence is built in the SAMPLE SEQUENCE for the autosampler.

C Chromatography of Ascorbic Acid

1. The extract is filtered through a 0.45 µm PVDF syringe filter into a sample vial.
2. Ascorbic acid in the sample is identified by comparing its retention time with the standard L-ascorbic acid.
3. The peak areas from each standard solution (0.5–50 µg/mL) are used to obtain a standard curve.
4. The ascorbic acid content of the sample is quantified by comparing the integrated chromatographic peak areas from the test samples to those of the known standard solutions. The total amount of ascorbic acid in the sample is expressed as mg per 100 g of sample.

Calculation

mg ascorbic acid per 100 g food =

$$\frac{\text{Peak area of sample}}{\text{Peak area of std.}} \times \text{Amount of std. in g} \times \frac{V1(\text{mL})}{\text{Inject volume in mL}} \times \frac{100}{\text{Wt. of food used}}$$

Reference

Phillips, K.M., Tarragó-Trani, M. T., Gebhardt, S. E., Exler, J., Patterson, K. Y., Haytowitz, D. B., Pehrsson, P. R., Holden, J. M. (1992). Stability of vitamin c in frozen raw fruit and vegetable homogenates. *Journal of Food Composition and Analysis*, 23:253-259.

3.3.5 DETERMINATION OF AMINO ACIDS

The determination of amino acids in foods is important to identify the various types of amino acids present so that consumers can make better choices. The analysis of amino acids comprises several steps: sample preparation (hydrolysis of protein to liberate amino acids), separation of individual amino acids, detection, and quantification of the amino acids.

The AOAC uses various methods and different kinds of instruments to analyse amino acids in food samples, depending on the types of amino acids and the food matrices.

3.3.5.1 DETERMINATION OF AMINO ACIDS IN FOOD (LC METHOD)

Principles

Amino acid composition in the food samples is detected and quantified using Liquid Chromatography (High Performance and Ultra Performance) with 3 types of detectors:

- Tuneable Ultraviolet (TUV) detector (TUV detector operates from 190 to 700 nm)
- Photodiode Array (PDA) detector (PDA detector operates between 190 and 500 nm)
- Fluorescence (FLR) detector (FLR detector operates from 200 to 890 nm)

Three procedures must be used for each food sample to quantify all amino acids present in the protein. The procedures are:

A. 6 N HCl Hydrolysis:

- For all amino acids except cysteine/cystine, methionine and tryptophan.

B. Performic Acid Oxidation followed by 6N HCl Hydrolysis:

- Cysteine/cystine (oxidised to cysteic acid) and methionine (oxidised to methionine sulphone).

C. Alkaline Hydrolysis:

- For tryptophan.

Chemicals/Reagents

1. Waters AccQ Tag Eluent A
2. Waters AccQ Tag Eluent B
3. Waters AccQ Fluor Reagents
4. Waters AccQ Amino acid standard
5. Waters AccQ 2 Amino butyric acid (AABA) as internal standard
6. Deionized water (DH₂O)
7. Acetonitrile, LC grade (ACN)
8. Hydrochloric acid (HCl)
9. L-methionine sulfone (L-MetS)
10. L-cysteic acid monohydrate (L-CyaH₂O)
11. Formic acid (CH₂O₂)
12. Hydrogen Peroxide (H₂O₂)
13. Hydrogen bromide (HBr)
14. Sodium acetate
15. Methanol
16. Tryptophan Standard
17. Sodium hydroxide (NaOH)

Apparatus/Instruments

1. Balance: analytical sensitivity ± 0.1 mg
2. Vortex mixer
3. Nitrogen gas blower
4. Oven
5. Rotary evaporator
6. Refrigerator

7. pH meter
8. Test tubes: 10 mL
9. Graduated bottle: 250 mL
10. Volumetric flasks: 10, 50, 100, 500 & 2000 mL
11. Measuring cylinder: 10 & 200 mL
12. Pasteur pipette
13. Micropipette
14. Test tubes: black screw-capped
15. Test tubes with stoppers
16. Filter funnel
17. Filter paper
18. Syringe filter: 0.45 µm cellulose acetate membrane
19. Syringe filter: 0.2 µm cellulose acetate membrane
20. Ultra filtration equipment: 0.45 µm, cellulose acetate membrane filters
21. Syringe
22. Centrifuge tube: 1.5 mL
23. Low-insert-volume tubes
24. Vials

Procedures

A. 6 N HCl Hydrolysis

Chromatographic Conditions:

The analysis is performed using the following UPLC system components:

- Waters Acquity UPLC H-Class Quaternary Solvent Manager
- Waters Acquity UPLC H-Class Sample Manager
- Waters Acquity UPLC H-Class PDA Detector

The column used is a Waters AccQ Tag Ultra C18 column (1.7 µm, 2.1 × 100 mm).

1. Mobile phase:

A: Waters AccQ Tag Eluent A

50 mL of Waters AccQ Tag Eluent A is measured and made up to 1 L with deionised water (DH₂O). The solution is filtered through a 0.2 µm nylon membrane filter.

B: Waters AccQ Tag Eluent B

Waters AccQ Tag Eluent B is taken directly and filtered through a 0.2 µm nylon membrane filter.

C: 10% ACN

100 mL of acetonitrile is measured and made up to 1 L with deionised water. The solution is filtered through a 0.2 µm nylon membrane filter.

D: 100% ACN

1 L of 100% acetonitrile is measured and filtered through a 0.2 µm nylon membrane filter.

2. Derivatisation is performed using Waters AccQ Fluor Reagents.
3. The amino acid standard hydrolysate used is Standard H (Pierce).
4. Flow rate is set at 0.7 mL/min.
5. The column temperature is maintained at 55°C.
6. Detection is carried out using a PDA detector.
7. The injection volume is set at 10 µL.

Table 3.3.5.1: Program Gradient

Time (min)	Flow (mL/min)	%A	%B	%C	%D	Curve
Initial	0.7	99.9	0.1	0	0	*
0.54	0.7	99.9	0.1	0	0	6
5.74	0.7	90.9	9.1	0	0	7
7.74	0.7	78.8	21.2	0	0	6
8.04	0.7	40.4	59.6	0	0	6
8.64	0.7	40.4	59.6	0	0	6
8.73	0.7	99.9	0.1	0	0	6
11.00	0.7	99.9	0.1	0	0	6

Table 3.3.5.2: Shut-down and Storage of Column

Time (min)	Flow (mL/min)	%C	%D	Curve
Initial	1.0	100.0	0.0	*
1.00	1.0	0.0	100.0	6
20.00	0.0	0.0	0.0	11

Procedures

A. Preparation of 6 N HCl

1. 523.56 mL of concentrated HCl is measured and made up to 1 L with deionised water (DH₂O).

B. Preparation of 0.1 N HCl

1. 0.1 N HCl is taken from the ampoule and diluted to 1 L with DH₂O.

C. Preparation of 2 Amino Butyric Acid (AABA) (internal standard) (50 µmole/mL)

1. A mass of 0.516 g of AABA is dissolved in 1 L of 0.1 N HCl.

D. Preparation of 2 Amino Butyric Acid (AABA) (internal standard) (25 µmole/mL)

1. 500 mL of 50 µmol/mL AABA solution is measured and made up to 1 L with 0.1 N HCl.

E. Preparation of Amino Acids Standard

1. A volume of 200 µL of 25 µmol/mL AABA internal standard is pipetted into a centrifuge tube and 800 µL of 0.1 N HCl is added (final concentration: 1 mL = 5 µmol/mL AABA).
2. A volume of 800 µL of the prepared 5 µmol/mL AABA solution is pipetted into a separate centrifuge tube, followed by the addition of 500 µL of Amino Acid Standard 'H' (final concentration: 1 mL = 2.5 µmol/mL of amino acids).

F. Sample Preparation and Addition of Internal Standard

1. A sample weighing 0.1–0.2 g is weighed and placed into a black screw-capped test tube.
2. 5 mL of 6 N HCl is added; the tube is vortexed and flushed gently with N₂ gas.
3. The sample is hydrolysed by heating in an oven at 110°C for 24 hours.
4. The tube is cooled to room temperature.
5. A 10 mL volume of 25 µmol/mL AABA internal standard is pipetted into a 100 mL volumetric flask.
6. A filter funnel is placed into the volumetric flask, and the hydrolysed sample from the test tube is poured in. The solution is made up to 100 mL with DH₂O.
7. The sample is filtered into a plastic test tube through filter paper (No. 541).
8. An aliquot of the filtrate is further filtered through a syringe filter into a final tube for analysis.

G. Derivatization

1. The Waters AccQ Fluor reagent is prepared (Moisture is kept away from the reagent at all times).
 - i. The reagent bottle (2A) is tapped gently until the reagent powder has settled.
 - ii. 1 mL of acetonitrile (2B) is added to bottle 2A; the bottle is capped quickly and vortexed.
 - iii. The solution is heated at 55°C for less than 10 minutes until the powder completely dissolves and the solution appears clear.
2. The following mixtures are then prepared:

Table 3.3.5.3: Derivatization

Solution	Derivatization Blank	Standard	Unknown	Remarks
Borate Buffer (µL)	80	70	70	In 1.5 mL centrifuge tube
Std / Unknown (µL)	-	10	10	Rinse the pipette tip & vortex
Vortex immediately				
Waters AccQ reagent (µL)	20	20	20	Vortex at once after adding. Add & vortex one by one
Vortex again				

- i. 10 µL of sample is pipetted into a 1.5 mL centrifuge tube
- ii. 20 µL of AccQ reagent is added, and the mixture is vortexed immediately.
- iii. Add 70 µL borate buffer, vortex immediately.
A volume of 70 µL of borate buffer is added, followed by immediate vortexing.
- iv. It is left for 1 minute.
- v. The tube is heated at 55°C for 10 minutes.
- vi. The derivatised sample is centrifuged at 4000 rpm for 20 minutes.
- vii. 100 µL of the supernatant is transferred into a low-insert volume tube.
- viii. The prepared sample is injected into the LC system

Table 3.3.5.4: The Weight of Amino Acids Injected

No	Amino acid	Weight injected (ng)	No	Amino acid	Weight injected (ng)
1	Asp	16.6375	11	AABA	1
2	Ser	13.1375			
3	Glu	18.2625	13	Tyr	22.65
4	Gly	9.3875	14	Val	14.6375
5	His	19.4			
6	NH3	1	16	Lys	18.275
7	Arg	21.775	17	Ile	16.4
8	Thr	14.8875	18	Leu	16.4
9	Ala	11.1375	19	Phe	20.65
10	Pro	14.3875			

B. Performic Acid Hydrolysis

Chromatographic Conditions:

1. The analysis is performed using the following UPLC system components:
 - Waters Acquity UPLC H-Class Quaternary Solvent Manager
 - Waters Acquity UPLC H-Class Sample Manager
 - Waters Acquity UPLC H-Class PDA Detector

2. The column used is a Waters AccQ Tag Ultra C18 column.

3. Mobile phase:

A: Waters AccQ Tag Eluent A

50 mL of Waters AccQ Tag Eluent A is measured and made up to 1 L with deionised water (DH₂O). The solution is filtered through a 0.2 µm nylon membrane filter.

B: Waters AccQ Tag Eluent B

Waters AccQ Tag Eluent B is taken directly and filtered through a 0.2 µm nylon membrane filter.

C: 10% ACN

100 mL of acetonitrile is measured and made up to 1 L with deionised water. The solution is filtered through a 0.2 µm nylon membrane filter.

D: 100% ACN

1L of 100% acetonitrile is measured and filtered through a 0.2 µm nylon membrane filter.

4. Derivatisation is performed using Waters AccQ Fluor Reagents.
5. The amino acid standard hydrolysate used is Standard H ('Pierce).
6. The flow rate is set at 0.7 mL/min.
7. The column temperature is maintained at 55°C.
8. Detection is carried out using a PDA detector.
9. The injection volume is set at 8 µL.

Table 3.3.5.5: Program Gradient

Time (min)	Flow (mL/min)	%A	%B	%C	%D	Curve
Initial	0.7	99.9	0.1	0	0	Initial
5.74	0.7	90.9	9.1	0	0	7
5.74	0.7	90.9	9.1	0	0	7
6.72	0.7	84.9	15.1	0	0	6
7.02	0.7	40.4	59.6	0	0	6
7.62	0.7	40.4	59.6	0	0	6
7.71	0.7	99.1	0.9	0	0	6
11.00	0.7	99.9	0.1	0	0	6

Procedures

A. Preparation of L-Methionine Sulfone (L-MetS) (25 µmole/mL)

1. A mass of 0.4530 g of L-MetS is dissolved in 100 mL of 0.1 N HCl.
2. The solution is filtered through a 0.2 µm nylon membrane filter.

B. Preparation of L-Cysteic Acid Monohydrate (L-CyaH₂O)

1. A mass of 0.4679 g of L-Cya·H₂O is dissolved in 100 mL of 0.1 N HCl.

C. Preparation of Standards

1. 500 µL of AABA internal standard (50 µmol/mL), 1 mL of L-Cya·H₂O (25 µmol/mL), 1 mL of L-MetS (25 µmol/mL), and amino acids standard 'H' are pipetted into a 10 mL volumetric flask.
2. The volumetric flask is filled to 10 mL with 0.1 N HCl (1 mL = 2.5 µmol of AA, AABA, L-MetS, and L-Cya·H₂O).
3. 500 µL of the prepared 2.5 µmol/mL solution is pipetted into a centrifuge tube and mixed with 500 µL of amino acids standard 'H' (2.5 µmol/mL), resulting in a final concentration of 1 mL = 1.25 µmol/mL of AA and AABA. The prepared standard mixture is stored at -20°C.
4. 80 µL of the 1.25 µmol/mL AA standard mixture is pipetted.
5. 10 µL of the 1000 µL solution (1 mL = 0.1 µmol/mL AA) is pipetted for derivatisation. Then, 70 µL of borate buffer and 20 µL of Waters AccQ reagent are added sequentially.
6. 0.8 µL (equivalent to 8 µmol AA and AABA per mL) is injected into the UPLC system.

Table 3.3.5.6: Preparation of Performic Acid

No. of samples	Formic acid (HCOOH) (mL)	Hydrogen Peroxide (H ₂ O ₂) (mL)
5	13.5	1.5
10	22.5	2.5
20	45.0	5.0
40	90.0	10.0

Sample Preparation

1. A mass of 0.1 g of sample is weighed into a stoppered tube.
2. The tube is placed in ice cubes to keep it chilled.
3. 2 mL of fresh, chilled performic acid is added.
4. The tube is transferred to a refrigerator and left for 16 hours.
5. 0.4 mL of chilled hydrogen bromide (HBr) is then added, and the solution is left to stand for 30 minutes.
6. The solution is transferred into a beaker.
7. The sample is dried on a steam bath at 80°C.
8. 6 N hydrolysis is performed.
9. A volume of 5 mL of 6 N HCl is added, and the tube is incubated at 110°C for 24 hours.
10. The solution is transferred into a 100 mL volumetric flask. A volume of 200 µL of 50 µmol/mL AABA internal standard is added.
11. The solution is made up to 100 mL with deionised water.

12. The sample is filtered through filter paper and a 0.45 µm nylon syringe filter.
13. 10 µL of the filtered solution is pipetted for derivatisation. 70 µL of borate buffer and 20 µL of Waters AccQ reagent are added.
14. 0.8 µL is injected into the UPLC system.

Table 3.3.5.7: The Weight of Amino Acid Injected

No.	Amino acid	Weight injected (ng)
1	L-CyaH ₂ O	0.9696
2	L-MetS	1.1936
3	AABA	1.0000

C. Analysis of Tryptophan (Alkaline Hydrolysis)

Chromatographic Conditions:

1. The analysis is performed using the following UPLC system components:
 - Waters Acquity UPLC H-Class Quaternary Solvent Manager
 - Waters Acquity UPLC H-Class Sample Manager
 - Waters Acquity UPLC H-Class PDA Detector
2. The column used is an Acquity UPLC BEH C18 column (1.7 µm, 2.1 × 100 mm).
3. Mobile phase: 0.0085 M sodium acetate at pH 4.0: Methanol (86.7 : 13.3)
 - A: 0.0085 M sodium acetate, pH 4.0
A 0.6973 g of sodium acetate is weighed and dissolved in deionised water. The solution is made up to 1 L with deionised water, and the pH is adjusted to 4.0 using acetic acid. The prepared solution is filtered through a 0.45 µm nylon membrane filter.
 - B: Methanol
Methanol is transferred into a 1 L bottle and filtered through a 0.45 µm nylon membrane filter.
4. The flow rate is set at 0.5 mL/min.
5. The column temperature is maintained at ambient conditions.
6. Detection is carried out using a PDA detector.
7. The injection volume for the standard is set at 2 µL.

Table 3.3.5.8: Program Gradient

Time (min)	Flow (mL/min)	%A	%B	%C	%D	Curve
Initial	0.5	86.7	13.3	0	0	Initial

Procedures

A. Preparation of Tryptophan (TRP) Standard:

a. Standard (5 ppm):

1. A mass of 0.05 g of TRP is weighed into a 50 mL volumetric flask.
2. The flask is made up to 500 mL with 0.1 N HCl (1 mL = 1000 µg TRP). The solution is ultrasonicated until fully dissolved.
3. 50 µL of the 1000 µg/mL solution is pipetted into a 10 mL volumetric flask and made up with mobile phase (0.0085 M sodium acetate at pH 4.0: methanol, 86.7:13.3).
4. 2 µL is injected into the HPLC system.
6. 0.8 µL (equivalent to 8 µmol AA and AABA per mL) is injected into the UPLC system.

b. Standard (4 ppm):

1. 800 µL of the 5 ppm standard is taken.
2. 200 µL of mobile phase (0.0085 M sodium acetate at pH 4.0: methanol, 86.7: 13.3) is added and mixed thoroughly.
3. 2 µL of the prepared solution is injected into the HPLC system.

c. Standard (3 ppm):

1. 600 µL of the 5 ppm standard is taken.
2. 400 µL of mobile phase (0.0085 M sodium acetate at pH 4.0: methanol, 86.7: 13.3) is added and mixed thoroughly.
3. 2 µL of the prepared solution is injected into the HPLC system.

d. Standard (2 ppm):

1. 400 µL of the 5 ppm standard is taken.
2. 600 µL of mobile phase (0.0085 M sodium acetate at pH 4.0: methanol, 86.7: 13.3) is added and mixed thoroughly.
3. 2 µL of the prepared solution is injected into the HPLC system.

e. Standard (1 ppm):

1. 200 µL of the 5 ppm standard is taken.
2. 800 µL of mobile phase (0.0085 M sodium acetate at pH 4.0: methanol, 86.7: 13.3) is added and mixed thoroughly.
3. 2 µL of the prepared solution is injected into the HPLC system.

B. Preparation of 4.3 N Sodium Hydroxide (NaOH):

1. A mass of 171.99 g of NaOH is weighed into a ceramic beaker.
2. The solution is made up to 1 L with deionised water.

C. Sample Preparation for Tryptophan (TRP):

1. Approximately 0.2 g of sample is weighed into a stoppered tube.
2. 15 mL of fresh 4.3 N NaOH is added.
3. The sample is sonicated until homogenised.
4. The stoppered tube is heated in an oven at 120°C for 16 hours.
5. The hydrolysate is transferred into a beaker and diluted with deionised water to less than 100 mL. The pH is adjusted to 4.5 using dilute HCl.
6. The solution is 100 mL with deionised water in a volumetric flask.
7. The sample is filtered through filter paper, and an aliquot of the filtrate is further filtered through a 0.2 µm nylon syringe filter.
8. 2 µL is injected into the HPLC system.

References

- 1) AOAC 2019
- 2) Otter, D. E. (2012). Standardised methods for amino acid analysis of food. *British Journal of Nutrition*, 108(S2), S230-S237.
- 3) Puwastien, P., Siong, T. E., Kantasubrata, J., Craven, G., Feliciano, R. R., & Judprasong, K. (2011). *Asean manual of food analysis*. Reg Cent Asean Netw Food Data Syst Inst Nutr Mahidol Univ Thai, 1-190.

3.3.6 DETERMINATION OF FATTY ACIDS

Principles

Fat and fatty acids are extracted from food by hydrolytic methods (acidic hydrolysis for most products, alkaline hydrolysis for dairy products, and a combination for cheese). Pyrogalllic acid is added to minimize oxidative degradation of fatty acids during analysis. Triglyceride, triundecanoin (C11:0), is added as an internal standard. Fat is extracted into ether, then methylated to fatty acid methyl esters (FAMES) using Boron trifluoride (BF₃) in methanol. FAMES are quantitatively measured by gas chromatography (GC) against C11:0 internal standard. Total fat is the sum of individual fatty acids expressed as triglyceride equivalents. Saturated and monounsaturated fats are calculated as the sum of respective fatty acids. Monounsaturated fat includes only the cis form. **Caution: Boron trifluoride may be fatal if inhaled.**

Chemicals/Reagents

1. Pyrogalllic acid
2. Hydrochloric acid – 12 and 8.3 M. To make 8.3 M HCl, add 250 mL of 12 M HCl to 110 mL of H₂O. Mix well. Store at room temperature (20°-25°C).
3. Ammonium hydroxide, NH₄OH – 58% (w/w)
4. Diethyl ether – Purity appropriate for fat extraction
5. Petroleum ether – Anhydrous
6. Ethanol – 95% (v/v)
7. Toluene – Nanograde
8. Chloroform
9. Sodium sulfate, Na₂SO₄ – Anhydrous
10. Boron trifluoride reagent – 7% BF₃ (w/w) in methanol, made from commercially available 14% BF₃ solution. Prepare in the hood.
11. Diethyl ether – petroleum ether mixture – 1+1 (v/v)
 - a. Triglyceride internal standard solution – C11:0-triundecanoin; 5.00 mg/mL in CHCl₃.
 - b. Accurately weigh 2.50 g C11:0- triundecanoin into a 500 mL volumetric flask.
 - c. Add ca 400 mL CHCl₃. Invert the flask at least 10 additional times.
 - d. Triglyceride internal standard solution is stable up to 1 month when stored in a refrigerator (2°-8°C).

Apparatus

1. Gas chromatograph with hydrogen flame ionization detector
2. Capillary column – Separating the FAME pair of adjacent peaks of C18:3 and C20:1 and the FAME trio of adjacent peaks of C22:1, C20:3, C20:4 with a resolution of 1.0 or greater, SP2560 100 m x 0.25 mm with 0.20 µm film is suitable
3. Mojonnier flasks
4. Stoppers – Synthetic rubber or cork
5. Mojonnier centrifuge basket
6. Hengar micro-boiling granules
7. Baskets – Aluminum and plastic
8. Shaker water bath – Maintaining 70°C – 80°C
9. Steam bath – Supporting common glassware
10. Water bath – With nitrogen stream supply, maintaining 40°C ± 5°C
11. Wrist action shaker – Designed for Mojonnier centrifuge baskets
12. Gravity convection oven – Maintaining 100°C ± 2°C
13. Vortex mixer
14. Gas dispersion tubes – 25 mm, porosity "A" extra coarse, 175 µm
15. Three-dram vials – About 11 mL
16. Phenolic closed top caps – With polyvinyl liner, to fit vials
17. Teflon / silicone septa – To fit vials

GC Condition

1. Capillary column
2. Split mode injector
3. Oven temperature programming is sufficient to implement a hold-ramp-hold sequence
4. Operating conditions:
 - a. Temperature (°C):
 - i. Injector, 225;
 - ii. Detector, 285;
 - iii. Initial temp, 100 (hold 4 mins);
 - iv. Ramp, 3°C/min;
 - v. Final temp 240; hold 15 mins.
 - b. Carrier gas, helium;
 - i. Flow rate, 0.75 mL/min;
 - ii. Linear velocity, 18 cm/s;
 - iii. Split ratio, 200:1.

Procedures

A. Standard Preparation

Fatty Acid Methyl Esters (FAMES) standard solutions:

1. A mixed FAMES standard solution (reference mixture containing a series of FAMES, including C18:1 cis and trans, e.g., GLC-85 from Nu Chek Prep or equivalent) is prepared.
 - a. The top of the glass vial is broken open, and the contents are carefully transferred into a 3-dram glass vial.
 - b. The original vial is washed with hexane to ensure complete transfer, and the washings are added to the 3-dram glass vial.
 - c. The volume is diluted to approximately 3 mL with hexane.
2. C11:0 FAME standard solution -C11:0 - Undecanoic methyl ester in hexane. Use only in preparation of individual FAME standard solutions (3).
 - a. To prepare the C11:0 FAME standard solution, the top of the glass vial is broken open, and the contents are carefully transferred into a 50 mL volumetric flask.
 - b. The original vial is then washed with hexane to ensure complete transfer, and the washings are added to the volumetric flask.
 - c. The solution is diluted to volume with hexane. The C11:0 FAME standard solution is stable for up to one week when stored at 0°C.
3. Individual FAME standard solutions – Prepare individual FAME standard solutions as follows:
 - a. The top of the glass vial is broken open, and the contents are carefully transferred into a 3-dram glass vial.
 - b. The original vial is washed with hexane to ensure complete transfer, and the washings are added to the 3-dram glass vial.
 - c. Then, 1.0 mL of the C11:0 FAME standard solution (b) is added, and the solution is diluted with hexane to a total volume of approximately 3.0 mL.
 - d. Individual FAME standard solutions are considered stable for up to one week when stored in a refrigerator at 2–8°C. Standard solutions are prepared for each of the following FAMES:
 1. C_{4:0} – tetraenoic methyl ester
 2. C_{8:0} –octanoic methyl ester
 3. C_{10:0} –decanoic methyl ester
 4. C_{12:0} - dodecanoic methyl ester
 5. C_{13:0} –tridecanoic methyl ester
 6. C_{14:0}-tetradecanoic methyl ester
 7. C_{14:1} -9-tetradecenoic methyl ester
 8. C_{15:0} – pentadecanoic methyl este
 9. C_{15:1} – 10-pentadecenoic methyl este
 10. C_{16:0} – hexadecanoic methyl ester
 11. C_{16:1} -9-hexadecenoic methyl ester
 12. C_{17:0} –heptadecanoic methyl ester
 13. C_{17:1} -10-heptadecenoic methyl ester
 14. C_{18:0} -octadecanoic methyl ester
 15. C_{18:1} -9-octadecenoic methyl ester

16. C_{18:2} 9, 12-octadecadienoic methyl ester
17. C_{18:3} -9,12,15-octadecatrienoic methyl ester
18. C_{20:0} -eicosanoic methyl ester
19. C_{20:1} -8-eicosenoic methyl ester
20. C_{20:2} -11,14-eicosatrienoic methyl ester, and
21. C_{22:0} - docosanoic methyl ester.

B. Extraction of Fat

Finely grind and homogenize test samples prior to the extraction of fat. [Note: With matrices of unknown composition, it may be necessary to analyze the test portion without adding an internal standard to ensure against interference. Should an interfering peak be found, the area of C11 internal standard peak must be corrected before calculating. Use 2.0 mL of chloroform instead of the internal standard solution.]

1. Foods excluding dairy products and cheese

- a. A ground and homogenised test portion (containing approximately 100–200 mg of fat) is accurately weighed into a labelled Mojonnier flask.
- b. The material is forced as far as possible into the flask.
- c. Approximately 100 mg of pyrogalllic acid and 2.00 mL of the triglyceride internal standard solution (11a) are added.
- d. A few boiling granules are introduced.
- e. Then, 2.0 mL of ethanol is added, and the mixture is stirred until the entire test portion is in solution.
- f. 10.0 mL of 8.3 M HCl is added and mixed well.
- g. The flask is placed into a basket in a shaking water bath at 70–80°C, set at moderate agitation speed, and maintained for 40 minutes.
- h. The contents are mixed on a vortex mixer every 10 minutes to incorporate any particulates adhering to the sides of the flask.
- i. After digestion, the flask is removed from the bath and cooled to room temperature (20–25°C).
- j. Sufficient ethanol is added to fill the bottom reservoir of the flask, and the contents are gently mixed.

2. Dairy products

- a. A ground and homogenised test portion (containing approximately 100–200 mg of fat) is accurately weighed into a labelled Mojonnier flask.
- b. The material is forced into the flask as far as possible
- c. Approximately 100 mg of pyrogalllic acid and 2.00 mL of the triglyceride internal standard solution (11a) are added.
- d. A few boiling granules are introduced.
- e. 2.0 mL of ethanol is added and mixed until fully dissolved in the solution.
- f. 4.0 mL of water is added. The flask is placed in a shaking water bath at 70–80°C for 10 minutes.
- g. The contents are mixed every 5 minutes with a vortex mixer to incorporate particulates adhering to the sides of the flask into the solution.
- h. After digestion, a few drops of phenolphthalein are added.
- i. The solution is kept basic (pink) by adding ammonium hydroxide.
- j. Ethanol is added to fill the bottom reservoir and mixed gently.

3. Cheese

- a. An accurately weighed test portion (ca 100–200 mg fat) is transferred into a labelled Mojonnier flask.
- b. The material is forced into flask as far as possible.
- c. Approximately 100 mg of pyrogalllic acid and 2.00 mL of triglyceride internal standard solution are added.
- d. A few boiling granules are added.
- e. 2.0 mL of ethanol is added and mixed until dissolved.
- f. 4.0 mL of H₂O is added and mixed well.
- g. 2.0 mL of ammonium hydroxide is added and mixed well.
- h. The flask is placed in a shaking water bath at 70–80°C for 20 minutes.

- i. The contents are mixed on a vortex mixer every 10 minutes to incorporate particulates adhering to the sides of the flask into the solution.
- j. 10.0 mL 12 M HCl is added, and the flask is placed in a boiling steam bath for 20 minutes, mixing every 10 mins using a Vortex mixer.
- k. The flask is removed and cooled to room temperature (20-25°C).
- l. Ethanol is added to fill the bottom reservoir and mixed gently.

4. Extraction

- a. 25 mL of diethyl ether is added to the Mojonnier flask. The flask is stoppered.
- b. The basket is placed in a wrist-action shaker, and the flask is secured using rubber tubing. The flask is shaken for 5 minutes.
- c. The stopper is rinsed into the flask with a diethyl ether–petroleum ether mixture.
- d. 25 mL of petroleum ether is added, the flask is stoppered, and shaken for 5 minutes more. The flask (in the basket) is centrifuged for 5 minutes at 600 rpm. (Note: If a centrifuge is not available, the contents are allowed to stand for at least 1 hour until the upper layer becomes clear.)
- e. The stopper is rinsed into the flask again with a diethyl ether–petroleum ether mixture.
- f. The ether (top) layer is decanted into a 150 mL beaker, and the flask's lip is carefully rinsed into the beaker with a diethyl ether–petroleum ether mixture.
- g. The ether is slowly evaporated on a steam bath, using a nitrogen stream to aid evaporation.
- h. The residue remaining in the beaker is retained as the extracted fat.

C. Methylation

- a. The extracted fat residue is dissolved in 2–3 mL of chloroform and 2–3 mL of diethyl ether.
- b. The mixture is transferred to a 3-dram glass vial and then evaporated to dryness in a 40°C water bath under a nitrogen stream.
- c. 25 mL of 7% BF₃ reagent and 1.0 mL toluene are added. The vial is sealed with a screwcap top containing a Teflon/silicone septum.
- d. The vial is heated in an oven for 45 minutes at 100°C and is gently shaken approximately every 10 minutes. (Note: Evaporation of liquid from the vial indicates inadequate sealing; if this occurs, the solution is discarded and the entire procedure is repeated.)
- e. The vial is allowed to cool to room temperature (20–25°C). Five millilitres of water, 1.0 mL hexane, and approximately 1.0 g sodium sulphate are added. The vial is capped and shaken for 1 minute.
- f. The layers are allowed to separate, and the top layer is carefully transferred to another vial containing approximately 1.0 g sodium sulphate. (Note: The top layer contains FAMES, including the FAME of the triglyceride internal standard solution.)
- g. The FAMES are injected onto the GC column or are transferred to an autosampler vial for GC analysis.

D. GC Determination

- a. Relative retention times (against the FAME of the triglyceride internal standard solution) and response factors of individual FAMES are obtained by GC analysis of the individual FAME standard solutions and the mixed FAMES standard solution. Approximately 2 µL of each FAME standard solution and 2 µL of the mixed FAMES standard solution are injected.
- b. The mixed FAMES standard solution optimises the chromatographic response before injecting any test solutions. Once all chromatographic conditions have been optimised, the test solutions from step C (Methylation) are injected.

Calculation

Total fat is defined as the sum of fatty acids from all sources, expressed as triglycerides. The expression of measured fatty acids as triglycerides is achieved through a mathematical equivalent of condensing each fatty acid with glycerol. For every 3 fatty acid molecules, 1 glycerol ($\text{HOCH}_2\text{CHOOCH}_2\text{OH}$) is required. Essentially, 2 methylene groups and 1 methine group are added to every 3 fatty acids.

Retention times for each FAME in individual FAME standard solutions are calculated by subtracting the retention time of the C11 peak from the retention time of the fatty acid peak. These retention times are then used to identify FAMEs in a mixed FAME standard solution. Additional FAME solutions from the same supplier are used when necessary for complete FAME identity verification.

1. The response factor (R_i) for each fatty acid i is calculated as follows:

$$R_i = \frac{PS_i}{PS_{C11:0}} \times \frac{W_{C11:0}}{W_i}$$

Where PS_i = peak area of individual in mixed FAMEs standard solution,
 $PS_{C11:0}$ = peak area of $C_{11:0}$ fatty acid in mixed FAMEs standard solution
 $W_{C11:0}$ = weight of internal standard in mixed FAMEs standard solution
and W_i = weight of individual FAME in the mixed FAMEs standard solution.

(Note: Peaks of known identity with known relative retention times are listed in Table 3.3.6A. When peaks of unknown identity are observed during the chromatographic run, attempt to identify such peaks using MS, FTIR, etc. The summation should not include peaks of unknown identity when quantifying fat in the test portion.)

2. The amount of individual triglycerides (WTG) in the test portion is calculated as follows:

$$W_{FAMEi} = \frac{Pt_i \times Wt_{C11:0} \times 1.0067}{Pt_{C11:0} \times R_i}$$

$$W_{TGi} = W_{FAMEi} \times f_{TGi}$$

Where Pt_i = peak area of fatty acid i in the test portion
 $Wt_{C11:0}$ = weight of $C_{11:0}$ internal standard added to test portion, g
1.0067 = conversion of internal standard from triglyceride to FAME
 $Pt_{C11:0}$ = peak area of $C_{11:0}$ internal standard in test portion
And f_{TGi} = conversion factor for FAMEs to triglycerides for individual fatty acids (see Table 3.3.6B)
(Note: If the procedure is followed exactly, $Wt_{C11:0}$ should be 0.010 g.)

3. The amount of total fat in the test portion [sum of all fatty acids, expressed as triglyceride equivalents (including cis and trans forms of monounsaturated acids)] is calculated as follows:

$$\text{Total fat, \%} = \left(\sum W_{TGi} \times 100 \right) / W_{\text{test portion}}$$

Where $W_{\text{test portion}}$ = weight of test portion, g.

4. The weight of each fatty acid (W_i) is calculated as follows:

$$W_i = W_{FAMEi} \times f_{FAi}$$

Where f_{FAi} = conversion factors for conversion of FAMES to their corresponding fatty acids (see Table 3.3.6B)

5. The percent of saturated fat in the test portion (w/w; expressed as saturated fatty acids; sum of C4:0, C6:0, C8:0, etc.) is calculated as follows:

$$\text{Saturated fat, \%} = \left(\sum \text{saturated } W/W_{\text{test portion}} \right) \times 100\%$$

6. The amount of monounsaturated fat in the test sample (w/w; expressed as the sum of only the cis form of monounsaturated fatty acids C16:1, C17:1, C18:1 cis, C20:1, etc.) is calculated as follows:

$$\text{Monounsaturated fat, \%} = \left(\sum \text{monounsaturated } W/W_{\text{test portion}} \right) \times 100\% \text{ Polysaturated fat, \%}$$

(Note: Test samples containing hydrogenated fat will yield complicated chromatograms due to the large number of isomers formed during the hydrogenation process. One general indication of hydrogenation is the presence of $C_{18:1}$ trans peak(s). For hydrogenated fat chromatograms, use the following guidelines to calculate FAME peak areas: trans peaks elute prior to cis; therefore, include all peaks between $C_{18:1}$ cis and $C_{18:2}$ cis, cis in the calculation of $C_{18:2}$ peak area. Often, $C_{18:1}$ trans "peak" consists of a broad series of peaks [due to positional isomers from hydrogenation]; include all of these in $C_{18:1}$ trans peak area.)

Reference

AOAC Official Method 996.06 (2019)

Table 3.3.6A: Retention time of fatty acids and methyl ester (FAME)

Fatty Acid	Retention time (min)	Relative retention times (to 11:0 int. std.)
4:0 Butyric	10.49	0.46
6:0 Caproic	12.36	0.54
8:0 Caprylic	15.69	0.68
10:0 Capric	20.39	0.89
11:0 Undecanoic	22.99	1.00
12:0 Lauric	25.58	1.11
13:0 Tridecanoic	28.15	1.22
14:0 Myristic	30.65	1.33
14:1 Myristoleic	32.63	1.42
14:1 trans-Myristelaidic	32.01	1.39
15:0 Pentadecanoic	33.04	1.44
15:1 Pentadecenoic	34.98	1.52
16:0 Palmitic	35.41	1.54
16:1 trans-Palmitelaidic	36.39	1.58
16:1 Palmitoleic	36.88	1.60
17:0 Margaric	37.54	1.63
17:1 Margaroleic	38.92	1.69
18:0 Stearic	39.78	1.73
18:1 trans 6-Petroselenic	40.50	1.76
18:1 trans-Elaidic	40.61	1.77
18:1 trans 11-Vaccenic	40.72	1.77
18:1 Petroselenic	40.90	1.78
18:1 Oleic	40.99	1.78
18:1 Vaccenic	41.18	1.79
18:1 Octadecenoic	41.54	1.81
18:2 trans-Linolelaidic	41.69	1.81
18:1 trans 9-Linolelaidic	42.11	1.83
18:2 trans 12-Linolelaidic	42.53	1.85
18:2 Linoleic	42.87	1.86
20:0 Arachidic	43.75	1.90
18:3 g-Linolenic	44.25	1.92
20:1 Eicosenic cis 5	44.42	1.93
20:1 Eicosenic trans 11	44.45	1.93
20:1 Eicosenic cis 8	44.67	1.94
20:1 Eicosenic cis 11	44.82	1.95
20:1 Eicosenic cis 13	44.99	1.96
18:3 Linolenic	45.02	1.96
18:2 Linoleic-conjugated	45.35	1.97
18:2 Linoleic-conjugated	45.40	1.97
21:0 Heneicosanoic	45.69	1.99
18:2 Linoleic-conjugated	46.18	2.01
18:4 Octadectetraenoic	46.39	2.02
20:2 Eicosadienoic	46.65	2.03
22:0 Behenic	47.46	2.06
20:3 g-Eicosatrienoic	47.94	2.09
22:1 Cetoleic	48.27	2.10
22:1 Erucic	48.50	2.11
20:3 Eicosatrienoic	48.68	2.12
20:4 Arachidonic	48.94	2.13

Fatty Acid	Retention time (min)	Relative retention times (to 11:0 int. std.)
23:0 Tricosanoic	49.22	2.14
22:2 Docosadienoic	50.17	2.18
24:0 Lignoceric	50.79	2.21
20:5 Eicosapentaenoic	50.96	2.22
24:1 Nervonic	51.92	2.26
22:3 Docosatrienoic	51.98	2.26
22:4 Docosatetraenoic	52.28	2.27
22:5 Docosapentaenoic	54.75	2.38
22:6 Docosahexaenoic	55.82	2.43

Table 3.3.6B Factors (f_{TG}) for conversion of FAMES to triglyceride equivalents.

Fatty Acid	F_{Ai} ^a	Tri/FAME (F_{TGi}) ^b
8:0 Caprylic	15.69	0.68
10:0 Capric	20.39	0.89
11:0 Undecanoic	22.99	1.00
12:0 Lauric	25.58	1.11
13:0 Tridecanoic	28.15	1.22
14:0 Myristic	30.65	1.33
14:1 Myristoleic	32.63	1.42
14:1 trans-Myristelaidic	32.01	1.39
15:0 Pentadecanoic	33.04	1.44
15:1 Pentadecenoic	34.98	1.52
16:0 Palmitic	35.41	1.54
16:1 trans-Palmitelaidic	36.39	1.58
16:1 Palmitoleic	36.88	1.60
17:0 Margaric	37.54	1.63
17:1 Margaroleic	38.92	1.69
18:0 Stearic	39.78	1.73
18:1 trans 6-Petroselenic	40.50	1.76
18:1 trans-Elaidic	40.61	1.77
18:1 trans 11-Vaccenic	40.72	1.77
18:1 Petroselenic	40.90	1.78
18:1 Oleic	40.99	1.78
18:1 Vaccenic	41.18	1.79
18:1 Octadecenoic	41.54	1.81
18:2 trans-Linolelaidic	41.69	1.81
18:1 trans 9-Linolelaidic	42.11	1.83
18:2 trans 12-Linolelaidic	42.53	1.85
18:2 Linoleic	42.87	1.86
20:0 Arachidic	43.75	1.90
18:3 g-Linolenic	44.25	1.92
20:1 Eicosenic cis 5	44.42	1.93
20:1 Eicosenic trans 11	44.45	1.93
20:1 Eicosenic cis 8	44.67	1.94
20:1 Eicosenic cis 11	44.82	1.95
20:1 Eicosenic cis 13	44.99	1.96
18:3 Linolenic	45.02	1.96
18:2 Linoleic-conjugated	45.35	1.97
18:2 Linoleic-conjugated	45.40	1.97
21:0 Heneicosanoic	45.69	1.99
18:2 Linoleic-conjugated	46.18	2.01

Fatty Acid	F_{Ai} ^a	Tri/FAME (F_{TGi}) ^b
23:0 Tricosanoic	0.9620	0.9964
24:0 Lignoceric	0.9963	0.9965
24:1 Nervonic	0.9635	0.9965

^a F_{Ai} is the conversion factor for the conversion of FAMES to the corresponding fatty acids

^b FT_{Gi} is the conversion factor for converting FAMES to triglycerides for individual fatty acids.

3.3.7 DETERMINATION OF CHOLESTEROL

3.3.7.1 DETERMINATION OF CHOLESTEROL IN FOODS

Cholesterol analysis usually renders clean chromatograms since most interferences are eliminated by saponification. Saponification segregates the molecule of interest from the saponifiable lipid fraction (e.g., acylglycerols) and hydrolyzes cholesterol esters. This action is critical for cholesterol analysis in food matrices (Cortés-Herrera et al., 2018).

Method A: Direct Saponification-gas Chromatographic

Principles

Lipid in the test portion is saponified at high temperature with ethanolic KOH solution. The unsaponifiable fraction containing cholesterol and other sterols is extracted with toluene. Cholesterol and other sterols are derivatized to trimethylsilyl (TMS) ethers and then quantified by gas chromatography.

Chemicals / Reagents

1. Dimethylformamide (DMF) – Distilled in glass
2. Hexamethyldisilane (HMDS)
3. 5 α - 5 α -Cholestane internal standard solution – 0.1 mg/mL in n-heptane
4. Cholesterol standard
 - a. Stock solution – 2.0 mg/mL dimethylformamide (DMF)
 - b. Working solution – The stock solution is diluted with DMF to obtain 6 solutions at concentrations of 0.0025, 0.005, 0.01, 0.05, 0.1, and 0.2 mg/mL.
5. Potassium hydroxide solutions
 - a. 50% KOH (w/w) – 500 g of KOH is dissolved in 500 g of H₂O.
 - b. 1 M KOH – 56 g of KOH is dissolved in approximately 800 mL of H₂O with cooling and diluted to the mark in a 1 L volumetric flask.
 - c. 0.5 M KOH – One part of 1 M KOH solution is diluted with one part of H₂O.
6. Trimethylchlorosilane (TMCS) – No. 88531, Pierce Chemical Co., or equivalent
7. Toluene – Distilled in glass
8. Sodium sulphate – Anhydrous
9. Glass wool

Apparatus/ Instruments

1. Centrifuge tubes – 15 mL, Pyrex No.13.
Silanize tubes as follows:
 - a. Tubes are filled with 10% hydrofluoric acid and allowed to stand for 10 minutes.
 - b. Tubes are thoroughly rinsed with H₂O, and then with anhydrous methanol.
 - c. Tubes are dried under a stream of nitrogen, filled with 10% hexamethyldisilane (HMDS) in toluene, and allowed to stand for 1 hour.
 - d. Tubes are thoroughly rinsed with toluene, and then with anhydrous methanol.
 - e. Tubes are dried in a 100°C oven before use.
 - f. Alternatively, a commercial silanizing reagent may be used.
 - g. Before each reuse, tubes are cleaned with H₂O, ethanol, hexane, and acetone, then dried in a 100°C oven. Tubes can be reused without re-silanization if a strong alkaline wash is avoided.
 - h. Tubes are re-silanized at least every 6 months.
2. Gas chromatography (GC)
 - a. Hydrogen flame ionization detector
 - b. Capillary column
 - c. Split-mode, 25 m x 0.32 mm x 0.17 μ m film thickness

- c. Split-mode, 25 m x 0.32 mm x 0.17 μ m film thickness
- d. Cross-linked 5% phenyl-methyl silicone or methyl silicone gum
- e. Split inlet liner filled with 10% SP 2100 on 80-100 mesh Supelco packing
- f. 2 ramp oven temperature programming

Operating conditions:

- a. Temperatures-injector 250°C,
 - b. Detector 300°C,
 - c. Column 190°C,
 - d. Hold 2 mins; increase 20°C/min to 230°C, hold 3 mins; increase 40°C/min to 255°C, hold 25 mins.
 - e. Flow rates: helium column ca mL/min, split vent ca 30 mL/min, purge vent ca 3 mL/min, auxiliary make-up gas ca 20 mL/min, hydrogen ca 35 mL/min, and air ca 280 mL/min.
3. Rotary evaporator with a glass condenser flask between the concentration flask and the metal shaft
 4. Magnetic stirrer - hot plate with variable speed and heat controls
 5. Micropipettes - Capable of delivering 100 and 200 μ L; metal body
 6. Test tube mixer
 7. Balance – Analytical, capable of weighing to 0.0001 g
 8. Glassware
 - a. Erlenmeyer flask, 125 and 250 mL
 - b. Volumetric flask and pipette
 - c. Graduated cylinders
 - d. Separatory funnels, 500 mL

Procedures

A. Saponification

1. A test portion of approximately 2–3 g (W1) is accurately weighed to the nearest 0.001 g into a 250 mL Erlenmeyer flask. The amount is chosen to contain ≤ 1 g of fat or ≤ 5 g of water (e.g., 1 g pure oils, 1.5 g salad dressings, and ≤ 5 g substances with high moisture content).
2. A magnetic stir bar is placed into the flask. 40 mL of 95% ethanol and 8 mL of 50% KOH solution are added to the flask. (Note: A portion of ethanol may be retained and used as a rinse after KOH addition to help prevent the ground-glass joints of the flask and condenser from freezing together.)
3. The flask is placed on a magnetic stirrer-hot plate, a condenser is attached, and stirring and heating are initiated. The mixture is refluxed for 70 ± 10 minutes. To ensure complete saponification, the test portion is occasionally checked and any clumps are dispersed with a glass rod or by adding more KOH solution while stirring.
4. Heating is stopped, and 60 mL of 95% ethanol is carefully added through the top of the condenser while stirring. (Caution: add carefully to avoid spurting alcohol.)
5. After approximately 15 minutes, the flask is removed from the condenser, closed with a stopper, and the solution is cooled to room temperature. The test solution remains stable for 24 hours.

B. Extraction

1. 100 mL of toluene (V1) is added to the saponified test portion while stirring. The flask is stoppered and stirred for at least 30 seconds.
2. The solution is poured into a 500 mL separatory funnel without rinsing.
3. 110 mL of 1 M KOH solution are added, and the funnel is shaken vigorously for 10 seconds. The layers are allowed to separate, and the aqueous (lower) layer is discarded (will appear turbid).
4. Approximately 40 mL of 0.5 M KOH solution is added, and the funnel is inverted and gently swirled for 10 seconds. The aqueous (lower) layer is discarded.
5. The toluene layer is washed with 40 mL of water by gently rotating the separatory funnel. The layers are allowed to separate, and the aqueous phase is discarded. This water wash is repeated at least three times, shaking more vigorously each time. If emulsification occurs, a small amount of 95% ethanol is added, the contents are swirled, and washing continues until the toluene layer appears crystal clear.
6. The toluene layer is then poured through a glass funnel containing a glass wool plug and approximately 20 g of Na_2SO_4 into a 125 mL Erlenmeyer flask containing around 2 g of Na_2SO_4 . The flask is stoppered, and the contents are swirled. The mixture is left to stand for at least 15 minutes. If tightly sealed, the test solution may be stored for up to 24 hours.

7. A 25 mL aliquot of the extract (V2) is pipetted into a 125 g flat-bottom boiling flask and the contents are evaporated to dryness on a rotary evaporator at $40 \pm 3^\circ\text{C}$.
8. Approximately 3 mL of acetone are added, evaporation to dryness is repeated.
9. The residue is dissolved in 3.0 mL of DMF (V3). The final cholesterol concentration in DMF is ensured to be within the range of the working standard solutions. If quantitation by gas chromatography indicates that the concentration falls outside the standard curve, the volume of toluene extract evaporated, or the volume of DMF used, or both, is adjusted. Hence, the final concentration falls within the range. If the test portion contains little or no cholesterol, 75 mL of toluene extract is evaporated and redissolved in 2 mL DMF to detect 1 mg cholesterol/100 g in a 1 g test portion.

C. Derivatization

1. Aliquots of 1.0 mL from the working standard solutions and the test solution are pipetted into separate 15 mL centrifuge tubes.
2. 0.2 mL of HMDS and 0.1 mL of TMCS are added to each tube. The tubes are stoppered and shaken vigorously on a test tube mixer or by hand for 30 seconds. The solutions are left undisturbed for 15 minutes.
3. Then, 1 mL of 5 α -cholestane internal standard solution and 10 mL of water are added to each tube. The tubes are stoppered, shaken vigorously for 30 seconds, and then centrifuged for ca 2 minutes.
4. A sufficient portion of the heptane (upper) layer is transferred to an injection vial, ensuring no aqueous layer is included. Derivatized standards and test solutions must be analysed within 24 hours.

GC Analysis

1. An injection of 1 μL , or other suitable volume, is made into the gas chromatograph. The areas of the 5 α -cholestane and cholesterol peaks are determined by height-width measurement or a digital integrator. (Note: 5 α -cholestane and cholesterol should elute at approximately 11–13 minutes and 16–18 minutes, respectively. If these retention times are not achieved, the carrier flow and temperature are adjusted).
2. The cholesterol peak area is divided by the internal standard peak area to obtain the standard response ratio. The response ratios of four high standards (0.01–0.20 mg/mL) are plotted against cholesterol concentrations. The standard response ratio plot should bracket the test solution response ratio. A low standard curve (0.0025–0.05 mg/mL) is plotted for low-level test solutions if necessary. High-level test solutions are diluted to bring them within the standard range.

Calculation

The grams of the test portion per milliliter derivatized are calculated as follows:

$$\frac{\text{g test portion}}{\text{mL derivatized}} = \frac{W_1}{V_1} \times \frac{V_2}{V_3}$$

Where W_1 = weight of test portion in g
 V_1 = volume of toluene used in extraction, 100 mL
 V_2 = aliquot of extract taken to dryness, 25 mL
 V_3 = volume of DMF used to dissolve residue, 3 mL.

The cholesterol content in the test portion is calculated as follows:

$$\frac{\text{mg cholesterol}}{100 \text{ g test portion}} = \frac{\frac{\text{mg}}{\text{mL cholesterol in test portion from standard curves}}}{\frac{\text{g test portion}}{\text{mL derivatized}}} \times 100$$

References

- 1) Official Methods of Analysis of AOAC INTERNATIONAL (2019) 21st Ed., AOAC INTERNATIONAL, Method 994.10
- 2) Cortés-Herrera, C., Artavia, G., Leiva, A., & Granados-Chinchilla, F. (2018). Liquid Chromatography Analysis of Common Nutritional Components, in Feed and Food. Foods (Basel, Switzerland), 8(1), 1.

Method B: HPLC With Photo Diode Array (PDA) Detector

Principles

The HPLC method is for the simultaneous quantifying retinol, α -tocopherol, and cholesterol in shrimp waste hydrolysate lipid fraction. The method includes microscale saponification and extraction with n-hexane. Liposoluble vitamins and cholesterol are quantified by HPLC with UV detection (HPLC-UV), on a 25 cm $\times$ 0.46 cm SS Exil ODS 5 μ m column, mobile phase 68:28:4 (v/v/v) methanol:acetonitrile: water; flow rate 1.4 mL/min; column temperature 36°C. The detection is operated using two channels of a diode-array spectrophotometer, 325 nm for retinol and 208 nm for α -tocopherol and cholesterol.

Chemicals / Reagents

1. Methanol, HPLC-grade
2. Hexane, HPLC-grade
3. Acetonitrile, HPLC-grade
4. Pyrocatechol, Analytical grade
5. Cholesterol, Analytical grade
6. Potassium hydroxide, KOH
7. Retinol and α -tocopherol
8. Ultrapure water
9. KOH solution prepared in methanol (0.5 M)

Note: The pyrocatechol solution (1 g in 5 mL of methanol) is prepared daily and stored at approximately 4°C away from light.

Apparatus/ Instruments

1. The HPLC-UV system:
 - a. Equipped with an auto-injector LC 1650
 - b. Online solvent degasser LC1460
 - c. system controller
 - d. pump LC1150
 - e. column oven LC1150
 - f. 20 μ L injection loop
 - g. photodiode-array detector LC5100
2. Balance: analytical sensitivity $\pm$ 0.1 mg
3. Vortex mixer
4. Centrifuge
5. Nitrogen gas blower
6. Test tube: screw capped
7. Micropipettes
8. Membrane-filtered (pore size 0.50 μ m; Whatman).
9. Ultra-filtration equipment

Chromatographic Conditions

1. Chromatographic analysis will be performed using an analytical scale (25 cm $\times$ 0.4 cm I.D.)
2. SS Exil ODS column with a particle size of 5 μ m
3. HPLC conditions are to be as follows:
 - a. mobile phase 68:28:4 (v/v/v) methanol: acetonitrile: water
 - b. flow rate of 1.4 mL/min
 - c. column temperature 36°C
 - d. The detection is operated using two channels of a diode-array spectrophotometer, 325 nm for retinol, and 208 nm for α -tocopherol and cholesterol. Retinol, α -tocopherol, and cholesterol are identified by retention and spectral data.

Standards and quantification

1. Stock standard solutions of retinol (0.5 mg/mL), α -tocopherol (0.5 mg/mL), and cholesterol (2.5 mg/mL) are prepared in 100% methanol and stored at -10°C away from light.
2. The purity of the reference standards is $\geq 97.0\%$ for retinol, $\geq 98.0\%$ for α -tocopherol, and $\geq 95.0\%$ for cholesterol.
3. Working solutions are prepared from these solutions and diluted with methanol prior to analysis.
4. The stock solution is always analysed together with the samples to determine retinol, α -tocopherol, and cholesterol in the lipid fraction.
5. Analyte concentrations in samples are estimated based on peak areas. The study team analysed the samples in triplicate. Retinol, α -tocopherol, and cholesterol contents are cited as means $\pm$ standard deviation.

Procedures

Saponification & Extraction

Retinol, α -tocopherol, and cholesterol are extracted from the lipid fraction by the method of Sanchez-Machado et al. (2002) with minor modification by Lopez-Cervantes et al. (2006).

1. A subsample of 0.40 g (± 0.001 g) is weighed into a screw-top assay tube.
2. An antioxidant, 200 μ L of pyrocatechol solution, is added.
3. 5 mL of KOH solution (0.5 M in methanol) are added and the contents are vortexed immediately for 20 seconds.
4. The tubes are placed in a water bath at 80°C for 15 minutes, removed every 5 minutes, and vortexed again for 15 seconds.
5. After cooling in iced water, 1 mL of distilled water and 5 mL of hexane are added. The mixture is vortexed rapidly for 1 minute and centrifuged for 2 minutes at 425 $\times$ g.
6. 3 mL of the upper phase is transferred to another test tube and dried under a nitrogen stream.
7. The residue is redissolved in 3 mL of the HPLC mobile phase (68:28:4, v/v/v methanol:acetonitrile:water) and filtered through a membrane filter (pore size 0.50 μ m; Whatman).
8. An aliquot of 20 μ L is injected into the HPLC column. (The extracts are kept at -10°C, protected from light.)

References

- 1) Albuquerque, T. G., Oliveira, M. B. P. P., Sanches-Silva, A., & Costa, H. S. (2016). Cholesterol determination in foods: Comparison between high performance and ultra-high performance liquid chromatography. *Food Chemistry*, 193, 18–25. <https://doi.org/10.1016/j.foodchem.2014.09.109>
- 2) Kolarič, L., & Šimko, P. (2020). Determination of cholesterol content in butter by HPLC: Up-to-date optimization, and in-house validation using reference materials. *Foods*, 9(10). <https://doi.org/10.3390/foods910137>
- 3) Ndhkala, A. R., Kavaz Yüksel, A., Çelebi, N., & Doğan, H. Ö. (2023). A General Review of Methodologies Used in the Determination of Cholesterol (C27H46O) Levels in Foods. In *Foods* (Vol. 12, Issue 24). Multidisciplinary Digital Publishing Institute (MDPI). <https://doi.org/10.3390/foods12244424>

3.4 METHOD PERFORMANCE CHARACTERISTICS AND QUALITY ASSURANCE

3.4.1 METHOD PERFORMANCE CHARACTERISTICS

The laboratory shall conform that it can properly operate standard methods before introducing the test or calibration through the analytical method validation consists of the following analytical performance characteristics: -

Accuracy

Closeness of agreement between the measured value and the accepted "true" or reference value.

Precision (Repeatability and Reproducibility)

Closeness of agreement (degree of scatter) among repeated measurements, either identical or different measurement conditions.

Recovery

The method measures the fraction of analyte added to the test sample (fortified or spiked) prior to analysis.

Specificity

Ability of the method to respond exclusively to the target analyte and not to any degradant, impurity, or other matrix component.

Limit of detection

The smallest quantity of analyte that can be shown to be significantly greater than the measurements (random) error of the blank at a prescribed level of confidence (usually 95%)

Limit of quantitation

Under previously established method conditions, the smallest amount of analyte in a test sample can be quantitatively determined with suitable precision and accuracy.

Linearity

The ability of the method to obtain test results proportional to the concentration of the analyte (within a given range).

Sensitivity

The change in the measuring instrument's response is divided by the corresponding change in the stimulus, represented by the calibration curve's slope.

Establishing these performance characteristics ensures the suitability of methods for their intended use and confirms that the results produced from the selected analytical methods are good-quality data.

By achieving Standard Method Performance Requirements (SMPRs®), that is, the minimum analytical performance requirements, the method can be classified either as a surveillance method or for dispute resolution, which require better accuracy, precision, and reproducibility, but time to determination is less important. These methods to ensure the maintenance of the quality throughout this study, methods are selected based on the following criteria: -

Predilection to the methods that have been recommended or adopted by international organizations such as the Association of Official Analytical Chemists (AOAC), International Organization for Standardisation (ISO) and European Standards(EN).

Predilection for the methods for reliability has been established by collaborative studies involving several laboratories, either locally or internationally. Predilection for the methods applicable to a wide range of food types and matrices rather than those focused only on specific foods.

3.4.2 QUALITY ASSURANCE

Quality Assurance (QA) refers to a completely separate and independent monitoring of laboratory studies and Quality Control (QC) activities. Every participating laboratory is recommended to establish the Quality Assurance according to the following requirements: -

Standard Operating Procedures (SOPs)

All test methods shall be documented and followed as part of standard operating procedures. These methods are periodically reviewed for updates.

Competency

Every participating laboratory involved in nutrient analysis will be tested for competence before engaging with the actual analysis. Tests are performed by trained staff members, and each staff member must meet performance criteria before performing a test.

Instrument

Each instrument is operated by trained staff members. Maintenance logbooks are kept for each instrument. The instruments are calibrated according to manufacturer guidelines and in accordance with the relevant SOPs. Balances, pipettes and other measuring devices are calibrated frequently.

System Suitability Tests are performed to ensure the complete analytical system (including instrument, reagent, column and analyst) is suitable for the intended application. They involve planned equipment performance checks during routine use to confirm that the same quality level is achieved. These tests are usually built into the analytical methods in terms of analytical response for blanks and calibration standards.

Metrological Traceability

The measurement result is only reliable if its traceability and uncertainty have been determined. Each participating laboratory shall ensure that all analytical standards used are traceable to certified reference materials. Internal reference materials are checked as far as is technically and economically practicable.

Quality Control (QC)

Quality Control (QC) refers to steps taken to ensure and monitor the precision and accuracy of test results. Quality Control practices include analysing internal and external QC with each set of samples.

Internal QC

The laboratory shall perform internal quality control in every batch to ensure analytical results fall within specified tolerances. These can be done using certified reference materials, reference materials, previous proficiency testing (PT) samples, in-house control material containing naturally found analyte (retained/routine test samples), spiking samples, duplicate samples and blanks. The system's statistical control status is checked, and the relative variability of repeating data is examined using a system of control charts.

External QC

The laboratory is encouraged to participate in the proficiency testing (PT) programme (e.g., FODAS by the Department of Chemistry Malaysia, FAPAS and APFAN) or Interlaboratory Comparison testing. Nevertheless, Internal Quality Control shall be performed to minimize deviations and eliminate outliers.

Quality Control Material

BLANKS - A reagent blank is analyzed with every set of extracted or digested samples. This reagent blank includes any and all reagents that are used in the analytical process and is carried through the entire process, including extraction and filtering or digestion.

DUPLICATES - Test samples are analyzed in duplicate and treated as quality control material. The results should fall within 5% of each sample unless sample homogeneity is problematic.

RETAINED/ROUTINE TEST SAMPLES—Routine test samples are helpful because they provide information on precision, interference, etc., which could realistically be encountered in day-to-day work. If the analyte content of test material, including PT samples, is accurately known, it can be used as a measurement bias.

SPIKE SAMPLES - Sample fortifications or spikes are used to verify the accuracy of tests requiring extensive sample manipulation (such as acid digestion) or for non-standard sample types.

REFERENCE MATERIALS - Reference material (RM) may take various forms, certified RM or RM, including pure substance RMs, matrix RMs and solutions or mixtures. They are used for calibration, method validation, measurement uncertainty evaluation, quality control, and training purposes. However, a specific RM can only be used for one measurement purpose, e.g., calibration or QA purposes. For quality control purposes, at least one standard reference item is examined with every set of samples. If a sample's results using a standard reference material fall beyond the permissible range, it is reanalyzed, including digestion or extraction.

Figure 1 shows a typical analytical process and illustrates the role of RMs in relation to calibration, method validation and QC.

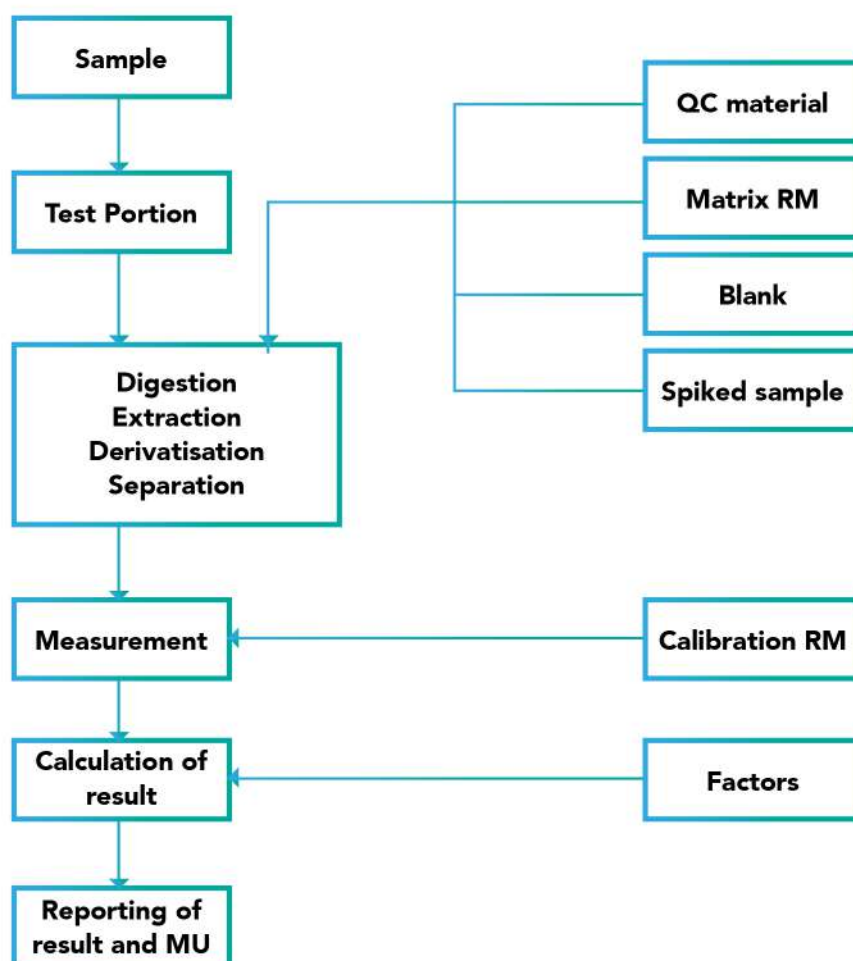


Figure 1 - Example of a typical analytical process, showing the role of RMs

Quality Control Acceptance Criteria

Table 3.4.2.1A: PROXIMATE

No.	Nutrient	Technique	Method Performance
1	Moisture	Air Oven / Vacuum Oven	Duplicate results should not differ by more than 5% of the mean.
2	Crude Protein	Kjedahl /Dumas	Duplicate results should not differ by more than 5% of the mean. The Mean concentration of duplicate In-house Food Reference Material results should be within ± 2 SD in the control chart based on established acceptance criteria.
3	Total Fat	Acid Hydrolysis	Duplicate results should not differ by more than 5% of the mean. The mean concentration of duplicate results of the reference materials should be within ± 2 SD in the control chart based on established acceptance criteria.
		Manual Extraction	Duplicate results should not differ by more than 5% of the mean. The mean concentration of duplicate results of the reference materials should be within ± 2 SD in the control chart based on established acceptance criteria
4	Total Dietary Fiber	Enzymatic - Gravimetric	Duplicate results should not differ by more than 10% of the mean. The mean concentration of duplicate results of the quality control sample or certified reference material should be within ± 2 SD in the control chart based on established acceptance criteria.
5	Ash	Gravimetric Method	Duplicate results should not differ by more than 5% of the mean

Table 3.4.2.1B: SUGAR

No.	Nutrient	Technique	Method Performance
1	Individual Sugar	HPLC (RI Detector/ELSD)	Duplicate results should not differ by more than 10% of the mean. The mean concentration of duplicate results of the quality control sample or certified reference material should be within ± 2 SD in the control chart based on established acceptance criteria
2	Total Sugar	HPLC	Duplicate results should not differ by more than 10% of the mean. The recovery test is between 80 to 110% The Mean concentration of duplicate quality control samples or certified reference material results should be within ± 2 SD in the control chart based on established acceptance criteria.

Table 3.4.2.1C: MINERALS

No.	Nutrient	Technique	Method Performance
1	Ca, Mg	AAS	Duplicate results should not differ by more than 10% of the mean.
			The accepted percent recovery of the spiked sample is 90-110%
3	Phosphorus	UV-Vis /ICPMS/ICPOES	Duplicate results should not differ by more than 10 % of the mean.
			The accepted percent recovery of the spiked sample is 90-110%
4	Sodium, Potassium	AAS	Duplicate results should not differ by more than 10% of the mean value
			Accepted per cent recovery of spiked sample is 90-110%
5	Iron, Copper, Zinc	AAS	The test sample is measured in duplicate and the values within the sample should not differ by more than 10% of the mean value.
			The accepted percent recovery of the spiked sample is 90-110%
6	Iodide	Spectrophotometric Method/ICPMS	The test sample is measured in duplicate and the values within the sample should not differ by more than 10% of the mean value.
			Recovery is performed by spiking a sample in every batch of analysis and the acceptance level is 80-120%
			In-house quality control should be within + 2 SD. Fish meal is suggested as one of the in-house quality control samples.
7	Iodide	Inverse Colorimetric Method Using Technicon Autoanalyzer	The recovery test should be between 80 to 120%
			Mean results of duplicate analysis should not vary by more than 10%.
			In-house control sample should not be more than + 2SD

Table 3.4.2.1D: VITAMINS

No.	Nutrient	Technique	Method Performance
1	A, E AND β -Carotene	HPLC	Duplicate results should have a difference of less than 10%
			The control chart should be within + 2 SD of the established mean value.
			% recovery should be 100 + 20%
			% CV should be less than 20%
2	B1, B2	Fluorometric Method	The control chart should be within + 2 SD of the established mean value.
			Duplicate results should have a difference of less than 5%
			The recovery test should be between 90 to 110%
		HPLC (Fluorescence/PDA Detector)	The control chart should be within + 2 SD of the established mean value
			Duplicate results should have a difference of less than 5%
			The recovery test should be between 90 to 110%
3	Water-soluble Vitamin, Niacin, B6, Folic Acid, B12	Microbiological Assay	Duplicate results should not be more than 15 % variation, and ideally be within 10%
			The control chart should be within + 2 SD of the established mean value.
			The recovery test should be between 90 to 110%
4	Vitamin C	Microfluorometric Method and HPLC	A recovery of 100 + 5%
		Titration Method	Results of duplicate analysis should not vary by more than 10%
			The recovery range should be between 70-120%

Table 3.4.2.1E: FATTY ACIDS AND CHOLESTEROL

No.	Nutrient	Technique	Method Performance
1	Fatty Acids	Gas Chromatography	Duplicate results should not differ by more than 10% of the mean
			The mean concentration of saturated fatty acid in the control sample should be within + 3SD in the control chart based on established acceptance criteria
2	Cholesterol	Gas Chromatography, add HPLC	The cholesterol level in the control sample must be within $\bar{x} \pm 2$ SD on the control chart.
			The different between duplicate results should be less than 10 %

Table 3.4.2.1F: AMINO ACIDS

No.	Nutrient	Technique	Method Performance
1	Amino Acid	Amino Acid Analyzer/HPLC	Duplicate results for the major component should not differ by more than 5 % of the mean. Standard solution is injected before a series of sample injections and at frequent regular intervals, such as every 10 runs. The result of the standard solution should not differ by more than 5 % from the first injection.

Reference

Regional Centre of ASEAN Network of Food Data System, 2011. ASEAN Manual of Food Analysis; Institute of Nutrition, Mahidol University; Thailand

Expected precision (repeatability) as a function of analyte concentration^a

Table 3.4.2.2A: Targets for RSDs for a range of analyte concentrations.

Analyte, %	Mass fraction (C)	Unit	RSD _r , %
100	1	100%	1.3
10	10 ⁻¹	10%	1.9
1	10 ⁻²	1%	2.7
0.1	10 ⁻³	0.1%	3.7
0.01	10 ⁻⁴	100 ppm (mg/kg)	5.3
0.001	10 ⁻⁵	10 ppm (mg/kg)	7.3
0.0001	10 ⁻⁶	1 ppm (mg/kg)	11
0.00001	10 ⁻⁷	100 ppb (µg/kg)	15
0.000001	10 ⁻⁸	10 ppb (µg/kg)	21
0.0000001	10 ⁻⁹	1 ppb (µg/kg)	30

^a Table excerpted from AOAC Peer-Verified Methods Program, Manual on Policies and Procedures (1998) AOAC INTERNATIONAL, Rockville, MD, USA.

Expected recovery as a function of analyte concentration^a

Table 3.4.2.3A: Target mean recovery ranges for analyte concentrations from 100% to 1 ppb.

Analyte, %	Mass fraction (C)	Unit	Mean recovery, %
100	1	100%	98–102
10	10 ⁻¹	10%	97–103
1	10 ⁻²	1%	95–105
0.1	10 ⁻³	0.1%	90–107
0.01	10 ⁻⁴	100 ppm (mg/kg)	80–110
0.001	10 ⁻⁵	10 ppm (mg/kg)	80–110
0.0001	10 ⁻⁶	1 ppm (mg/kg)	80–110
0.00001	10 ⁻⁷	100 ppb (µg/kg)	80–110
0.000001	10 ⁻⁸	10 ppb (µg/kg)	60–115
0.0000001	10 ⁻⁹	1 ppb (µg/kg)	40–120

^a Table excerpted from AOAC Peer-Verified Methods Program, Manual on Policies and Procedures (1998) AOAC INTERNATIONAL, Rockville, MD, USA.

Table 3.4.2.4: Predicted relative standard deviation of reproducibility

Analyte, %	Mass fraction (C)	Unit	RSD _r , %
100	1	100%	2
10	10 ⁻¹	10%	3
1	10 ⁻²	1%	4
0.1	10 ⁻³	0.1%	6
0.01	10 ⁻⁴	100 ppm (mg/kg)	8
0.001	10 ⁻⁵	10 ppm (mg/kg)	11
0.0001	10 ⁻⁶	1 ppm (mg/kg)	16
0.00001	10 ⁻⁷	100 ppb (µg/kg)	22
0.000001	10 ⁻⁸	10 ppb (µg/kg)	32
0.0000001	10 ⁻⁹	1 ppb (µg/kg)	45

a Table excerpted from *Definitions and Calculations of HorRat Values from Intralaboratory Data*, HorRat for SLV.doc, 2004-01-18, AOAC INTERNATIONAL, Rockville, MD, USA.

Reference

AOAC Official Methods of Analysis (2019) Guidelines for Standard Method Performance Requirements Appendix F, p. 9

Table 3.4.2.5: Nutrient integrity checks comprise Crosschecks and Edit Limit Checks

No	Description	Notes
Nutrient crosschecks		
1.	Total sugar (g) > carbohydrate (g)	
2.	Retinol (mcg) > vitamin A as retinol activity equivalents (mcg)	
3.	Folic acid (mcg) > folate as dietary folate equivalents (mcg)	
4.	Food folate (mcg) > folate as dietary folate equivalents (mcg)	
5.	Vitamin B12, added (mcg) > vitamin B12 (mcg)	
6.	Vitamin E, added (mg) > vitamin E as alpha tocopherol (mg)	
7.	Sum of moisture, protein, carbohydrate, total fat, ash, and alcohol in 100 g of food <97 g or >103 g for meat and meat products <99 g or >101 g for all other foods	
8.	Energy value compared with energy calculated using the algorithm (4 protein+ 4 carbohydrate+9 total fat+7 alcohol) percent difference > ± 25%	
9.	Total fat compared with the sum of total saturated fatty acids, total monounsaturated fatty acids, and total polyunsaturated fatty acids percent difference > ± 25%	
10.	Total saturated fatty acids compared with the sum of individual saturated fatty acid values (4:0, 6:0, 8:0, 10:0, 12:0, 14:0, 16:0, and 18:0) percent difference > ± 25%	
11.	Total monounsaturated fatty acid value compared with the sum of individual monounsaturated fatty acid values (16:1, 18:1, 20:1, and 22:1), percent difference > ± 25%	

No	Description	Notes
Nutrient crosschecks		
12.	Total polyunsaturated fatty acid value compared with the sum of individual polyunsaturated fatty acids (18:2, 18:3, 18:4, 20:4, 20:5, 22:5, and 22:6) percent difference $> \pm 25\%$	
13.	Vitamin A as retinol activity equivalents compared with vitamin A calculated using the algorithm (retinol+1/12 beta-carotene+1/24 (alpha-carotene+beta-cryptoxanthin)) percent difference $> \pm 25\%$	
14.	Ash compared with the sum of mineral values (calcium, iron, magnesium, phosphorus, potassium, sodium, zinc, copper, and selenium): $>25\%$ or $<500\%$. A broad range is used because some minerals are not quantified in the database.	
No	Description	Notes
Edit limit checks		
1.	Define and identify extreme values for nutrients newly added to the database.	
2.	Vitamin E, added >75 mg (based on twice the adult RDA in an average serving of ready-to-eat cereals)	
3.	Vitamin B12, added >12 mcg (based on twice the adult RDA in an average serving of ready-to-eat cereals)	
4.	Vitamin B12, added or Vitamin E, added >0 in food groups other than infant formulas, cereals, and meal replacement.	
5.	Caffeine >0 in food groups other than beverages and chocolate-containing foods	
6.	Theobromine >0 in foods other than tea and chocolate containing foods	

Reference

- 1) Journal of Food Composition and Analysis 21 (2008) S119–S124, Quality control procedures for the USDA Food and Nutrient Database for Dietary Studies nutrient values, Jaspreet K.C. Ahuja, Betty P. Perloff
- 2) USDA-ARS, Beltsville Human Nutrition Research Center, Beltsville, MD 20705, USA
Received 20 December 2006; received in revised form 15 May 2007; accepted 31 May 2007

3.5 MANDATORY NUTRIENTS

Table 3.5.1: Mandatory Nutrients

No	Nutrient	Method of Analysis	Reference	Method Performance
1.	Energy	Energy by calculation JKM F 1205	Guide to Nutrition Labelling and Claims by Food Safety and Quality Division, MOH, 2006	
2.	Water	Determination of Moisture In Air Oven (JKM F 0932) or Vacuum Oven (JKM F 0933)	1) AOAC, 15th Ed. 2)FAO Food and Nutrition Paper, Manual of Food Quality Control, No. 7	QC based on replicate testing. Repeatability: CV: 2.4 – 2.6 %
3.	Protein	Protein Determination by the Kjeldahl Digestion Method JKM F 0908 or Total Protein by Dumas Method JKM F 0931	1) Pearson's Chemical Analysis of Foods,8th Ed. 2) JAOAC 73, 849	QC-based Reference Material Repeatability: CV: 2.5 % (JKM F0908) CV: 1.6 % (JKM F0931) Mean Recovery: 97%
4.	Fat	Total Fat in Food by Automatic Soxhlet Extraction JKM F 0925	Pearson, D. (1970). The Chemical Analysis of Food, pp 14.	QC based on replicate testing. Repeatability: CV: 1.69% Measurement Uncertainty: 0.04x Conc. of Fat
5.	Available Carbohydrate	Available Carbohydrate by calculation JKM F 1204	Guide to Nutrition Labelling and Claims by Food Safety and Quality Division, MOH, 2006	
6.	Total Dietary Fiber TDF	Total dietary fiber in foods by Enzymatic-gravimetric Method –(phosphate buffer) JKM F 1206	Ref: AOAC 985.29 (1995)	QC based on replicates / Reference Material Repeatability: CV: 14.9% Measurement Uncertainty: 0.35 x % DF
7.	Ash	Determination of Total Ash and Acid Insoluble Ash JKM F 0902	Pearson's Chemical Analysis of Food (8th Edition)	QC based on replicate Repeatability: CV = 6.5% (total ash) CV = 12% (ash insoluble in acid)
8.	Calcium, Ca	Determination of Minerals (Calcium, Sodium, Magnesium & Iron) in Food Products by ICP-OES after Dry Ashing JKM F1209	Journal of AOAC International Vol. 87, No.1, 2004. Comparison of Partial Digestion Procedures of Ca, Cr, Cu, Fe, K, Mg, Mn, Na, P and Zn in Milk by Inductively Coupled Plasma-Optical Emission Spectrometry	QC based on spiked sample/reference material. Repeatability: CV: 13 % Mean Recovery: 109% Measurement Uncertainty: 0.15 x Conc. of Ca
9.	Iron, Fe	Determination of Minerals (Calcium, Sodium, Magnesium & Iron) in Food Products by ICP-OES after Dry Ashing JKM F1209	1) Journal of AOAC International Vol. 87, No.1, 2004. Comparison of Partial Digestion Procedures of Ca, Cr, Cu, Fe, K, Mg, Mn, Na, P and Zn in Milk by Inductively Coupled Plasma-Optical Emission Spectrometry	QC based on spiked sample/reference material. Repeatability: CV: 15 % Mean Recovery: 102% Measurement Uncertainty: 0.28 x Conc. of Fe

No	Nutrient	Method of Analysis	Reference	Method Performance
10.	Magnesium, Mg	Determination of Minerals (Calcium, Sodium, Magnesium & Iron) in Food Products by ICP-OES after Dry Ashing JKM F1209	1) Journal of AOAC International Vol. 87, No.1, 2004. Comparison of Partial Digestion Procedures of Ca, Cr, Cu, Fe, K, Mg, Mn, Na, P and Zn in Milk by Inductively Coupled Plasma-Optical Emission Spectrometry	QC based on spiked sample/reference material. Repeatability: CV: 10% Mean Recovery: 117% Measurement Uncertainty: 0.15 x Conc of Mg
11.	Phosphorus, P	-	-	-
12.	Potassium, K	-	-	-
13.	Sodium, Na	Determination of Minerals (Calcium, Sodium, Magnesium & Iron) in Food Products by ICP-OES after Dry Ashing JKM F 1209	1) Journal of AOAC International Vol. 87, No.1, 2004. Comparison of Partial Digestion Procedures of Ca, Cr, Cu, Fe, K, Mg, Mn, Na, P and Zn in Milk by Inductively Coupled Plasma-Optical Emission Spectrometry	QC based on spiked sample/reference material. Repeatability: CV: 9 % Mean Recovery: 108% Measurement Uncertainty: 0.11 x Conc. of Na
14.	Zinc, Zn	Determination of Trace Metals (Pb, Cd, Zn & Cu) in Food by Atomic Absorption Spectrophotometry after Dry Ashing JKM F 0501	JAOAC July/August 1993, Volume 76, No. 4. Determination of Metals in Foodstuffs by Atomic Absorption Spectrophotometry after Dry Ashing	QC based on spiked sample/reference material. Repeatability: CV: 5 % Mean Recovery: 97%
15.	Copper, Cu	Determination of Trace Metals (Pb, Cd, Zn & Cu) in Food by Atomic Absorption Spectrophotometry after Dry Ashing JKM F 0501	JAOAC July/August 1993, Volume 76, No 4. Determination of Metals in Foodstuffs by Atomic Absorption Spectrophotometry after Dry Ashing	QC based on spiked sample/reference material. Repeatability: CV: 4 % Mean Recovery: 98%
16.	Selenium, Se	-	-	-
17.	Manganese, Mn	-	-	-
18.	Iodine	-	-	-
19.	Ascorbic Acid (Vitamin C)	Vitamin C in Fruit Juices and Related Products by Liquid Chromatography JKM F 1202	JAOAC Vol 86, No.2,2003	QC based on spiked sample. Repeatability: CV: 5.9 % Mean Recovery: 97.7% Measurement Uncertainty: 0.12 x Conc of Vit C
20.	Thiamine (B1)	Vitamin B Rapid Determination of B1,B2,B3,B6 and B9 in Food by Liquid Chromatography JKM F 1201	Pearson's Chemical Analysis of Food, 8th Ed., pg 73-74 JAOAC Vol 85, No 4, 2002	QC based on spiked sample/reference material. Repeatability: CV: 13.69 % Mean Recovery: 77.6% Measurement Uncertainty: 0.3 x Conc. of B1

No	Nutrient	Method of Analysis	Reference	Method Performance
21.	Riboflavin (B2)	Vitamin B Rapid Determination of B1,B2.B3,B6 and B9 in Food by Liquid Chromatography JKM F 1201	1) Pearson's Chemical Analysis of Food, 8th Ed., pg 73-74 2) JAOAC Vol 85, No 4, 2002	QC based on spiked sample /reference material. Repeatability: CV: 16.98 % Mean Recovery: 61.3% Measurement Uncertainty: 0.36 x Conc of B2
22.	Niacin (B3)	Vitamin B Rapid Determination of B1,B2.B3,B6 and B9 in Food by Liquid Chromatography JKM F 1201	Pearson's Chemical Analysis of Food, 8th Ed., pg 73-74 JAOAC Vol 85, No 4, 2002	QC based on spiked sample /reference material. Repeatability: CV: 22.29 % Mean Recovery: 68.5% Measurement Uncertainty: 0.5 x Conc. of B3
23.	Folic Acid (B9)	Vitamin B Rapid Determination of B1,B2.B3,B6 and B9 in Food by Liquid Chromatography JKM F 1201	Pearson's Chemical Analysis of Food, 8th Ed., pg 73-74 JAOAC Vol 85, No 4, 2002	QC based on spiked sample /reference material. Repeatability: CV: 11.09 % Mean Recovery: 45.8% Measurement Uncertainty: 0.27 x Conc. of B9
24.	Vitamin A (Retinol)	Vitamin A (Retinol) and Vitamin E (alpha-Tocopherol) in Food by HPLC JKM F 1203	In-house method based AOAC Vol 85, No 2, 2002, pg 424-434	QC based on spiked sample /reference material. Repeatability: CV: 3.8 % Mean Recovery: 115.2% Measurement Uncertainty: 0.13 x Conc. of Vit A
25.	Carotenoid	-	-	-
	α-carotene			
	β-carotene			
	Lycopene			
	Lutein			
26.	Vitamin D	-	-	-
27.	Vitamin E	Vitamin A (Retinol) and Vitamin E (alpha-Tocopherol) in Food by HPLC JKM F 1203	In-house method based AOAC Vol 85, No 2, 2002, pg 424-434	QC based on spiked sample /reference material. Repeatability: CV: 7.9 % Mean Recovery: 98.4% Measurement Uncertainty: 0.25 x Conc. of Vit E
28.	Vitamin KD	-	-	-
29.	Total sugar	Determination of Total Sugars by HPLC ELSD JKM F 0923	In-house method	QC based on Reference Material. Repeatability: CV: 5.0 % Measurement Uncertainty: 0.10 x Conc of Total Sugars
	Sucrose			
	Glucose			
	Fructose			
	Lactose			
	Maltose			

3.6 OPTIONAL NUTRIENTS

Table 3.6.1: Optional Nutrients

No	Nutrient	Method of Analysis	Reference	Method Performance
1.	Pantothenic Acid (B5)	-	-	-
2.	Pyridoxine (B6)	Vitamin B Rapid Determination of B1,B2,B3,B6 and B9 in Food by Liquid Chromatography JKM F 1201	Pearson's Chemical Analysis of Food, 8th Ed., pg 73-74 JAOAC Vol 85, No 4, 2002	QC based on spiked sample /reference material. Repeatability: CV: 9.66 % Mean Recovery: 92.0% Measurement Uncertainty: 0.21 x Conc. of B6
3.	Cobalamin (B12)	-	-	-
4.	Choline	-	-	-
5.	Biotin (B7)	-	-	-
6.	Fatty acid, total saturated fat	Analysis of saturated, monounsaturated, polyunsaturated and trans fatty acids in food by GC JKM F 1210	AOAC Official Method of Analysis, Method 996.06	QC based on replicates Repeatability: CV: 5.5 % Mean Recovery: 91.0% Measurement Uncertainty: 0.34 x Conc. of sat. fat
	4:0			
	6:0			
	8:0			
	10:0			
	12:0			
	14:0			
	16:0			
	18:0			
7.	Fatty acid, total monounsaturated fat	Analysis of saturated, monounsaturated, polyunsaturated and trans fatty acids in food by GC JKM F 1210	AOAC Official Method of Analysis, Method 996.06	QC based on replicates Repeatability: CV: 5.0 % Mean Recovery: 92.2%
	16:1			
	18:1			
	20:1			
	22:1			
8.	Fatty acid, total polyunsaturated fat	Determination of Minerals (Calcium, Sodium, Magnesium & Iron) in Food Products by ICP-OES after Dry Ashing JKM F1209	Journal of AOAC International Vol. 87, No.1, 2004. Comparison of Partial Digestion Procedures of Ca, Cr, Cu, Fe, K, Mg, Mn, Na, P and Zn in Milk by Inductively Coupled Plasma-Optical Emission Spectrometry	QC based on spiked sample/reference material. Repeatability: CV: 13 % Mean Recovery: 109% Measurement Uncertainty: 0.15 x Conc. of Ca
	18:2			
	18:3			
	18:4	-	-	-
	20:4	Analysis of saturated, monounsaturated, polyunsaturated and trans fatty acids in food by GC JKM F 1210	AOAC Official Method of Analysis, Method 996.06	QC based on replicate Repeatability: CV: 6.3 % Mean Recovery: 65.6% Measurement Uncertainty: 0.16 x Conc of polyunsat. Fat
	20:5	-	-	-

No	Nutrient	Method of Analysis	Reference	Method Performance
9.	Trans fatty acids	Analysis of saturated, monounsaturated, polyunsaturated and trans fatty acids in food by GC JKM F 1210	AOAC Official Method of Analysis, Method 996.06	QC based on replicate Repeatability: CV: 6.3 % Mean Recovery: 99.5% Measurement Uncertainty: 0.16 x Conc of trans fatty acid
10.	Cholesterol	Determination of Cholesterol in Food by Direct Saponification JKM F 1208	AOAC Official Method of Analysis (2000). Method 994.10	QC based on spiked sample Repeatability: CV: 10.15 % Mean Recovery: 102.2% Measurement Uncertainty: 0.22 x Conc of cholesterol
11.	Amino Acid: Tryptophan Threonine Isoleucine Leucine Lysine Methionine Phenylalanine Valine	-	-	-



PART IV

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PROTOCOL FOR SAMPLING AND METHODS OF ANALYSIS FOR MALAYSIAN FOOD COMPOSITION DATABASE TECHNICAL COMMITTEE

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