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ASEAN

Manual of Food Analysis

Regional Centre of ASEAN Network of Food Data System
Institute of Nutrition, Mahidol University
THAILAND

2011

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First Edition

ASEAN Manual of Food Analysis

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The ASEAN Manual of Food Analysis

is a compilation of methods submitted by members of the ASEAN Network of Food Data Systems (ASEANFOODS), in collaboration with the Thai Technical Team.

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Associate Professor Prapasri Puwastien, PhD.
Organiser of the ASEANFOODS Workshop-2002
ASEANFOODS Coordinator

FOREWORD

The ASEAN Network of Food Data System (ASEANFOODS) was established in 1986, with six member countries, namely Brunei Darussalam, Indonesia, Malaysia, the Philippines, Singapore and Thailand. The Institute of Nutrition, Mahidol University, Thailand, was designated as the Regional Centre. Vietnam joined in 1996 and Cambodia, Laos and Myanmar in 2001. The main activity of ASEANFOODS is to promote and support the development of high quality national and regional food composition databases, accessible to users in ASEAN and other regions, in order to contribute towards improving food and nutritional security and sustainability of diets.

To fulfill these aims, laboratories with well trained analysts using standard, fully documented methods and quality control systems are necessary. ASEANFOODS Regional Centre, collaborating with other regional networks, including APFAN, OCEANIAFOODS and INDOFOODS, organised training courses on analytical methods and quality assurance programmes. Since 1989, developing ASEAN food reference materials with various matrices and organising laboratory performance studies or proficiency testing have been a major activity of the Regional Centre.

An International Graduate Course on the Production and Use of Food Composition Data in Nutrition (FoodComp-Asia 2002) was organised in Thailand in 2002. The participants of the course included participants from ASEANFOODS, NEASIAFOODS and SAARCFOODS and they issued a strong recommendation that an ASEAN Manual of Food Analysis should be compiled. Accordingly, an ASEANFOODS Workshop was organised in 2002 to select and identify the standard methods to be incorporated into the ASEAN Manual of Food Analysis. The compilation was achieved by the hard work and contributions of experts and the technical team (eleven experts from six ASEANFOODS member countries and the fifteen-strong technical team from Thailand). The names and address of the contributors with their areas of expertise are given in **Appendix 1**.

This first edition of the ASEAN Manual of Food Analysis covers methods for analysis of proximate composition, starch, sugars, cholesterol, minerals, vitamins, fatty acids and amino acids. It is expected that the Manual will serve the needs of laboratories in ASEANFOODS member countries and in other regions. It is one of the essential documents required in the accreditation process of analytical laboratories according to ISO 17025 and we hope that using these standard methods of food analysis will improve the quality of the generated food composition databases.

Associate Professor Songsak Srianujata, PhD
Chairman of the ASEANFOODS Workshop 2002
Director (2000-2004), Institute of Nutrition, Mahidol University, Thailand

DETERMINATION OF MOISTURE BY AIR OVEN

1. PURPOSE/SCOPE

The method is used for the quantitative determination of moisture in all foods except food samples high in sugar and fat (> 10%).

2. SAFETY

Use tongs when handling drying containers.

3. REFERENCE

3.1 Horwitz W (2000) (editor). Official Method of Analysis of AOAC International. 17th Edition. AOAC International, Maryland, USA.
3.2 Greenfield H and Southgate DAT (1992). Food Composition Data: Production, Management and Use. Elsevier Applied Science, UK.
3.3 Kirk RS and Sawyer R (1991). Pearson's Composition and Chemical Analysis of Foods, 9th Edition. Longman Scientific & Technical, Essex, England.

4. DEFINITION

Moisture in this method refers to the amount of free water and volatile substances that are lost by drying the food under controlled temperature in an air oven. It is expressed in g per 100 g sample.

5. PRINCIPLE

The method is based on the drying of food sample under controlled temperature until constant weight is obtained. Moisture content is required to express the nutrient content per dry weight basis. In some foods, moisture is used to indicate their quality. Standard values of moisture are indicated in food notification or regulation.

6. MATERIAL

Sand, acid washed, 40 mesh

7. APPARATUS

7.1 Air oven, capable of being controlled at 100±5°C.
7.2 Analytical balance, 200 g capacity and 0.1 mg sensitivity.
7.3 Desiccator with desiccant such as silica gel. Ensure that the desiccant is activated prior to use by heating in an oven at 100°C until blue.
7.4 Aluminum dishes or porcelain crucibles or weighing bottles or other appropriate drying containers.
7.5 Tongs.
7.6 Stirring rod.
7.7 Boiling water bath with removable rings to hold the drying containers.

8. PROCEDURES

8.1 Sample preparation

8.2.1 Preparation of drying container: place container in the drying oven at 100±5°C until constant weight (1 - 2 h). Cool in a desiccator for about 30 min and weigh (***W_1***). For liquid or semisolid sample, prepare drying container with 15 - 20 g acid washed sand and a stirring rod.

8.2.2 Preparation of sample: grind or blend sample until homogenous. Analyse sample immediately after preparation. If sample cannot be analysed on the same day, keep in screw-cap bottle in a freezer. For samples intended for analysis of vitamins or other labile nutrients, flush sample with nitrogen before storing.

8.2 Analysis

8.2.1 For dry sample

Thaw sample to room temperature. Mix sample thoroughly by turning the tightly closed bottle up and down three (3) times. Weigh accurately 3 – 4 g sample, in duplicate, into a pre-weighed drying container (***W_2***).

8.2.2 For liquid/wet/slurried sample

Mix sample thoroughly and weigh 5–15 g slurried sample and 20 g for liquid sample into a pre-weighed drying container with acid-washed sand and stirring rod (W_2). For liquid samples, dry to a consistency of a thick paste over a boiling water bath before drying in an oven.

8.2.3 Place container with sample in the air oven pre-heated to 100±5°C for 2 – 3 hours.

8.2.4 Transfer the container with the dried sample into a desiccator, cool for 30 min and weigh (***W_3***).

8.2.5 Repeat the heating procedure until constant weight. Difference in weight between two consecutive weighing should not be more than 5 mg.

9. CALCULATION

$$\text{Moisture (g/100 g)} = \frac{(W_2 - W_3)\ x\ 100}{(W_2 - W_1)}$$

Total solid (%) = 100 - % moisture (w/w)

where: W_1 = weight of container or empty dish (g)
W_2 = weight of container + sample before drying (g)
$W_2 - W_1$ = weight of sample (g)
W_3 = weight of container + sample after drying (g)
$W_2 – W_3$ = loss of weight (g)

Report test results (in g per 100 g sample) to one decimal place.

10. ACCEPTANCE OF RESULTS

Duplicate results should not differ by more than 5% of the mean.

DETERMINATION OF MOISTURE BY VACUUM OVEN

1. PURPOSE/SCOPE

The method is used for the quantitative determination of moisture in food products high in sugar and fat (> 10%).

2. SAFETY

Use tongs when handling drying containers.

3. REFERENCE

3.1 Horwitz W (2000) (editor). Official Method of Analysis of AOAC International. 17th Edition. AOAC International, Maryland, USA.

3.2 Greenfield H and Southgate DAT (1992). Food Composition Data: Production, Management and Use. Elsevier Applied Science, UK.

3.3 Kirk RS and Sawyer R (1991). Pearson's Composition and Chemical Analysis of Foods, 9th Edition. Longman Scientific & Technical, Essex, England.

4. DEFINITION

Moisture in this method refers to the amount of free water and volatile substances that are lost by drying the food under vacuum and controlled temperature. It is expressed in g per 100 g sample.

5. PRINCIPLE

The method is based on the drying of food sample under controlled pressure and temperature until constant weight is obtained. Moisture content is required to express the nutrient content per dry weight basis. In some foods, moisture is used to indicate their quality. Standard values of moisture are indicated in food notification or regulation.

6. MATERIALS

Sand, acid washed, 40 mesh

7. APPARATUS:

7.1 Vacuum oven, capable of being controlled at 60-110°C, equipped with suitable thermometer. To dry the inlet air to the oven, use suction flask half-filled with appropriate desiccant.

Note: If concentrated sulfuric acid is used as desiccant, use with extreme precaution. Understand the toxicity and safety hazards of sulfuric acid before starting the method

.

7.2 Vacuum pump, capable of providing up to a pressure of 30 mm Hg.

7.3 Drying oven, capable of being controlled at 50-100°C, equipped with a suitable thermometer.

7.4 Analytical balance, 200 g capacity, 0.1 mg sensitivity

7.5 Boiling water bath with removable rings to hold the drying containers.

7.6 Desiccator with desiccant such as silica gel. Ensure that the desiccant is activated prior to use by heating in an oven at 100°C until blue.
7.7 Aluminum dishes or porcelain crucibles or weighing bottles or other appropriate drying containers.
7.8 Tongs.
7.9 Stirring rod.

8. PROCEDURES

8.1 Sample preparation

8.2.1 Preparation of drying container: place container in the drying oven at 100±5°C until constant weight (1-2 h). Cool in a desiccator for about 30 min and weigh (W_1). For liquid or semisolid sample, prepare drying container with 15-20 g acid washed sand and a stirring rod.

8.2.2 Preparation of sample: grind or blend sample until homogenous. Analyse sample immediately after preparation. If sample cannot be analysed on the same day, keep in screw-cap bottle in a freezer. For samples intended for analysis of vitamins or other labile nutrients, flush sample with nitrogen before storing.

8.3 Analysis

8.2.1 For dry sample

Thaw out sample to room temperature. Mix sample thoroughly by turning the tightly closed bottle up and down three (3) times. Weigh accurately 3–4 g sample, in duplicate, into a pre-weighed drying container (W_2).

8.2.2 For liquid/wet/slurried sample

Mix sample thoroughly and weigh 5–15 g slurried sample and 20 g for liquid sample into a pre-weighed drying container with acid-washed sand and stirring rod (W_2). For liquid samples, dry to a consistency of a thick paste over a boiling water bath before drying in an oven.

8.2.3 Place container with sample in the vacuum oven at 60–70°C < 25 mm Hg for 5–6 hours.

8.2.4 Transfer the container with the dried sample into a desiccator, cool for 30 min and weigh (W_3).

8.2.5 Repeat the heating procedure until constant weight. Difference in weight between two consecutive weighing should not be more than 5 mg.

9. CALCULATION

$$\text{Moisture (g/100 g)} = \frac{(W_2 - W_3)\ x\ 100}{(W_2 - W_1)}$$

Total solid (%) = 100 - % moisture (w/w)

where: W_1 = weight of container or empty dish (g)
W_2 = weight of container + sample before drying (g)
$W_2 - W_1$ = weight of sample (g)
W_3 = weight of container + sample after drying (g)
$W_2 - W_3$ = loss of weight (g)

Report test results (in g per 100 g sample) to one decimal place.

10. ACCEPTANCE OF RESULTS

Duplicate results should not differ by more than 5% of the mean.

11. APPENDIX/SUPPLEMENTARY NOTE

This method may also be used for all food samples by drying under vacuum at 70-100°C.

DETERMINATION OF CRUDE PROTEIN BY KJELDAHL METHOD

1. PURPOSE/SCOPE

The method is used for the quantitative determination of crude protein in all types of foods.

2. SAFETY/PRECAUTION

2.1 Always wear protective devices, e.g. face mask, goggles and gloves when handling toxic and corrosive chemicals.
2.2 Understand the toxicity and safety of the reagents used before starting the method.
2.3 Use pipette aides in handling acids.
2.4 Digest sample under a hood to reduce inhalation of acid fumes.
2.5 Analysis should be done in an ammonia-free environment.

3. REFERENCES

3.1 Horwitz W (2000) (editor). Official Method of Analysis of AOAC International. 17th Edition. AOAC International, Maryland, USA.
3.2 WHO (1973). Report of a Joint FAO/WHO Ad Hoc Expert Committee on Energy and Protein Requirements, WHO Technical Report Series No. 522, WHO, Geneva.
3.4 Greenfield H and Southgate DAT (1992). Food Composition Data: Production, Management and Use. Elsevier Applied Science, UK.
3.5 Kirk RS and Sawyer R (1991). Pearson's Composition and Chemical Analysis of Foods, 9th Edition. Longman Scientific & Technical, Essex, England.

4. DEFINITION

4.1 Crude protein is total nitrogen multiplied by protein factor. It is expressed in g per 100 g sample.
4.2 Total nitrogen content includes nitrogen primarily from proteins and to a lesser extent from all organic nitrogen containing non-protein substances. For practical purposes, non-protein nitrogen is assumed to be of little significance.

5. PRINCIPLE:

The method is based on the digestion of proteins and other organic food components in the sample with sulfuric acid in the presence of catalyst e.g. sodium or potassium sulfate to release nitrogen from protein and retain it as ammonium salt. Ammonia gas is liberated upon addition of excess alkali (concentrated sodium hydroxide) and is distilled into a boric acid solution to form ammonium-borate complex. The ammonia liberated from the complex is titrated with standardised hydrochloric acid. The amount of nitrogen in the sample is determined from the milligram equivalent of the acid used. Crude protein is determined by multiplying the nitrogen content with a conversion factor specific to the food matrix (see Appendix).

6. REAGENTS

6.1. Sulfuric acid, concentrated, A.R. grade or equivalent

6.2. Catalysts: Potassium sulfate and or copper sulfate with or without selenium (IV) dioxide

Note: For environmental and health concerns mercury-containing catalysts should not be used.

6.3. Sodium hydroxide solution, 50%. Weigh 500 g sodium hydroxide, dissolve in water and make up to 1 L with H_2O.

Note: The reaction of NaOH with water is exothermic. Place reaction vessel in a basin of ice cold water when dissolving it.

6.4. Boiling chips.

6.5. Ammonium sulfate, A.R.

6.6. Hydrochloric acid, 0.1 N standard solution. Pipette 8.3 mL of concentrated hydrochloric acid to approximately 500 mL distilled H_2O in 1 L volumetric flask soaked in ice cold water. Allow to cool and make up to volume with distilled H_20. Pour the solution in 1 L brown bottle and let the solution stand for 2 or 3 days with occasional shaking before standardisation.

6.7. Standardisation of HCl solution stated in 6.6.

Weigh accurately about 0.47 g AR sodium tetraborate decahydrate (borax) into a 250mL conical flask, dissolve in about 50mL water and add a few drops of methyl red indicator solution. Titrate with the hydrochloric acid from a burette until the colour changes to pink at the endpoint. [Methyl red indicator solution: dissolve about 1g of methyl red in 600mL alcohol and dilute with 400 mL water.]

$$\text{Normality} = \frac{\text{wt borax (g)} \times 1000}{\text{mLs HCl titrant} \times 190.72}$$

6.8. Methyl red/bromcresol green indicator solution. Dissolve 0.2 g methyl red and dilute to 100 mL with 95% ethanol. Dissolve 1.0 g bromcresol green and dilute to 500 mL with 95% ethanol. Mix one (1) part methyl red solution with five (5) parts bromcresol green solution.

Note: Methylene blue, 0.2% in 95% ethanol and methyl red, 0.2% in ethanol (1:2) indicator can also be used.

6.9. Boric acid solution, 4% with indicator. Dissolve 40 g boric acid in hot distilled water, cool and make up to 1 L with distilled water and add 3 mL methyl red/bromcresol green indicator solution. Solution will be light orange in color.

7. APPARATUS

7.1 Kjeldahl and other commercial systems (such as Kjeltec/Tecator System). The system consists of three units, namely

7.1.1 Digestion unit.

7.1.2 Distillation unit.

7.1.3 Titration unit.

7.2 Digestion tubes.
7.3 Erlenmeyer flask, 250 mL, 500 mL
7.4 Magnetic stirrer and magnetic bar.
7.5 Drying oven, capable of being controlled at 100-120°C and equipped with a suitable thermometer.
7.6 Balance, analytical, 200 g capacity with 0.1 mg sensitivity

8. PROCEDURE

8.1 Preparation of sample: grind or blend sample until homogenous. If sample cannot be analysed on the same day, keep in screw-cap bottle in a freezer. For samples intended for analysis of vitamins or other labile nutrients, flush sample with nitrogen before storing.

8.2 Analysis

8.2.1 Blank: include two reagent blanks (containing all reagents used in nitrogen analysis except the sample) in every batch of analysis to subtract reagent nitrogen from the sample nitrogen.

8.2.2 Test sample

8.2.2.1 Thaw out sample to room temperature and mix the sample thoroughly.

8.2.2.2 Weigh in duplicate 2–10 g sample (depending on the nitrogen content of the sample) into the digestion tube.

8.2.2.3 Add 5-7 g catalyst and 1 glass bead to prevent solution from bumping and 10–20 mL sulfuric acid.

8.2.2.4 Place digestion tube in the digestor. Digest mixture initially at low temperature to prevent frothing and boil briskly until the solution is clear and is free of carbon or until oxidation is complete.

Note: the digestion time and volume of sulfuric acid required depends on the material to be digested. If the digest is still yellowish, cool the digest and add an additional 5-10 mL sulfuric acid.

8.2.2.5 Continue digestion until a clear digest is obtained.

8.2.2.6 Heat for another hour after the liquid has become clear to complete breakdown of all organic matter.

8.2.2.7 Place a 250-500 mL Erlenmeyer flask containing 50 mL of 4% boric acid with indicator as receiver on the distillation unit.

Note: the tip of the condenser should extend below the surface of the acid solution.

8.2.2.8 Add 100 mL of water and 70 mL of 50% sodium hydroxide to the digests and start distillation.

Note: Make sure excess NaOH is added to neutralize sulfuric acid to ensure complete release of ammonia.

8.2.2.9 Distill until all ammonia has been released or approximately ≥ 150 mL distillate is obtained.

Note: Use condenser with ice cold water to effectively capture all distilled ammonia.

8.2.2.10 Lower the receiver flask so that the delivery tube is above the liquid surface and continue the distillation for 1-2 minutes.

8.2.2.11 Finally, rinse the delivery tube with water and allow the washings to drain into the flask.

8.2.2.12 Titrate the distillate with the standardised 0.1 N hydrochloric acid until the first appearance of the pink colour.

8.2.2.13 Record volume of acid used to the nearest 0.05 mL.

Note: Always check the normality of the standardised 0.1N HCl.

9. CALCULATION

$$N\ (g\%) = \frac{(mL\ 0.1N\ HCl\ sample - mL\ 0.1N\ HCl\ \ blank) \times 0.0014 \times N\ HCl \times 100}{Weight\ of\ sample}$$

Protein (g per 100g) = % total nitrogen x appropriate nitrogen conversion factor (see Appendix)

Report test results (in g per 100 g sample) to one decimal place.

10. ACCEPTANCE OF TEST RESULTS

Accept test results if one or more of the following conditions are satisfied:

10.1 Duplicate results should not differ by more than 5% of the mean.

10.2 Mean concentration of duplicate results of In-house Food Reference Material should be within ± 2 SD in the control chart based on established acceptance criteria.

11. APPENDIX

Factors for the conversion of nitrogen to protein

Food Items	Factor
Meat and Fish	6.25
Gelatin	5.55
Milk and milk products	6.38
Casein	6.40
Human milk	6.37
Eggs: Whole	6.25
Vitellin	6.32
Albumin	6.12
Wheat : Whole	5.83
Bran	6.31
Embryo	5.80
Endosperm	5.70
Rice and rice flour	5.95
Barley, oats, rye and flour	5.83
Millet	6.31
Corn/Maize, beans	6.25
Soybean	5.71
Castor bean	5.30
Nuts and seeds	
Peanuts, Brazil nuts, ground nuts	5.46
Coconuts, cashew nuts & other nuts	5.30
Sesame seeds, sunflower seeds and all other seeds	5.30
All other foods	6.25

Source: Reference 3.2

DETERMINATION OF TOTAL FAT BY ACID HYDROLYSIS METHOD

1. PURPOSE/SCOPE

The method is used for the quantitative determination of total fat in foods including milk and milk products, raw and cooked foods, processed food products. It is also suitable for the analysis of processed food with low fat content.

2. SAFETY

2.1 Understand the toxicity and safety of the reagents used before starting the method.
2.2 Wear face mask or eye protection when handling solvents.
2.3 Use gloves or hot hand when handling hot digestion tubes.
2.4 Perform all operations under a hood.

3. REFERENCES

3.1 Horwitz W (2000) (editor). Official Method of Analysis of AOAC International. 17th Edition. AOAC International, Maryland, USA.
3.2 Greenfield H and Southgate DAT (1992). Food Composition Data: Production, Management and Use. Elsevier Applied Science, UK.
3.3 Kirk RS and Sawyer R (1991). Pearson's Composition and Chemical Analysis of Foods, 9th Edition. Longman Scientific & Technical, Essex, England.

4. DEFINITION

Fat includes fatty acids, triglycerides, esters, long chain alcohols, hydrocarbons, other glycol esters and sterols determined by the method. It is expressed as g fat per 100 g sample.

5. PRINCIPLE

The method involves digestion of sample with dilute HCl to free the bound lipid fractions and subsequent extraction with organic solvents such as petroleum ether or diethyl ether either manually or in a solvent extraction unit. Addition of ammonia prior to acid treatment is advisable with foods such as dried milk, processed cheese and those containing high proportion of sugar.

6. REAGENTS AND MATERIALS

6.1 Hydrochloric acid, A.R.
6.2 Hydrochloric acid, 4 or 6 or 8 N
6.3 Diethyl ether or petroleum ether, A.R.

Note: Moist ether should not be used in fat extraction since it will dissolve sugars and other materials resulting in higher fat content. Test ether for presence of peroxide before use (refer to appendix 1)

7. APPARATUS

7.1 Soxhlet extractor with heating mantle or hot plate. Commercial systems (such as Soxtec System) may also be used.
7.2 Analytical balance, 200 g capacity, with 0.1 mg sensitivity.
7.3 Fume hood.
7.4 Drying oven can be maintained at 50° - 100°C.
7.8 Water bath with removable rings to hold the round flat bottom flask.
7.5 Desiccator with desiccant such as silica gel. Ensure that the desiccant is activated prior to use by heating in an oven at 100°C until blue.
7.6 Tongs.
7.7 Wash bottle.
7.8 Graduated cylinder, 100 mL capacity.
7.9 Beaker, 100 mL.
7.10 Round flat bottom flask (if a conventional soxhlet is used) or extraction cup (if a commercial system is used)
7.11 Erlenmeyer flask.
7.12 Rubber policeman.
7.13 Spatula.

8. PROCEDURE

8.1 Preparation of sample: grind or blend sample until homogenous. Analyse sample immediately after preparation. If sample cannot be analysed on the same day, keep in screw-cap bottle in a freezer. For samples intended for analysis of vitamins or other labile nutrients, flush sample with nitrogen before storing.
8.2 Preparation of analytical sample
Thaw the test sample to room temperature. Mix thoroughly; use a high-speed mixer if necessary.
8.3 Acid hydrolysis.
8.3.1 Weigh, in duplicate, 1-5 g of homogeneous sample or 10 mL liquid into a container (W_1). If this sample is to be used for fatty acids determination an antioxidant such as pyrogallic acid, BHT and BHA is to be added.
8.3.2 Add 50–100 mL of 4, 6 or 8 N HCl to the sample in the digestion tube or flask.
8.3.3 Put in some glass beads. Connect the flask to an air condenser and reflux with gentle boiling for 30 min - 1 h.
8.3.4 If a commercial extractor system is used, filter the digestion mixture. Wash residue with warm water until the filtrate is free from acid (test by pH paper).
8.3.5 Dry the filter paper containing the residue in an oven at 50-60°C for 4-6 h or overnight. Then transfer it into an extraction thimble. Place the thimble in the reservoir part of the soxhlet apparatus.
8.3.6 Dry a round flat bottom flask or extraction cup in an oven at 100°C for 1 h. Cool in a desiccator and weigh (W_2).
8.3.7 Add 50 mL diethyl ether or petroleum ether into the pre-weighed round flat bottom flask or extraction cup.

8.3.8 Place the flask or extraction cup into the fat extraction system.

8.3.9 Extract the sample in the thimble by immersing it in warmed solvent for 30 min if a commercial system is used. When conventional soxhlet apparatus is used, continuous distillation should be carried out for at least 3 h or more (or until extraction of fat is complete).

8.3.10 Evaporate the solvent in each round flat bottom flask or extraction cup on a water bath in a fume hood.

8.3.11 Dry the flask or extraction cup in an oven at 100 ± 5°C for 30 min and cool in a desiccator. Then re-heated and weigh again every 30 min until constant weight is obtained (***W_3***).

Note: If the lipid obtained is to be used for fatty acids determination the heating temperature has to be decreased to 50-60℃ (refer to the method of fatty acids determination).

9. CALCULATION

$$\text{Total Fat (g/100 g)} = \frac{(W_3 - W_2)\ x\ 100}{W_1}$$

where: W_1 = Weight of sample
W_2 = Weight of dried extraction cup before fat extraction
W_3 = Weight of dried extraction cup after fat extraction

10. ACCEPTANCE OF TEST RESULTS

10.1 Duplicate results should not differ by more than 5% of the mean.

10.2 Mean concentration of duplicate results of the reference materials should be within ± 2 SD in the control chart based on established acceptance criteria.

11. APPENDIX / SUPPLEMENTARY NOTES

11.1 Test method for presence of peroxide in ether

11.1.1 Weigh 5 g potassium iodide (KI)

11.1.2 Dissolve in 25 mL distilled water and dilute to 50 mL (10% KI).

11.1.3 Pipette 1 mL of the KI solution (freshly prepared) and 10 mL ether into a 25 mL graduated cylinder.

11.1.4 Shake for 1 minute then take note of the colour:
colorless – no peroxide
yellowish – presence of peroxide, do not use

DETERMINATION OF TOTAL FAT BY MANUAL EXTRACTION

1. PURPOSE/SCOPE

The method is used for the quantitative determination of total fat in foods using manual extraction.

2. SAFETY/SPECIFIC PRECAUTION

Petroleum ether is toxic (boiling point 35-60°C), and flammable, handle with care.

3. REFERENCES

Horwitz W (2000) (editor). Official Method of Analysis of AOAC International. 17th Edition. AOAC International, Maryland, USA, 920.39, p 33.

4 DEFINITION

Fat includes fatty acids, triglycerides, esters, long chain alcohols, hydrocarbons, other glycol esters and sterols determined by the method. It is expressed as g fat per 100g sample.

5. PRINCIPLE

The sample is hydrolised by hydrochloric acid at 70-80°C. Protein, if any, can be dissolved in the acid, crude fat is then manually extracted by diethyl and petroleum ether. The solvent is removed by evaporation and the oil residue is dried and weighed.

6. MATERIALS

6.1 Petroleum ether (boiling point 35-60°C)
6.2 Ethyl alcohol 95 %
6.3 4 N Hydrochloric acid
6.4 Diethyl ether, free from residue on evaporation.
6.5 Petroleum ether, b.p.35-60 C°

7. APPARATUS

7.1 Round flat bottom flask or beaker
7.2 Thimble
7.3 Cotton wool
7.4 Condenser
7.5 Extraction glassware (separating funnel or Rohring tubes or Majonnier tubes or equivalent extracting container)
7.6 Glass funnel
7.7 Hot air oven
7.8 Cylinder
7.9 Water bath
7.10 Desiccator
7.11 Analytical balance
7.12 Filter paper (Whatman No. 541)

8. PROCEDURE

8.1 Place 2 g dried sample (***W₁***) in a 250 mL Erlenmeyer flask or extraction tube, add 2 mL alcohol. Stir to moisten all particles (moistening of sample with alcohol prevents lumping on addition of acid).

8.2 Add 10 mL of the diluted 4N HCl and mix well. Set the flask on the heater and reflux for 30 min. If the tube is used, place the tube in water bath held at 70–80°C and stir at frequent intervals until sample is completely hydrolysed (usually 30–40 min).

8.3 Add 10 mL alcohol and cool.

8.4 If the hydrolysis has taken place in a flask, transfer the digested mixture to extraction glassware. Rinse the flask and pour into the extraction tube with 25 mL diethyl ether in three portions.

8.5 Close the tube with cork and shake vigorously for 1 min. Add 25 mL petroleum ether and again shake vigorously for 1 min.

8.6 Let stand until upper liquid is practically clear.

8.7 Transfer as much as possible of the ether-fat solution into a pre-weighed 125 mL flask by filtering it through a funnel containing a plug of cotton packed firmly in the stem part. Allow free passage of ether into the flask.

8.8 Before weighing the flask, dry it in drying oven at 100±5°C and then let cool in a desiccator and weigh (***W₂***).

8.9 Repeat extraction of the liquid sample remaining in tube twice using the same solvent. Each time, transfer the clear ether solutions through the same funnel into the same flask. When finished, rinse inside and outside of the funnel into the same flask.

8.10 Evaporate solvents completely on a water bath at 70-80°C

8.11 Dry fat in an oven at 100±5°C until constant weight is obtained.

8.12 Allow the flask to cool in a desiccator and weigh (***W₃***).

9. CALCULATION

$$\text{Total Fat (g/100 g)} = \frac{(W_3 - W_2)\ x\ 100}{W_1}$$

where: W_1 = Weight of sample
W_2 = Weight of dried flask before fat extraction
W_3 = Weight of dried flask after fat extraction

10. ACCEPTANCE OF TEST RESULTS

10.1 Duplicate results should not differ by more than 5% of the mean.

10.2 Mean concentration of duplicate results of the reference materials should be within ± 2 SD in the control chart based on established acceptance criteria.

DETERMINATION OF TOTAL DIETARY FIBRE BY ENZYMATIC- GRAVIMETRIC METHOD

1. PURPOSE/SCOPE

The method is used for the quantitative determination of total dietary fibre (TDF) in foods with low sugar and fat content (≤ 10% fat). Pre-treatment prior to analysis is required for samples high in fat, sugar or moisture.

2. SAFETY/PRECAUTIONS

Understand the toxicity and safety of the reagents used before starting the method. Use fume hood to reduce the inhalation of solvents. Ensure that all enzymes used are not expired.

3. REFERENCES

3.1 Horwitz W (2000) (editor). Official Method of Analysis of AOAC International. 17th Edition. AOAC International, Maryland, USA. Methods 985.29 and 991.43.

3.2 Greenfield H and Southgate DAT (1992). Food Composition Data: Production, Management and Use. Elsevier Applied Science, UK.

3.3 Sungsoo Cho, Jonathan W.De Vries and Leon Prosky (1999). Dietary Fibre Analysis and Application. AOAC International Gaithersburg, Maryland, USA.

4. DEFINITION

Dietary fibre is the edible part of plant or their extracts, or analogous carbohydrates that are not easily digested or absorbed in the human small intestine, but are partly or completely fermented in the large intestine. The term includes polysaccharides, oligosaccharides, lignin and associated plant substances.

5. PRINCIPLE

The method involves sequential enzymatic digestion of dried, defatted samples (containing ≤ 10% fat) by heat stable alpha-amylase, protease and amyloglucosidase to remove starch and protein present in the sample. Ethanol is added to the digest to precipitate soluble dietary fiber. The total dietary fiber (TDF) is the residue left after subsequent washing of the insoluble residue and the precipitate with alcohol and acetone, dried, weighed and corrected for protein and ash content.

6 REAGENTS

6.1 Ethanol solution

6.1.1 Ethanol solution, 95% V/V

6.1.2 Ethanol solution, 85%

Place 895 mL 95% ethanol into 1 L volumetric flask and dilute to volume with deionised water.

6.1.3 Ethanol solution, 78%
Place 821 mL 95% ethanol into 1 L volumetric flask and dilute to volume with deionized water.

6.2 Acetone, AR

6.3 Heat stable alpha-amylase solution, Sigma No. A 3306 or Termamyl 300L, Cat, No. 361-6282 or equivalent. Store at 2-8°C (refrigerator).

6.4 Protease, Sigma No. P3910 or equivalent. Store at 2-8°C (refrigerator).

6.5 Protease solution, 50 mg/mL.
Dissolve 50 mg protease in 1 mL solution in MES/TRIS buffer. Prepare fresh each time of use.
Note: an appropriate time to do this is during incubation with alpha-amylase.

6.6 Amyloglucosidase solution, Sigma No. AMG A9913 or equivalent. Store at 2-8°C (refrigerator). Test enzyme activity for each new lot of enzyme used or at a maximum interval of 6 months by analyzing TDF content of standards listed in Table 6.1 to ensure absence of undesirable enzymatic activities.

6.7 Celite, acid washed, Sigma, No. C8656 or equivalent.

6.8 MES.–2-(N-Morpholino)ethanesulfonic acid, Sigma No. M-8250, or equivalent.

6.9 TRIS.-Tris(hydroxymethyl)aminomethane, Sigma No.T-1503, or equivalent.

6.10 MES/TRIS Buffer solution, 0.05 M, pH 8.2 at 24°C.
Dissolve 19.52 g MES and 12.2 g TRIS in 1.7L H_2O. Adjust pH to 8.2 at 24°C with 6 N NaOH and dilute to 2L with H_2O.
Note: It is important to adjust pH to 8.2 at 24°C. However, if buffer temperature is 20°C, adjust pH to 8.3; if temperature is 28°C, adjust pH to 8.1.

6.11 Hydrochloric acid, AR

6.11.1 Hydrochloric acid, 6N.
Add 50 mL conc. HCl to about 40 mL water and dilute to 100 mL

6.11.2 Hydrochloric acid, 0.561 N.
Add 93.5 mL 6 N HCl to about 700 mL H_2O in 1 L volumetric flask. Dilute to 1 L with H_2O.

6.12 Standard buffer solutions, pH 4.0, 7.0 and 10.0

7. APPARATUS

7.1 Beaker, 400 or 600 mL, tall-form

7.2 Fritted crucible, porosity #2, coarse ASTM 40-60 μm (Pyrex No. 32940 or Corning No. 36060 Buchner, 60 mL, or equivalent). Clean crucibles thoroughly, heat one hour at 525°C and cool; soak and rinse in water; air dry. Add 0.5 g of Celite* to each crucible and dry at 130°C to constant weight (one hour or more). Cool in a desiccator and weigh to nearest 0.1 mg. Store in the desiccator until needed.

7.3 Shaking water bath, with cover, capable of being maintained at 60° and 98 ± 2°C.
7.4 Vacuum pump or water aspirator, with regulating device.
7.5 Analytical balance, 200 g capacity with 0.1 mg sensitivity.
7.6 Muffle furnace, capable of being maintained at 525±5°C
7.7 Drying oven, capable of being maintained at 105°C and 130±3°C.
7.8 Fume hood.
7.9 Timer.
7.9 Desiccator with desiccant such as silica gel. Ensure that the desiccant is activated prior to use by heating in an oven at 100°C until blue.
7.10 pH meter, with temperature compensation
7.11 Auto-pipette with disposable tips, 100-300 μL and 5 mL capacity
7.12 Graduated cylinder, 100 mL, 500 mL
7.13 Hot plate stirrer and magnetic bar
7.14 Rubber spatula

8. PROCEDURE

8.1 Sample Preparation:

8.1.1 Total dietary fibre should be determined on dried, low fat or fat free sample. If fat content is unknown, defat before determining dietary fibre.

8.1.2 Weigh accurately in duplicate 1.0±0.005 g dry, ground sample into 400 mL (or 600 mL) tall-form beakers.

8.1.3 If samples with > 10% fat:
Defat by stirring with 25 mL petroleum ether. Centrifuge and discard the petroleum ether portion. Repeat the defatting step twice. Evaporate the residual petroleum ether from the defatted sample under fume hood.
If the Soxhlet is used for defatting sample, record loss of weight due to fat removal and make appropriate correction to the final dietary fibre content.

8.1.4 For wet samples:
Dry overnight in 70°C vacuum oven or in an air oven at 100±5°C or freeze-dried before grinding.

8.1.5 For dry samples high in sugars:
Extract accurately weighed sample 3 times with 85% ethanol. Use 10 mL ethanol per g sample for extraction. Decant and dry overnight in an air oven at 40^{o}C.

8.2 Blank sample
Run 2 blanks (reagents without sample) per batch of analysis along with samples to measure any contribution from reagents to the residue.

8.3 Digestion of sample

8.3.1 Add 40 mL of MES/TRIS buffer solution, pH 8.2 to each beaker. Put in a magnetic bar and place beaker on magnetic stirrer. Mix until sample is completely dispersed. This will prevent lump formation, which would otherwise make the sample inaccessible to the enzymes.

8.3.2 Add 50 μL of alpha-amylase solution and stir at low speed.

8.3.3 Cover each beaker with aluminum foil, place in shaking water bath at 95-100°C, and incubate for 30 min with continuous agitation. Start timing once bath temperature reaches 95°C.

8.3.4 Remove beakers with sample from hot water bath, and cool to 60°C.

8.3.5 Remove foil cover and scrape any adhering particles from the inside wall of the beaker. Disperse any gels at the bottom of the beaker with spatula.

8.3.6 Rinse the sides of the beaker and spatula with approximately 10 mL water using a wash bottle.

8.3.7 Add 100 μL of protease solution to each sample in the beaker and cover with aluminum foil.

8.3.8 Incubate in shaking water bath with continuous agitation at 60±1°C, for 30 min. Start timing once the temperature of the water bath reaches 60°C.

8.3.9 Remove beakers with sample from water bath and add 5 mL 0.561 N HCl solution while stirring.

8.3.10 Adjust pH to 4.0–4.7 using 1N NaOH solution or 1 N HCl solution.

Note: It is important to check/adjust pH while the temperature of the solution is 60°C. The pH of the solution will increase at a lower temperature. Most cereals, grains and vegetable products do not require pH adjustment. Once verified for each laboratory, pH checking procedure can be omitted. As a precaution, check pH of blank routinely. Check also the pH of the samples if pH of blank is outside the desirable range.

8.3.11 Add 300 μL of amyloglucosidase solution while stirring. Cover with aluminum foil.

8.3.12 Incubate in shaking water bath with constant agitation at 60±1°C for 30 min. Start timing once the temperature of the water bath reaches 60°C.

8.4 Precipitation and filtration

8.5.1 To each digest, add 225 mL 95% ethanol, pre-heated to 60°C (ratio of ethanol to sample volume should be 4:1). Let the solution stand and allow the precipitate to form at room temperature for 60 min.

8.5.2 Wet crucible with 15 mL 78% ethanol. Apply suction to allow Celite to form an even mat onto the fritted glass crucible.

8.5.3 Filter the alcohol-treated enzyme digest through the pre-weighed crucible containing Celite. With the aid of rubber spatula, use 78% ethanol to wash and quantitatively transfer all remaining particles to the crucible.

8.5.4 Wash residue two times with 15 mL portions of each of the following:
a. 78% ethanol
b. 95% ethanol
c. Acetone

Filter under vacuum.

Note: In some samples, gum is formed, trapping the liquid. If this occurs, break layer of film with spatula without disturbing the Celite bed.

8.5.5 Dry crucibles containing residue in an oven, at 105°C overnight or until constant weight.

8.5.6 Cool crucible in a desiccator for about 1 hr and weigh.

8.5.7 Analyse residue from one sample of the duplicate set for protein by Kjeldahl method, using N x 6.25 as conversion factor.

8.5.8 Determine the ash content by incinerating the residue from the other duplicate in a muffle furnace at 525°C for 5 h. Switch off furnace and allow cooling to 180°C. Transfer crucible in a desiccator, cool for 1 h and weigh.

9. CALCULATIONS

Blank = blank residue – blank protein residue – blank ash residue

Total dietary fiber (g TDF per 100g)

$$= \frac{\text{(sample residue – sample protein residue – sample ash residue – blank) x 100}}{\text{weight of sample}}$$

Report test results (in g per 100 g sample) to one decimal place.

10. ACCEPTANCE OF RESULTS:

Accept test results if the following condition is satisfied.

10.1 Duplicate results should not differ by more than 10% of the mean.

10.2 Mean concentration of duplicate results of quality control sample or certified reference material should be within ± 2 SD in the control chart based on established acceptance criteria.

11. APPENDIX/SUPPLEMENTARY NOTES

11.1 USFDA and the US Department of Agriculture's (USDA) have recognised the AOAC methods 985.29 and 991.43 as suitable methodologies for the analysis of dietary fibre for nutrition labelling purposes.

11.2 The phosphate buffer used in the AOAC 985.29 is 0.08 M, pH 6.0. Preparation: dissolve 1.4 g Na phosphate dibasic, anhydrous (Na_2HPO_4) or 1.753 g dihydrate and 9.68 g Na phosphate monobasic monohydrate (NaH_2PO_4) or 10.94 g dihydrate in 700 mL water. Dilute to 1 L with water. Check pH with pH meter.

11.3 The MES -TRIS buffer-based method has advantages over the phosphate-based method, because of the following:

11.3.1 The pH of MES-TRIS changes with temperature because the pK_a (the pH for optimum buffering capacity for any buffering system) of organic buffers decreases at higher temperatures. Therefore MES-TRIS which has been adjusted to pH 8.2 at 24°C shifts to pH 6.9-7.2 at 85-90°C which is the optimum pH for starch digestion by the heat-stable α-amylase. The pH of this buffer also shifts to 7.4-7.6 at 60°C, thereby eliminating the need to adjust the pH for the protease digestion.

11.3.2 MES-TRIS is less sensitive than phosphate buffer to differences in acidity or alkalinity of sample matrices because of the high buffering capacity of the system. The enzymatic digestion is carried out at the pH closed to the pK_a of the buffer system (pK_a = 8.2 at 24°C - the pK_a being the pH point at which optimum buffering occurs in a system).

11.3.3 Less 95% ethanol is used to precipitate the soluble dietary fibre. This reduces the total filtration volumes.

11.4 Although the substitution of organic buffers for phosphate buffers results in faster filtration times with less handling for most samples, foods containing highly viscous fibres such as psyllium are still difficult to filter by either method. Reducing the sample size and using sonication followed by high speed centrifugation before filtration step are suggested to reduce the filtration time for highly viscous fibre samples.

11.5 In-house food reference materials:

Food samples with different level of dietary fibre should be used as in-house quality control sample. Examples are oats, defatted soybean flour, breakfast cereal, and brown rice.

Weigh accurately in duplicate 1 g defatted control sample into 400 mL (or 600 mL) tall-form beakers and analyse simultaneously with the sample.

11.6 Standards for checking enzyme activity are given in the following table:

Standards for testing enzyme activity

Standard	Activity Tested	Weight of Standard, g	Expected Recovery, (%)
Citrus pectin	Pectinase	0.1-0.2	95-100
Arabinogalactan	Hemicellulase	0.1-0.2	95-100
β-Glucan	β-Glucanase	0.1-0.2	95-100
Wheat starch	α-Amylase+AMG	1.0	0-1
Corn starch	α-Amylase+AMG	1.0	0-1
Casein	Protease	0.3	0-1

DETERMINATION OF STARCH BY ACID HYDROLYSIS METHOD

1. PURPOSE/SCOPE

The method is used for the quantitative determination of starch in foods.

2. SAFETY

2.1 Understand the toxicity and safety of the reagents used before starting the method.

2.2 Use safety devices such as tong or gloves in handling hot containers.

3. REFERENCE

3.1 Horwitz W (2000) (editor). Starch in baking powders, 25.1.11, Official Method of Analysis of AOAC International. 17th Edition. AOAC International, Maryland, USA.

3.2 Greenfield H and Southgate DAT (1992). Food Composition Data: Production, Management and Use. Elsevier Applied Science, UK.

3.3 Kirk RS and Sawyer R (1991). Pearson's Composition and Chemical Analysis of Foods, 9th Edition. Longman Scientific & Technical, Essex, England.

4. DEFINITION

Starch constitutes the major component of the polysaccharides in most foods and consists mainly of amylose and amylopectin.

5. PRINCIPLE

The test sample is dispersed in water and heated in acidic solution to hydrolyse the starch, releasing the sugars. The resulting sugars are then determined by titration with Fehling solution according to the method of Lane-Eynon (see Determination of Total Sugars by the Lane-Eynon Method).

6. REAGENTS

6.1 Hydrochloric acid (HCl), 35%

6.2 Sodium hydroxide pellet (NaOH)

6.3 50% Sodium hydroxide solution: dissolve 50 g of NaOH in 100 mL of distilled water.

6.4 For other reagents, refer to the list for sugar determination by the Lane-Eynon method

7. APPARATUS

7.1 Round bottom flask 250 mL

7.2 Reflux condenser

7.3 Glass beads

7.4 Volumetric flask 250 and 500 mL

7.5 Filter paper No. 541

7.6 Litmus paper

7.7 Hotplate

7.8 For other apparatus, refer to the list for sugar determination by the Lane-Eynon method

8. PROCEDURE

8.1 Preparation of sample: grind or blend sample until homogenous. If sample cannot be analysed on the same day, keep in screw-cap bottle in a freezer.

8.2 Sample gelatinisation and hydrolysis

8.2.1 Test sample is mixed thoroughly and about 3 g is weighed (with an accuracy of 0.01 g) into a 250 mL round bottom flask. 200 mL of distilled water is added, followed by 20 mL of concentrated HCl and 3-4 glass beads.

8.2.2. Then the round bottom flask is equipped with a reflux condenser. The heat is applied until boiled and clear solution is obtained. Cool to room temperature.

8.2.3. The test solution is neutralised by adding 50% NaOH (checked by litmus paper) and the test solution is filtered through Whatman No 541 into a 250 mL volumetric flask. Dilute to the mark with distilled water.

8.3 For the analysis of sugars using Fehling solution, follow the method of Lane-Eynon.

9. CALCULATION

Calculate the starch content as follow:

Starch, g per 100 g = % Total sugar x 0.9

Total sugar is obtained from the method of Lane-Eynon.

Report test results (in g per 100 g sample) to one decimal place.

10. ACCEPTANCE OF RESULTS

Accept test results if the following condition is satisfied.

10.1 Duplicate results should not differ by more than 10% of the mean.

10.2 Mean concentration of duplicate results of quality control sample or certified reference material should be within $\pm$ 2 SD in the control chart based on established acceptance criteria.

11. APPENDIX/SUPPLEMENTARY NOTES

11.1 Methods based on acid hydrolysis also measure non-starch polysaccharide (NSP), limiting their use to situations in which a low degree of accuracy is permissible or the amount of NSP are known to be small.

11.2 The preferred methods for the analysis of starch are based on the use of enzymatic hydrolysis coupled with a specific glucose assay of the hydrolysate.

11.3 The AOAC (reference 3.1) also contains enzymatic hydrolysis methods for the analysis of starch in a variety of other foods, such as cereal and cereal products (32.2.05 and 32.2.05A) and in condensed or dry milk products (4.7.03).

DETERMINATION OF STARCH BY SPECTROPHOTOMETER METHOD

1. PURPOSE/SCOPE

The method is used for the quantitative determination of starch in foods.

2. SAFETY

2.1 Understand the toxicity and safety of the reagents used before starting the method.

2.2 Use safety devices such as tong or gloves in handling hot containers.

3. REFERENCE

3.3 Horwitz W (2000) (editor). Starch in Baking powders, 25.1.11, Official Method of Analysis of AOAC International. 17th Edition. AOAC International, Maryland, USA.

3.4 Greenfield H and Southgate DAT (1992). Food Composition Data: Production, Management and Use. Elsevier Applied Science, UK.

3.5 Li BW. (1996) Determination of Sugars, Starches, and Total Dietary Fiber in Selected High-Consumption Foods. J AOAC Inter. 79: 718

3.6 Helrich K. 1990. Official Methods of Analysis of the Association of Official Analytical Chemists. 15th edi. vol. 2, No. 985.29 Association of Official Analytical Chemists, Inc. Virginia pp. 1105-6.

4. DEFINITION

Starch constitutes the major component of the polysaccharides in most foods consists mainly of amylose and amylopectin.

5. PRINCIPLE

The test sample is dispersed in water and hydrolyses with enzyme to release the sugars. The resulting sugars are then determined by titration with Fehling solution according to the method of Lane-Eynon (see Determination of Total Sugars by the Lane-Eynon Method).

6. REAGENTS

6.1 Calcium carbonate, AR

6.2 Ethanol, 85% (v/v), in distilled water, AR

6.3 Acetone

6.4 0.08 M Sodium phosphate buffer pH 6.0 : Dissolve 1.400 g Na_2HPO_4 and 9.68 g NaH_2PO_4 in 700 ml H_2O, dilute to 1 L. Check pH.

6.5 0.325 M HCl

6.6 0.275 M NaOH

6.7 95 % Ethanol

6.8 12 M H_2SO_4

6.9 Termamyl 120 L or α-amylase

6.10 Protease

6.11 Amyloglucosidase

6.12 1 mM calcium carbonate in 0.5 M NaOH

6.13 4 % PAHBAH (p-Hydroxybenzoic acid hydroxide) in 0.5 M HCl
6.14 Working reagent: mix 4 volumes of reagent 1 with 1 volume of reagent 2, prepare fresh before use
6.15 Standard : 20, 40, 60, 80, 100 μg/mL glucose in 0.25 M H_2SO_4

7. APPARATUS

7.1 Erlenmeyer flask.
7.2 Analytical balance
7.3 Shaking water bath maintain at 60°C
7.4 Flat-bottom flask.
7.5 Centrifuge
7.6 Spectrophotometer

8. PROCEDURE

8.1 Preparation of sample
Grind or blend sample until homogenous. If sample cannot be analysed on the same day, keep in screw-cap bottle in a freezer.

8.2 Starch extraction.
8.2.1 Weigh accurately 1-5 g (***W_1***) finely ground sample into 125 mL Erlenmeyer flask.
8.2.2 Add 1-3 g $CaCO_3$ to neutralise sample. Add 25 mL of 85% ethanol to sample, cap flask with aluminum foil, place on shaking water bath at 60°C for 1 h.
8.2.3 Remove sample from the shaking water bath and immediately filter the solution through a pre-weighed filter paper into a 250 mL flat-bottom flask.
8.2.4 Repeat the extraction with 25 mL boiling 85% ethanol three times.
8.2.5 Dry the residue on filter paper with 2 X 25 mL acetone and put in the oven 105°C for 10 min.
8.2.6 Cool in desiccator and weigh (***W_2***).

8.3 Hydrolysis of Starch using enzyme.
8.3.1 Weigh 0.13 g sample from the previous step (***W_3***), add 6.5 mL sodium phosphate buffer, suspend thoroughly.
8.3.2 Add 15 μL Termamyl, incubate in a boiling water bath for 15 min (with stirring).
8.3.3 Cool, add 1.3 ml NaOH, mix.
8.3.4 Add 15 μL protease
8.3.5 Incubate in 60° C for 30 min.
8.3.6 Add 1.3 mL HCl, mix.
8.3.7 Add 40 μL amyloglucosidase, incubate in 60°C for 30 min.
8.3.8 Add 37 mL 60°C 95 % Ethanol, place in ice bath for 30 min, centrifuge 3000 rpm, 15 min.
8.3.9 Remove supernatant for analysis of starch by sugar determination.

8.4 Sugar determination
- 8.4.1 Dilute sample 1:20 with distilled water.
- 8.4.2 Mix 0.15 mL sample with 2.5 mL PAHBAH solution.
- 8.4.3 Boil 5 min, cool, read at 410 nm spectrophotometer.
- 8.4.4 Determine sugar content against glucose standards

9. CALCULATIONS

Starch (g / 100 g) = %Total sugars x 0.9 x W_2 / W_3 x $1 / W_1$

where W_1 = weight of sample
W_2 = weight of starch extracted
W_3 = weight of starch taken for hydrolysis

10. ACCEPTANCE OF RESULTS

Accept test results if the following condition is satisfied.

10.1 Duplicate results should not differ by more than 10% of the mean.

10.2 Mean concentration of duplicate results of quality control sample or certified reference material should be within $\pm$ 2 SD in the control chart based on established acceptance criteria.

11. APPENDIX/SUPPLEMENTARY NOTES

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DETERMINATION OF INDIVIDUAL SUGARS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

1. PURPOSE/SCOPE

The method is used for the quantitative determination of individual sugars in beverages (malt beer, soft drink, lemonade, table wine, orange juice), honey, marmalade, foods and foods products.

2. SAFETY

2.1 Understand the toxicity and safety of the solvents used before starting the method.

2.2 Acetonitrile is carcinogenic; wear gloves to reduce contact with skin.

3. REFERENCES

3.1. AOAC International (1993). Methods of Analysis for Nutrition Labelling. Chapter 33. Sugars (Mono & Di), Glucose, Fructose, Sucrose and Maltose in Presweetened Cereals Liquid Chromatographic Method (982.14); Sugars (Mono & Di), Separation of Sugars in Honey Liquid Chromatographic Method (977.20).

3.3. Will RBH and Greenfield H (1984). Laboratory instruction manual for food composition studies. Department of Food Science and Technology, The University of New South Wales, p 59.

3.4. Waters (1987). Choosing the Right Column Chemistry for carbohydrate Analysis, Notes Food & Beverage, Waters Chromatography Division Millipore Corporation, 2:4-6.

4. DEFINITION

Sugars determined by this method consist of mono-, di- and trisaccharides.

5. PRINCIPLE

5.1. Beverages and honey are appropriately diluted with water, filtered if required, then chromatographed on HPLC column to separate the individual sugars.

5.2. Foods and food products are extracted with aqueous ethanol. After evaporation of the ethanol, the sugars left in aqueous solution are separated by HPLC.

5.2.1. Samples with high fat content should be defatted using petroleum ether before extraction step.

5.2.2. For samples with high protein content, the protein should be precipitated by potassium hexacyanoferrate and zinc sulfate before extraction step.

6. REAGENTS

6.1. Calcium carbonate, AR

6.2. Ethanol, 85% (v/v), in distilled water, AR

6.3. Standard sugars: glucose, fructose, sucrose, maltose and lactose

6.4. Distilled water, ultra pure
6.5. Ethylenediaminetetraacetic acid (EDTA), Disodium-calcium salt.
6.6. Petroleum ether, AR
6.7. Acetonitrile, HPLC grade
6.8. Ethanol, HPLC grade
6.9. Potassium hexacyanoferrate, 15 % (v/v) in distilled water
6.10. Zinc sulfate, 30 % (v/v) in distilled water

7. APPARATUS

7.1. High pressure liquid chromatograph system
7.2. Hypersil ® (APS_2) NH_2, 5 μm (Thermo Hypersil-Keystone) with guard column, 250 x 4.6 (id) mm or equivalent
7.3. Analytical balance
7.4. Whatman™ filter paper No. 41 and 42
7.5. Funnels
7.6. Flat-bottom flask, 250 mL
7.7. Ultrafiltering equipment with membrane filter 0.45 μm, diameter 47 mm
7.8. Sample Clarification Kit with cellulose acetate membrane filter 0.45 μm, diameter 13 mm
7.9. Rotary evaporator
7.10. Sample vials
7.11. Erlenmeyer flask, 125 mL
7.12. Volumetric flask: 25, 50, 100, 500 and 1000 mL
7.13. Shaking water bath
7.14. Beaker, 50 mL
7.15. Pipette, 2, 5, 10, 20, 25 and 50 mL
7.16. Spatula

8. PROCEDURE

8.1. Sample preparation procedures prior to injection into the HPLC
8.1.1 Beverages such as orange juice, malt beer, soft drink, lemonade, table wine are filtered through 0.45 μm membrane.
8.1.2 Honey is dissolved in hot water, approximately 1 g in 10 mL water, and filtered through 0.45 μm membrane.
8.1.3 Marmalade is dissolved in hot water & insoluble parts were centrifuged off. The clear portion is filtered through 0.45 μm membrane.
8.1.4 Foods and food products
8.1.4.1 Samples containing high fat.
Weigh accurately 1-5 g finely ground sample into 50 mL centrifuge tube. Add 30 mL petroleum ether, vortex and discard petroleum ether. Repeat extraction 3 times. Dry sample in oven 60°C for 1 h to remove any residual petroleum ether. Transfer defatted sample into 125 mL Erlenmeyer flask. Add 1-3 g $CaCO_3$ to neutralise

sample. Add 25 mL 85% ethanol to sample, cap flask with aluminum foil, place on shaking water bath at 60°C for 1 h. Remove sample from the shaking water bath and immediately filter the solution through a filter paper into a 250 mL flat-bottom flask. Repeat the extraction with 25 mL boiling 85% ethanol for three times. Evaporate the ethanol on rotary evaporator at 45°C until the remaining aqueous solution is approximately 3 mL. The aqueous solution is transferred by using a pipette to a 10 mL volumetric flask, made up to volume with distilled water. The solution is filtered through an ultrafilter (0.45 μm). Keep samples in a sample vial and inject into HPLC.

8.1.4.2 Samples containing high protein.

Weigh accurately 1-5 g finely ground sample into 25 mL volumetric flask. For solid sample, add 5 mL distilled water to disperse the sample. Pipette 1.25 mL 15% potassium hexacyanoferrate and 1.25 mL 30% zinc sulfate. Mix well and stand for 15 min. Add 5 mL acetonitrile and make to volume with distilled water. Mix well and stand overnight. The solution is filtered through an ultrafilter (0.45 μm). Keep samples in a sample vial and inject into HPLC.

8.1.4.3. Samples containing high fat and high protein.

Weigh accurately 1-5 g finely ground sample into 50 mL centrifuge tube. Add 30 mL petroleum ether, vortex and discard petroleum ether. Repeat extraction 3 times. Dry sample in oven 60°C for 1 h to remove any residual petroleum ether. Transfer defatted sample into 25 mL volumetric flask. For solid sample, add 5 mL distilled water to disperse the sample. Pipette 1.25 mL 15% potassium hexacyanoferrate and 1.25 mL 30% zinc sulfate. Mix well and stand for 15 min. Add 5 mL acetonitrile and make to volume with distilled water. Mix well and stand overnight. The solution is filtered through an ultrafilter (0.45 μm). Keep samples in a sample vial and inject into HPLC.

8.1.4.4 Samples containing low fat and low protein.

Weigh accurately 1-5 g finely ground sample into 125 mL Erlenmeyer flask. Add 1-3 g $CaCO_3$ to neutralise sample. Add 25 mL 85% ethanol to sample, cap flask with aluminum foil, place on shaking water bath at 60°C for 1 h. Remove sample from the shaking water bath and immediately filter the solution through a filter paper into a 250 mL flat-bottom flask. Repeat the extraction

with 25 mL boiling 85% ethanol three times. Evaporate the ethanol on rotary evaporator at 45°C until the remaining aqueous solution is approximately 3 mL. The aqueous solution is transferred by using a pipette to a 10 mL volumetric flask, made up to volume with distilled water. The solution is filtered through an ultrafilter (0.45 μm). Keep samples in a sample vial and inject into HPLC.

8.1.4.5 Samples containing high salt (e.g. fish sauce and seasonings).
Weigh accurately 1-5 g homogenised sample into 25 mL volumetric flask. Make up to volume with 85% ethanol. Mix well and filter through an ultrafilter (0.45 μm). Keep samples in a sample vial and inject into HPLC.

8.2 Standard sugar preparation
- 8.2.1 Prepare 2% of each individual sugar for determination of retention time
- 8.2.2 Prepare 2% solution of sugar mixture for quantitative determination

8.3 HPLC equipment and condition:

Column:	Hypersil ® (APS_2) NH_2, 5 μm (Thermo Hypersil-Keystone) with guard column, 250 x 4.6 (id) mm or equivalent
Mobile phase:	Acetonitrile : distilled water : ethanol = 82 : 17.5 : 0.5
Detector:	RI detector
Conditions:	Flow rate 1.5 mL/min Injector volume 20 μL Column temperature 25-30 °C RI detector temperature 25-30°C

8.4. Determination
- 8.4.1. Inject sugar standards and sample solution (10 –20 μL), into column with appropriate conditions as indicate above.
- 8.4.2. Measure areas or peak heights of each sugar peak in sample and standard.

9. CALCULATION

$$\text{Amount of each sugar (g/100g)} = \frac{A_{SPL} \times C_{STD}}{A_{std}} \times \frac{V}{W}$$

where: A_{sp} = area/peak height of each sugar in sample solution
A_{std} = area/peak height of sugar standard
C_{std} = concentration of sugar standard (g/100 mL)
V = total volume of prepared sample solution (mL)
W = weight of sample (g)

Results are reported to the nearest 0.1 g/100 g

10. ACCEPTANCE OF RESULTS

Accept test results if the following condition is satisfied.

10.1 Duplicate results should not differ more than 10% of the mean.

10.2 Mean concentration of duplicate results of quality control sample or certified reference material should be within ± 2 SD in the control chart based on established acceptance criteria.

11. APPENDIX/SUPPLEMENTARY NOTES

Alternative HPLC equipment and conditions:

11.1. Chromatographic condition I:

Column: Sugar-Pak (cation exchanger) or SC-1011 with guard column, 300 x 6.5 (id) mm or equivalent
Mobile phase: Distilled water containing Ca-EDTA 50 mg/l
Detector: RI detector
Conditions: Flow rate 0.5 mL/min
Injection volume 10 μL
Column temperature 80-90 °C
RI detector temperature 40 °C

11.2. Chromatographic condition II:

11.2.1 Column: Radial-Pak Silica cartridge (10 cm x 8 mm ID) in WATERS RCM-100 radial compression module.

11.2.2 Preparation of conditioning reagent and modification of column

11.2.2.1 Mix 5 vials of WATERS™ SAM reagent 1 with 15 mL H_2O.

11.2.2.2 Add 385 mL acetonitrile, mix well.

11.2.2.3 Flow 5-10 mL of the above conditioning reagent through the capillary pipe of HPLC equipment.

11.2.3 Install the new silica pak column and pump all of the conditioning reagent through the column with the flow rate of 3 mL/min. Column is ready for use.

11.2.4 Mobile phase is prepared as follows:

11.2.4.1 Mix 1 vial of WATERS™ SAM reagent 1 with 210 mL H_2O

11.2.4.2 Add 770 mL acetonitrile, mix well.

11.2.4.3 Filter the solution through membrane filter FHUP 04700

11.2.4.4 Shake and digest the solution to eliminate the bubble from the mobile phase.

11.2.4.5 Pass the mobile phase through the modified column prepared in point 11.2.2 and 11.2.3 with the flow rate of 3 mL/min.

11.2.4.6 Discard the first 100 mL of the eluate

11.2.4.7 Collect the rest of the eluate for use as a recycled eluant.

11.2.5 Detector: RI detector, at room temperature (25 -30^{o}C)

11.2.6 Conditions: Flow rate 3 mL/min
Injection volume 20 μL
Column temperature 25-30 ^{o}C
RI detector temperature 25-30^{o}C

11.3. Chromatographic condition III:

Column: NH_2 Rad-Pak used with RCM 8 x 10 cartridge holder.

Mobile phase: Add ½ bottle of PIC A/liter instead of SAM 1 (this gives a conc. of 2.5 mM PIC A)

Note: This condition is used for solutions with high salt (>150 mg NaCl/100 g of food)

DETERMINATION OF TOTAL SUGAR BY VOLUMETRIC METHOD (THE LANE–EYNON METHOD)

1. PURPOSE/SCOPE

The method is used for the quantitative determination of total sugar in sugar and sugar products. Results are expressed as percentage of sugar in 100 g of sample.

2. SAFETY

2.1 Understand the toxicity and safety of the reagents used before starting the method.

2.2 Use safety devices such as tong or gloves in handling hot containers.

2.3 Always use eye protection during the analysis especially in the titration.

3. REFERENCES

3.1 Horwitz W (2000) (editor). Official Method of Analysis of AOAC International. 17th Edition. AOAC International, Maryland, USA.

3.2 Greenfield H and Southgate DAT (1992). Food Composition Data: Production, Management and Use. Elsevier Applied Science, UK.

3.7 Kirk RS and Sawyer R (1991). Pearson's Composition and Chemical Analysis of Foods, 9th Edition. Longman Scientific & Technical, Essex, England.

4. DEFINITION

Total sugar in this method refers to all sugar that undergoes sugar inversion with hydrochloric acid

5. PRINCIPLE

The method involves the inversion of sugars present in food samples with hydrochloric acid. The sugar present in a specified volume of the hydrolysed solution is used to reduce copper in the Fehling's solution previously standardised with working standard invert sugar solution. Excess copper is back titrated with the standard sugar solution. The difference in the volume of standard sugar used for the standardisation and for back titration is a measure of total sugar content of the sample.

6. REAGENTS

6.1 Hydrochloric acid, AR.

6.2 Sucrose, AR.

6.3 Copper sulfate solution: dissolve 34.639 g of copper sulfate $Cu_2SO_45H_2O$, AR, in water, dilute to 500 mL and filter through glasswool or filter paper.

6.4 Alkaline tartrate solution: dissolve 173 g potassium sodium tartrate $4H_2O$, AR and 50 g NaOH in H_2O, dilute to 500 mL, let stand for 2 days and filter through fiberglass.

6.5 Fehling's solution: prepare by mixing equal volume of reagents in 6.3 and 6.4.
6.6 Sodium hydroxide, AR: prepare a 20% solution
6.7 Sugar standard solution
6.7.1 Stock solution (10 mg/mL)
6.7.1.1 Dry sucrose in an air oven at 100°C for 1 h.
6.7.1.2 Weigh 5 g dried sucrose. Add 2.5 mL conc. HCl and dilute with water to approximately 100 mL.
6.7.1.3 Store for 3 days at room temperature, then dilute to 500 mL.
6.7.2 Working standard solution (2.5 mg/mL)
6.7.2.1 Pipette 125 mL stock solution into 500 mL volumetric flask.
6.7.2.2 Add few drops phenolphthalein, and neutralise with 20% NaOH.
6.7.2.3 Dilute to volume and mix well. Prepare fresh daily.

Note: Acidified 1% invert sugar solution is stable for several months.

7. APPARATUS:

7.1 Electric heater-stirrer, with white top and continuous temperature control
7.2 Burette, 50 mL graduated in 0.1 mL
7.3 Analytical balance, 200 g capacity, with 0.1 mg sensitivity
7.4 Fume hood
7.5 Shaking water bath, maintained at 60±5°C.
7.6 Drying oven, maintained at 100°C.
7.7 Desiccator with desiccant such as silica gel. Ensure that the desiccant is activated prior to use by heating in an oven at 100°C until blue.
7.8 Tongs
7.9 Wash bottle
7.10 Graduated cylinder, 100 mL
7.11 Erlenmeyer flasks, 250 mL
7.12 Beaker, 100 mL and 2 L
7.13 Glass pipettes, 50 mL, 25 mL, 5 mL, 10 mL
7.14 Volumetric flasks, 100 mL, 500 mL, 1000 mL
7.15 Spatula

8. PROCEDURE

8.1 Preparation of sample
Thaw the sample to room temperature.
8.1.1 Liquid products.
Shake the sample container well. Take care to incorporate in the sample any fat or other constituent adhering to the wall of the container. If the sample contains lumps or pieces of ingredients homogenize in an appropriate blender and mix all samples thoroughly until the sample is homogenous.

8.2.2 Viscous or pasty products. Mix the sample with a spatula. If the product still contains lumps or solid particles, homogenize in a blender and stir well until the sample is homogenous.

8.2.3 Dried product
Grind to a fine powder in a blender and mix well.

8.2 Preparation of analytical sample

8.2.1 For liquid or viscous/pasty products
Weigh sample and transfer to a 250 mL volumetric flask and dilute to volume with water, mix well and filter.

8.2.2 For dried products

8.2.2.1 Weigh, in duplicate, test sample containing approximately 30% total sugar in a 250 mL Erlenmeyer flask.

8.2.2.2 Add 150 mL water preheated to 60°C $\pm$ 5°.

8.2.2.3 Mechanically shake flask for 30 minutes in a water bath maintained at 60°C $\pm$ 5°.

8.2.2.4 Let stand in water bath for another 30 minutes and cool to room temperature.

8.2.2.5 Transfer to a 250 mL volumetric flask, dilute to volume, mix well and filter or centrifuge then filter.

Note: Discard the first 25 mL filtrate. Cover funnel during filtration to avoid evaporation

8.3 Recovery test
Weigh sample (refer to 8.2.1 or 8.2.2) and add approximately 1 g sucrose previously dried in an oven at 100°C for 1 h. Proceed as in step 8.2.1 or 8.2.2

8.4 Sugar inversion with acid at room temperature

8.4.1 Pipette 50 mL filtrate into 100 mL volumetric flask.

8.4.2 Add 2.5 mL conc. HCl. Let stand overnight

8.4.3 Add few drops of phenolphthalein and neutralise with 20% NaOH solution.

8.4.4 Add a few drops of dilute HCl (0.5 N HCl) until the red colour disappears.

8.4.5 Dilute to volume with water and mix well.

8.5 Standardisation of Fehling's solution

8.5.1 Fill burette with working standard solution.

8.5.2 Accurately pipette 5 mL of each reagent in 6.3 and 6.4 into 250 mL Erlenmeyer flask.

8.5.3 Mix, and add 30 mL water.

8.5.4 Add approximately 19 mL working standard solution from a burette.

8.5.5 Put into the solution a magnetic stirring bar and place on electric heater-stirrer.

8.5.6 Regulate heat so that boiling will begin in approximately 3 minutes.

8.5.7 Maintain moderate boiling for 2 minutes, reducing heat if necessary to prevent bumping.

8.5.8 Without removing flask from heater, add 4 drops 1% methylene blue.

8.5.9 Stir moderately and complete titration within 3 minutes by dropwise addition of working standard solution at intervals of 10 seconds until mixture resumes bright orange appearance.

8.5.10 Repeat titration 3 times.

Note: Maintain continuous evolution of steam while titrating to prevent re-oxidation of copper by air.

8.6 Approximate titration of sample

8.6.1 Accurately pipette 5 mL each of reagent 6.3 and 6.4 into 250 mL Erlenmeyer flask.

8.6.2 Pipette an aliquot of hydrolysed sample solution into the Erlenmeyer flask and dilute with distilled water to about 50 mL.

8.6.3 Pre-heat electric heater-stirrer so that boiling of solution can start in approximately 3 minutes.

8.6.4 Mix solution, put a magnetic bar into the solution and place on the pre-heated heater.

8.6.5 After boiling for 10 to 15 seconds, observe colour of the solution. If blue colour persists, add working sugar standard solution, 0.5 to 1 mL at a time, with few seconds interval, until disappearance of blue colour.

8.6.6 Add 3 drops of methylene blue indicator and continue adding standard sugar solution at intervals of 10 seconds until the indicator is completely decolorised.

Note: the titration should be completed within 3 minutes. Volume used for titration should be within 15 – 50 mL.

8.7 Titration of sample

8.7.1 Follow steps given in 8.6.1-8.6.4.

8.7.2 While rapidly boiling, add working sugar standard from burette. The volume used is 0.5 to 1 mL less than that is used in the approximate titration carried out in 8.6.

8.7.3 Continue as in step 8.6.6.

9. CALCULATION

9.1 Total sugar (g per 100 g) = (F-M) x I x 250 x 100 x100 / (W x A x 50)

where: F = volume of standard sugar solution required to reduce 10 mL mixed Fehling's solution

M = volume of standard sugar solution used in back titration of the sample

I = gram sugar per mL working standard solution.

W = weight of sample

9.2 % Recovery = $\frac{(\%\text{sugar in spiked sample} - \%\text{sugar in sample}) \times 100}{(\text{amount of sugar added/weight of sample} \times 100)}$

10. ACCEPTANCE OF RESULTS

Accept test results if one or more of the following condition is satisfied.

10.1 Duplicate results should not differ by more than 10% of the mean.

10.2 Recovery test is between 80 to 110%

10.3 Mean concentration of duplicate results of quality control sample or certified reference material should be within $\pm$ 2 SD in the control chart based on established acceptance criteria.

DETERMINATION OF ASH BY GRAVIMETRIC METHOD

1. PURPOSE/SCOPE

The method is used for the quantitative determination of ash in raw, cooked and processed foods.

2. SAFETY

2.1 Always use tongs and gloves in handling crucibles.
2.2 Wear face/eye protection when opening hot furnace.

3. REFERENCES

3.1 Horwitz W (2000) (editor). Official Method of Analysis of AOAC International. 17th Edition. AOAC International, Maryland, USA.
3.2 Greenfield H and Southgate DAT (1992). Food Composition Data: Production, Management and Use. Elsevier Applied Science, UK.
3.3 Kirk RS and Sawyer R (1991). Pearson's Composition and Chemical Analysis of Foods, 9th Edition. Longman Scientific & Technical, Essex, England.

4 DEFINITION

Ash content refers to the total mineral residue left after incineration of organic matter. It has no nutritional significance per se, but the value for ash is a useful check in summing up the proximate composition of food and a measure of its mineral content. It is expressed as g ash per 100 g sample.

5. PRINCIPLE

The method involves oxidation of all organic matter by incineration in a furnace at a specified temperature (<550°C). Ashing above 650°C will volatilise inorganic salts like alkali chloride and a portion of ash will fuse and enclose some carbon, preventing them from being ignited. The residue left after incineration is the ash content of the sample.

6. MATERIALS

4 N Nitric acid or dilute HCl (1:2.5)

7. APPARATUS

7.1 Furnace, capable of being controlled at 100° to 600°C
7.2 Air oven
7.3 Hotplate or Bunsen burner or electric coil
7.4 Analytical balance, 200 g capacity with 0.1 mg sensitivity
7.5 Desiccator with desiccant such as silica gel. Ensure that the desiccant is activated prior to use by heating in an oven at 100°C until blue.
7.6 Boiling water bath with removable rings to hold the containers.
7.7 Tongs
7.8 Porcelain crucible or silica dish

7.9 Spatula

8. PROCEDURE

8.1 Heat marked crucible in a furnace at 500 - 550ºC for 2 - 3 h.

8.2 Lower the furnace temperature to 180ºC and transfer the crucibles into a desiccator, cool for 30 min and weigh (***W*****$_1$**).

8.3 Weigh sample in duplicate into the pre-weighed crucible dish (***W*****$_2$**), 2 – 4 g for dry samples and 10 g for wet samples.

8.4 For dry samples, char over a hotplate, initially at low temperature to avoid spattering. Increase the temperature gradually until smoking ceases.

8.5 For wet or liquid samples, pre-dry over a boiling water bath until samples are dry. Char the sample as in 8.4.

8.6 Incinerate the charred samples in a furnace at 500-550ºC until the residue is uniformly white or nearly white.

8.7 If sample is not completely white, moist ash with a few drops of water or diluted acid. Evaporate on water bath and repeat heating in the muffle furnace for 30 – 60 min until constant weight is obtained.

NOTE: If ash samples are to be used for mineral analysis, temperature of the furnace should not exceed 450℃. Too high a temperature may cause the volatilisation of certain elements particularly Fe, K, Na, S, Cl & P. It may also cause the mineral matter to fuse and melt.

8.8 Decrease the temperature of the furnace to 180ºC and transfer the crucibles into a desiccator, cool for 30 min and weigh (***W*****$_3$**).

9. CALCULATIONS

$$\text{Ash, g per 100 g} = \frac{(W_3 - W_1)\ x\ 100}{(W_2 - W_1)}$$

where: W_1 = weight of crucible
W_2 = weight of crucible + sample
W_3 = weight of crucible + ash

Report test results (in g per 100 g sample) to one decimal place.

10. ACCEPTANCE OF RESULTS

Duplicate results should not differ by more than 5% of the mean.

DETERMINATION OF MINERALS: SAMPLE PREPARATION FOR MINERAL ANALYSIS

1. PURPOSE/SCOPE

The method includes dry ashing and/or wet digestion (closed or open system), in the destruction of organic matters in food sample before mineral determination.

2. SAFETY/PRECAUTION

2.1 To avoid contamination, crucible with smooth surface or platinum dish must be used in dry ashing.

2.2 Ashing temperature should be between 450-525°C. Temperature exceeding 525°C can cause loss of trace elements particularly Cu and Zn due to volatilization.

2.3 Extreme caution is required in using acids, especially perchloric acid. Avoid heating test solutions to dryness as this may lead to explosion.

2.4 Wear gloves and safety glasses and carry out all acid digestion in a fume hood.

2.5 All activities should be done in a clean, dust free room to avoid contamination.

3. REFERENCES

3.1 Horwitz W (2000) (editor). Official Method of Analysis of AOAC International. 17th Edition. AOAC International, Maryland, USA. (Methods 9.1.09 and 50.1.14)

3.2 Greenfield H and Southgate DAT (1992). Food Composition Data: Production, Management and Use. Elsevier Applied Science, UK.

3.3 Kirk RS and Sawyer R (1991). Pearson's Composition and Chemical Analysis of Foods, 9th Edition. Longman Scientific & Technical, Essex, England.

4. DEFINITION

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5. PRINCIPLE

The method involves the separation of minerals from the food matrix by destruction of the organic matter of the sample through dry ashing or wet digestion. The mineral content in diluted acid is then determined either by atomic absorption spectrophotometer, AAS (Ca, Cu, Fe, K, Mg, Na, Zn), or gravimetric method (P) or inductively coupled plasma, ICP (all minerals).

6. REAGENTS

NOTE: Water used in this analysis is deionised (DI) water.

6.1 Nitric acid, 65%

Nitric acid, 1N: Add 139.5 mL 65% HNO_3 to approximately 1.5L DI water. Shake, allow to cool and dilute to 2 L.

6.2 Sulfuric acid, 98%
6.3 Hydrochloric acid, 37%
6.4 Perchloric acid, 70%

7. APPARATUS

NOTE: All glassware must be soaked overnight in 20% HNO_3 (v/v), cleaned and rinsed with deionised water before use.

7.1 For dry ashing

7.1.1 Muffle furnace with temperature range of 250-600^{0}C
7.1.2 Measuring pipet, 10 ml
7.1.3 Volumetric pipet, 1, 2, 3, 4, 5, 10 ml
7.1.4 Volumetric flask, 50, 100 mL
7.1.5 Analytical balance, 200 g and 0.1 mg sensitivity.
7.1.6 Porcelain crucible, 50 mL, capable of withstanding temperatures to 600^{0}C, or platinum crucible or equivalent
7.1.7 Hot plate
7.1.8 Filter paper, Whatman No. 541.
7.1.9 Water bath with removable rings to hold crucibles
7.1.10 Crucible tong, stainless steel

7.2 For wet digestion

7.2.1 Kjeldahl flask, 300 mL or beaker, 250 mL
7.2.2 Measuring pipets, 10 ml
7.2.3 Volumetric pipets, 1, 2, 3, 4, 5, 10 mL
7.2.4 Volumetric flask, 50, 100 mL
7.2.5 Analytical balance, 200 g and 0.1 mg sensitivity
7.2.6 Heating mantle or hot plate
7.2.7 Glass beads: soak overnight in 20% v/v nitric acid before use
7.2.8 Ice bath
7.2.9 Filter paper, Whatman® No. 541

8. PROCEDURE / ACTIONS

8.1 Preparation of Test Sample

Grind or blend sample until homogenous. If sample cannot be analyzed on the same day, keep in screw-cap bottle and store in freezer. The test samples can be prepared by dry ashing (8.3) or wet digestion (8.4).

8.2 Blank Test

Include one blank test per sample by adding in a beaker, crucible or Teflon cup, without sample, the same amount of acid/reagents added to the samples.

8.3 Dry ashing

8.3.1 Accurately weigh homogenous sample, in duplicate, 2–4 g for dry samples or 10 g for wet samples, in a crucible. For liquid sample, water is evaporated in a steam bath before charring.

8.3.2 Char the dry sample over a hotplate until smoking ceases.

8.3.3 Place charred samples in a furnace and incinerate at 525^0C for 3-4 h. Some types of foods, such as meat, may require longer ashing time, e.g. 16 hours.

8.3.4 If ashing is not complete, remove crucible, cool and moisten with 2-3 mL of DI water and add 0.5–3 mL of HNO_3. Dry on a water bath or hot plate.

8.3.5 Return the sample in the furnace and continue ashing until uniformly white or gray ash is obtained.

8.3.6 Turn off the furnace and allow the temperature to drop to 250^0C. Remove the crucible and cool. Add 5 mL 1N HNO_3 to dissolve the ash and transfer into a 50 mL volumetric flask.

8.3.7 Wash the crucible several times with 1N HNO_3 to ensure complete removal of the ash and filter using Whatman® filter paper No. 541.

8.3.8 Dilute to mark with 1N HNO_3. If the analysis cannot be done on the same day, keep the test solution in a screwed-cap, acid-washed polyethylene bottle and store in refrigerator.

8.4 Wet Digestion

8.4.1 Closed system

8.4.1.1 Accurately weigh, in duplicate, 0.5-1.5 g dry sample or 3-5 g wet sample or 5-10 g liquid sample (depending on the mineral concentration in the test sample) in a Teflon cup with screw cap.

8.4.1.2 For dry sample, add 5 mL conc. HNO_3 and 1mL conc. $HClO_4$. For wet sample, add 5-10 mL HNO_3 and 1-2 mL $HClO_4$ (HNO_3: $HClO_4$ = 5:1).

8.4.1.3 Leave the tightly closed cups overnight at room temperature to predigest the sample.

8.4.1.4 Place the cup in an oven at 100°C for 5-8 hours.

8.4.1.5 Cool to room temperature in a fume hood. Open the teflon cup carefully and, if the sample is not clear, add 5 mL conc. HNO_3. Close the cup and continue digestion in the oven for 2-3 h.
Note: Do not open the cup before it is completely cool.

8.4.1.6 Cool and transfer the digested samples quantitatively to a 50 mL or 100 mL volumetric flask (depending on the concentration of minerals in the sample). Dilute to mark with DI water and mix well.

8.4.1.7 Filter solution through Whatman® filter paper No. 541 and transfer to Nalgene® plastic bottles for mineral detection.

8.4.2 With HNO_3 and H_2O_2: Open system

8.4.2.1 Accurately weigh 1-2 g test portion of homogenized sample in a 250 mL beaker, with 3-4 glass beads. If material is liquid, weigh 10 g or pipette 10 mL and evaporate to small volume.

8.4.2.2 Cautiously, add 10 mL 50% HNO_3. Cover beaker with watch glass (ribbed watch glass allows evaporation and prevents contamination of samples), heat to 95^{o}C, and let the solution reflux for 10-15 minutes.

8.4.2.3 Cool and add 5 mL conc. HNO_3. Reflux for another 30 minutes at 95^{o}C.

8.4.2.4 Cool, add 5 mL conc. HNO_3, cover with watch glass, and let the solution reflux for another 30 minutes at 95^{0}C.

8.4.2.5 Evaporate the solution to about 5 mL. Do not allow the solution to dry.

8.4.2.6 Cool and add 2 mL DI water and 3 mL 30% H_2O_2. Cover with watch glass, and heat slowly (to avoid losses by excessive reaction) to initiate peroxide reaction.

8.4.2.7 Continue heating the beaker until effervescence subsides.

8.4.2.8 Cool and add 7 mL 30% H_2O_2 (1mL at a time) while heating so that all samples will receive 10 mL 30% H_2O_2.

8.4.2.9 Cool and add 5 mL conc. HCl, and 10 mL DI water, cover with watch glass and let the solution reflux for an additional 15 minutes without boiling.

8.4.2.10 Cool and transfer the digest into a a 50 mL or 100 mL volumetric flask (depending on the concentration of minerals in the sample) while filtering with Whatman® filter paper No. 541.

8.4.2.11 Dilute the sample to the mark with DI water.

Note: The acid-digested solutions from the closed and the above mentioned open system can be used for both macro and trace mineral measurement in AAS/ICP.

8.4.3 With HNO_3 and H_2SO_4: Open system

8.4.3.1 Accurately weigh, in duplicate, 1-2 g test portion of homogenized dry samples into a 300 or 500 mL Kjeldhal flask, with 3-4 glass beads. If sample is liquid, weigh 10 g or pipette 10 mL and evaporate to small volume.

8.4.3.2 Add 5 mL conc. HNO_3 and cautiously heat until first vigorous reaction subsides.

8.4.3.3 Add 2 mL conc. H_2SO_4 and continue heating to maintain oxidizing condition by adding conc. HNO_3 in small increment until solution is colorless.

8.4.3.4 Continue heating until there is no dense fume of H_2SO_4 and all HNO_3 is removed.

8.4.3.5 Cool, wash with 20 mL DI water, filter with Whatman filter paper No. 541 and transfer quantitatively into a 50 mL or 100 mL volumetric flask (depending on the concentration of minerals in the sample). Dilute to volume with DI water.

Note: The acid-digested solution can be used for trace and macro elements determination except for calcium. Sulfuric acid will form calcium sulfate precipitation, resulting in inaccurate determination of calcium.

8.4.4 With HNO_3 and $HClO_4$: Open system

8.4.4.1 Accurately weigh 1-2 g test portion of homogenized dry sample into a 300 mL Kjeldhal flask, with 3-4 glass beads. For liquid samples, weigh 10 g or pipette 10 mL and evaporate to small volume.

8.4.4.2 Add 20 mL 65% HNO_3 and 10mL 70% $HClO_4$.

8.4.4.3 Predigest the sample overnight in a fume hood at ambient temperature.

8.4.4.4 Before starting digestion, prepare an ice bath for cooling the flask. HNO_3 should also be readily available. To start digestion, place each flask on a heating mantle set at a low temperature.

8.4.4.5 Increase the temperature gradually to about 120°C. Once boiling starts, dense red-orange fumes of NO_2 appear. Maintain the temperature until the fume is replaced by the white fumes (HNO_3 and H_2O are removed). *At this point effervescent reaction occurs between sample and $HClO_4$.*

8.4.4.6 Remove the flask from the heating mantle and let digestion proceed with occasional heating.

Note: It is important that the reaction between sample and $HClO_4$ do not go rapidly, to prevent drying and eventual charring, as this may lead to an explosion. If drying occurs, immediately place flask in ice bath to stop digestion, add 1 mL HNO_3 and resume gentle heating.

8.4.4.7 After complete reaction of test solution with $HClO_4$ (identified by cessation of effervescence), increase the temperature and heat the sample for 2 minutes.

Note: Do not allow sample to dry!! This may lead to an explosion.

8.4.4.8 Remove the flask from heating mantle and cool. Quantitatively transfer the digested solution into a 50 mL or 100 mL volumetric flask (depending on the concentration of minerals in the sample).

8.4.4.9 Rinse the flask several times and dilute to volume with DI water. Some precipitation is likely to occur (especially in samples with high salt) after dilution. Shake the solution to prevent precipitation.

8.4.4.10 Leave the diluted solution overnight and filter through Whatman® filter paper No. 541 before analysis.

9. SUPPLEMENTARY NOTE:

9.1 Store crucibles in the dessicator after ashing.

9.2 If borosilicate glassware and silica basins are used, pre-clean with diluted HNO_3 and rinse in distilled water before use.

9.3 All activities should be done in a clean, dust free room to avoid contamination.

DETERMINATION OF CALCIUM AND MAGNESIUM BY ATOMIC ABSORPTION SPECTROPHOTOMETER

1. PURPOSE/SCORE

The method is used for the quantitative determination of Ca and Mg in foods by atomic absorption spectrophotometer (AAS).

2. SAFETY/PRECAUTION

2.1 Be careful when using $HClO_4$ in sample digestion. Avoid heating test solution to dryness as this may lead in explosion during digestion.

2.2 Wear gloves and safety glasses and carry out all acid digestion in a fume hood.

2.3 All activities should be done in a clean, dust-free environment to avoid mineral contamination.

3. REFERENCES

3.1 Horwitz W (2000) (editor). Official Method of Analysis of AOAC International. 17th Edition. AOAC International, Maryland, USA. (Methods 9.1.09 and 50.1.14)

3.2 Greenfield H and Southgate DAT (1992). Food Composition Data: Production, Management and Use. Elsevier Applied Science, UK.

3.3 Kirk RS and Sawyer R (1991). Pearson's Composition and Chemical Analysis of Foods, 9th Edition. Longman Scientific & Technical, Essex, England.

4. DEFINITION

4.1 *Test sample* refers to the laboratory sample before sample separation.

4.2 *Test solution* refers to a solution of analytical sample taken for analysis.

4.3 *Blank sample* refers to a sample, which contains no detectable level of the analyte or a complete analysis without the analyte.

4.4 *AAS* refers to atomic absorption spectrophotometer, which will be used in the determination of minerals at a specific wavelength.

5. PRINCIPLE

Calcium and magnesium in the sample, prepared by either dry ashing or wet digestion, are quantitatively measured by atomic absorption spectrophotometer (AAS) at a specific wavelength.

6. REAGENTS

Deionised water (DI water) will be used in this analysis.

6.1 Nitric acid, 1N

Add 139.5 mL 65% HNO_3 to approximately 1.5 L DI water. Shake, cool and dilute to 2 L.

6.2 Lanthanum chloride ($LaCl_3$), 1% (w/v).
Weigh 11.7±0.1g La_2O_3 (AAS grade), add 50 mL conc. HCl and dissolve in DI water. Transfer to a 1 L volumetric flask. Dilute to volume with DI water and mix. Prepare fresh solution every six months.

6.3 Standard Ca and Mg, 1 mg/mL, certified AA standards

6.3.1 Working standard, 20 μg/mL
Pipette accurately 1 mL certified AA standard (6.3) into a 50 mL volumetric flask and dilute to volume with 1N HNO_3. Mix the solution by slowly inverting the flask 10 times.

6.3.2 Calibration standard: 0, 0.4, 0.8, 1.2, 1.6, 2.0 μg/mL
Pipette 0 (reagent blank), 1, 2, 3, 4, and 5 mL working standard solution (6.3.1) into 50 mL volumetric flasks. Add 1% w/v $LaCl_3$ solution (6.2) to make a final concentration of 0.1% w/v $LaCl_3$ and dilute to the volume with 1N HNO_3.

7. APPARATUS

Atomic Absorption Spectrophotometer (AAS) should be well maintained with good response per unit concentration, e.g. 0.0200 O.D. or above for 4 mg/L Ca solution.

Note: List of apparatus is presented in the procedure of determination of minerals: sample preparation for mineral analysis.

8. PROCEDURE/ACTION

Sample preparation for mineral analysis is presented in the previous procedure (Determination of minerals: sample preparation for mineral analysis).

9. AAS DETERMINATION

9.1 Pipette an aliquot of the test solution into a volumetric flask of appropriate size depending on the concentration of the minerals in the sample.

9.2 Add 1% w/v $LaCl_3$ solution (6.2) to make a final concentration of 0.1% w/v $LaCl_3$.

9.3 Dilute the solution to an appropriate volume with 1N HNO_3 or, if the sample was prepared from wet digestion, with DI water. The solution is ready for calcium and magnesium determination by AAS.

9.4 Measurement of Ca and Mg
Both flame AAS or ICP-OES can be used for measurement of these minerals. Specific wavelength for each mineral is shown below:

Element	*Wavelength (nm)*	
	AAS	ICP-OES
Ca	422.7	317.93
Mg	285.2	279.08

9.4.1 Set up the instrument to optimum conditions according to the instrument's manufacturer.

9.4.2 Measure the absorbance or intensity of the prepared standards and the test solutions against reagent blank. The measurement should be carried out according to the following order: water, reagent blank (0 ppm, to set zero), sample blank, standards (from the lowest concentration to the highest), and test solution. Wash the system with water after reading each test solution. Measure the absorbance or intensity of a working standard every after ten measurements to check the stability of the instrument.

10. CALCULATION

$$\text{Ca or Mg (mg/100g)} = \frac{(C_0) \times \text{total volume (mL)} \times \text{dilution} \times 100}{\text{wt. of sample (g)} \times 1000}$$

where: C_0 = concentration of the sample in mg/L from the calibration curve (mg/L)

1000 = conversion of mL to L

Report test results in mg per 100 g sample with no decimal place.

11. ACCEPTANCE OF RESULTS

Duplicate results should not differ by more than 10% of the mean.

12. METHOD VALIDATION

Example of calcium determination by dry ashing at 450°C and AAS.

Test material: in-house reference material: IFRM-5 milk powder

Precision as repeatability (*r*), within a day

Parameter	In house IFRM-5
Number of analyses (n)	7
Mean Ca (mg/100g)	799
Standard deviation (SD)	7.8
Relative standard deviation (% RSD)	0.98
Repeatability at 95% confidence limit	27.1

Precision as reproducibility (*R*), on different days

Parameter	In house IFRM-5
Number of analyses (n)	8 days (21)
Mean Ca (mg/100g)	800
Standard deviation (SD)	27.5
Relative standard deviation (RSD)	3.44
HORRAT Value (RSD/RSDp)	0.83

Accuracy

Parameter	SRM 1846 Infant Formula
Number of analyses (n)	7
Mean Ca (mg/100g)	368 (wet)
Standard deviation (sd)	5.3
Relative standard deviation (RSD)	1.4
Certified value, (mg/100g)	367 $\pm$ 20

Linearity range (standard)

Parameter	Standard
Ca Conc. range	0.4 – 2.0 ppm
Correlation (R mean)	0.999
Slope (mean)	0.06
Line intercept (mean)	-0.003

13. SUPPLEMENTARY NOTE

Atomic absorption spectrophotometer (AAS) or inductively coupled plasma optical emission spectrophotometer (ICP-OES) can be used for the determination of calcium and magnesium.

14. QUALITY CONTROL

14.1 Use in-house reference material, e.g. rice flour (IFRM-1), milk powder (IFRM-5) for checking precision.

14.2 Prepare quality control chart and/or use certified reference material (CRM), e.g., infant formula (NIST 1846), or reference material with consensus values of nutrients, e.g., weaning food (ASFRM-5) for checking accuracy.

14.3 Accepted percent recovery of spiked sample is 90-110%.

14.4 Participate regularly in laboratory performance study.

DETERMINATION OF PHOSPHORUS BY GRAVIMETRIC METHOD

1. PURPOSE/SCOPE

The method is used in determination of phosphorus (P) in food products by gravimetric method

2. SAFETY

2.1 Consult Material and Safety Data Sheet (MSDS) for appropriate reagent handling/safety precautions.
2.2 Use safety devices such as tong and gloves in handling hot containers.
2.3 Use gas masks to minimize inhalation of diethyl ether vapor.

3. REFERENCE

3.1 Kolthoff IM, Sandell EB, Meehan EJ and Bruckenstein S (1969). Quantitative chemical analysis, New York, Macmillan Company.
3.2 Horwitz W (2000) (editor). Official Method of Analysis of AOAC International. 17th Edition. AOAC International, Maryland, USA.
3.3 Greenfield H and Southgate DAT (1992). Food Composition Data: Production, Management and Use. Elsevier Applied Science, UK.
3.4 Kirk RS and Sawyer R (1991). Pearson's Composition and Chemical Analysis of Foods, 9th Edition. Longman Scientific & Technical, Essex, England.

4. DEFINITION

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5. PRINCIPLE

Phosphorus in the sample, prepared by either dry ashing or wet digestion, is precipitated by ammonium molybdate solution and determined gravimetrically. The gravimetric method is used for samples containing more than 10 mg P.

6. REAGENTS

Water used in the analysis is deionized (DI) water.

6.1 Nitric acid, 4N: add 25 mL of 65% HNO_3 in DI water and dilute to 100 mL
6.2 Ammonium nitrate (NH_4NO_3), 5%
6.3 Ammonium molybdate solution
  6.3.1 Solution A: dissolve 100 g ammonium molybdate in a mixture of 400 mL of DI water and 80 ml of conc. ammonium hydroxide
  6.3.2 Solution B: dilute 400 mL of conc. HNO_3 with 600 mL of DI water.
  6.3.3 Immediately before use, prepare a 1 (6.3.1) : 2 (6.3.2) ratio and mix.
6.4 Ethanol, 95% (v/v)
6.5 Diethyl ether, anhydrous

7. APPARATUS

7.1 Porcelain crucible
7.2 Volumetric flask, 50, 100, 500 and 1000 mL
7.3 Beaker, 250 mL
7.4 Volumetric pipette, 10 and 25 mL
7.5 Watch glass
7.6 Glass filter crucible
7.7 Desiccator, activate desiccant prior to use by heating in an oven at 100°C until blue
7.8 Filter paper, Whatman® No 541
7.9 Analytical balance, 200 g and 0.01 mg sensitivity.
7.10 Muffle furnace
7.11 Vacuum pump

8. PROCEDURE

8.1 Prepare sample by dry ashing as described in sample preparation for mineral analysis
8.2 Dissolve the ash in 2.5 mL 4N HNO_3 and filter with Whatman® filter paper No. 541. Wash the crucible several times with DI water and dilute to 25 mL.
8.3 Transfer 5 mL test solution into a 250 mL beaker. The amount of the test solution aliquot varies depending on the amount of P present in the test solution (recommended >10mg P).
8.4 Add DI water to make the volume to 40 mL. Boil for 3 min.
8.5 Add 10 mL conc. HNO_3. Heat the test solution to boiling and swirl the solution for 10 second.
8.6 Add 50 mL ammonium molybdate solution. Cover with watch glass. Stand and swirl every 30 min for 2h.
8.7 Leave to precipitate at room temperature overnight.
8.8 Clean glass filter crucible with 95% ethanol and ether. Dry in a desiccator under vacuum for 1 h and weigh (***W1***).
8.9 Quantitatively transfer the precipitate from the beaker to the glass filter crucible and filter using a vacuum pump. Rinse the beaker with 5% NH_4NO_3 with the use of a policeman.
8.10 Rinse the precipitate with 95% EtOH and diethyl ether.
8.11 Dry the precipitate in the desiccator under vacuum for 1h and weigh (***W2***)

9. CALCULATION

$$P \text{ (mg /100g)} = \frac{(W2-W1) \times Y \times 1559.4557}{\text{weight of test sample (g)}}$$

where W1: weight of dry glass filter (g)
W2: weight of glass filter with the precipitate (g)
Y: dilution factor (if applicable)

1559.4557: factor for calculate P in the precipitate
(0.015594557 x 100x1000)

Report test results, in mg P per 100 g sample, as round number.

10. ACCEPTANCE OF RESULTS

Duplicate results should not differ by more than 10 % of the mean.

11. METHOD VALIDATION

Follow the methods for calcium and magnesium analysis.

12. SUPPLEMENTARY NOTE

12.1 If (*W2-W1*) is more than 1.5 g, repeat the analysis using a smaller volume of the digested solution or the initial sample weight.

12.2 In cleaning the glass filter crucible: wash first with deionised water, followed by 4N NaOH. Rinse with deionised water, and by 4N HNO_3. Rinse with distilled water.

13. QUALITY CONTROL

13.1 Use in-house reference material, e.g. rice flour (IFRM-1), milk powder (IFRM-5) for checking precision.

13.2 Prepare quality control chart and/or use certified reference material (CRM), e.g., infant formula (NIST 1846), or reference material with consensus values of nutrients, e.g., weaning food (ASFRM-5) for checking accuracy.

13.3 Accepted percent recovery of spiked sample is 90-110%.

13.4 Participate regularly in laboratory performance study.

DETERMINATION OF PHOSPHORUS BY UV VISIBLE SPECTROPHOTOMETRIC METHOD

1. PURPOSE/SCOPE

This method is used for the determination of phosphorus as phosphates (PO_4) in food products using spectrophotometric method

2. SAFETY

2.1 Consult Material and Safety Data Sheet (MSDS) for appropriate reagent handling/safety precautions.
2.2 Use safety devices such as tong and gloves in handling hot containers.
2.3 Perform analysis in a fume hood when using acids.

3. REFERENCES

3.1 Horwitz W (2000) (editor). Official Method of Analysis of AOAC International. 17th Edition. AOAC International, Maryland, USA. (Methods 50.1.12 and 50.1.15)
3.2 Greenfield H and Southgate DAT (1992). Food Composition Data: Production, Management and Use. Elsevier Applied Science, UK.
3.3 Kirk RS and Sawyer R (1991). Pearson's Composition and Chemical Analysis of Foods, 9th Edition. Longman Scientific & Technical, Essex, England.

4. DEFINITIONS

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5. PRINCIPLE

Phosphorus, as phosphate, in the test solution is complexed with molybdovanadate reagent. The yellow colour formed is directly related with the amount of P in the sample and the absorbance is measured by UV-VIS spectrophotometer.

6. REAGENTS

6.1 Deionised (DI) water
6.2 Hydrochloric acid, 4N
Add 250 mL conc. HCl to approximately 500 mL DI water. Shake, allow to cool and dilute to 1L.
6.3 Nitric Acid
6.3.1 Nitric acid, 6N
Dilute 38 mL conc. HNO_3 to 100 mL of DI water.
6.3.2 Nitric Acid, 1N
Add 139.5 mL conc. HNO_3 to 1.5 L DI water. Shake, cool and dilute to 2 L DI water.
6.4 Ammonium monovanadate, 0.25%
Dissolve 0.25 g of ammonium monovanadate in 2 mL conc. HNO_3 and dilute to 100 mL with DI water.

6.5 Ammonium molybdate, 5% (w/v)
Weigh 5 g of ammonium molybdate and dilute to 100 mL with DI water

6.6 Potassium dihydrogen phosphate (KH_2PO_4)

6.6.1 Phosphate stock solution, 2 mg P/mL
Dissolve 8.7874 g KH_2PO_4, previously dried at 105°C for 2h, in 750 mL deionised water. Dilute to 1000 mL with DI water and store in refrigerator.

6.6.2 Working standard solution, 0.1mg P/mL (freshly prepared)
Dilute 50mL stock standard solution (6.6.1) to 1000mL with DI water.

7. APPARATUS

7.1 Volumetric flasks, class A, 50, 100 and 1,000 mL
7.2 Volumetric pipettes, class A, 5 and 10 mL
7.3 UV-VIS Spectrophotometer, capable of operation near 400 nm
7.4 Analytical balance, capable of weighing to the nearest 0.1 mg
7.5 Filter paper, Whatman® No. 42

8. PROCEDURE

8.1 Prepare sample by dry ashing as described in "Sample preparation for mineral analysis".
8.2 Dissolve ash in 5 mL 1N HNO_3. Heat in water bath or on hot plate for 2-3 min to aid dissolving.
8.3 Carefully transfer the ash solution into a 10 mL volumetric flask.
8.4 Rinse the crucible by washing with 1N HNO_3 twice, dilute to 100 mL with 1N HNO_3 and filter through Whatman® filter paper No. 42.
8.5 Determination of phosphorus

8.5.1 Pipette 10 mL of 0.1 mg/mL phosphate standard solution (6.6.2) to obtain 1 mg P and 5 mL test solution into 100 mL volumetric flask.
8.5.2 To each flask, add 10 mL of 6N HNO_3 (6.3).
8.5.3 Add 10 mL 0.25% ammonium monovanadate (6.4) and 10 mL 5% ammonium molybdate (6.5).
8.5.4 Dilute to mark with DI water. Mix well and stand for exactly 15 min to allow complete color development.
8.5.5 Measure the absorbance of each solution in 1cm cell at 400 nm and use reagent blank for autozero.

9. CALCULATION

$$\text{P mg /100g} = \frac{(Abs_{sam} \times 1 \times V_o \times 100)}{(Abs_{std} \times V_p \times W)}$$

where Abs_{sam}: absorbance sample
Abs_{std}: absorbance standard (1mg/mL)
V_o: total volume (mL)
V_p: volume of diluted sample (mL)

W: sample weight (g)

Report test results, in mg P per 100 g sample, as round number.

10. ACCEPTANCE OF RESULTS

10.1 Duplicate results should not differ by more than 10 % of the mean.

10.2 Accepted percent recovery of spiked sample is 90-110%

11. METHOD VALIDATION

Follow the methods for calcium and magnesium analysis.

12. SUPPLEMENTARY NOTE

Be aware that the color intensity of the test solution after adding ammonium molybdate increases with time.

13. QUALITY CONTROL

13.1 Use in-house reference material, e.g. rice flour (IFRM-1), milk powder (IFRM-5) for checking precision.

13.2 Prepare quality control chart and/or use certified reference material (CRM), e.g., infant formula (NIST 1846), or reference material with consensus values of nutrients, e.g., weaning food (ASFRM-5) for checking accuracy.

13.3 Participate regularly in laboratory performance study.

DETERMINATION OF SODIUM AND POTASSIUM BY ATOMIC ABSORPTION SPECTROPHOTOMETRY METHOD

1. PURPOSE/SCOPE

The method is used for the quantitative determination of Na and K in foods.

2. SAFETY

2.1 Wear gloves to reduce contact with acids and chemical reagents.
2.2 Wear gas mask to reduce inhalation of acid fumes.
2.3 Work in fumehood when using acids.

3. REFERENCE

3.1 Horwitz W (2000) (editor). Official Method of Analysis of AOAC International. 17th Edition. AOAC International, Maryland, USA. Methods 9.1.09 and 50.1.14.
3.2 Greenfield H and Southgate DAT (1992). Food Composition Data: Production, Management and Use. Elsevier Applied Science, UK.
3.3 Kirk RS and Sawyer R (1991). Pearson's Composition and Chemical Analysis of Foods, 9th Edition. Longman Scientific & Technical, Essex, England.

4. DEFINITION

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5. PRINCIPLE

Sodium and potassium in the sample, prepared by dry ashing or wet digestion, are determined by atomic absorption spectrophotometer (AAS) at specific wavelength. Flame photometry may also be used but sensitivity is less than AAS.

6. REAGENTS

NOTE: Prepare fresh solution every 6 months.

6.1 Deionised water (DI water)
6.2 Nitric Acid, 1N
Add 139.5 mL conc. HNO_3 to approximately 1.5L DI water. Mix thoroughly. Cool and dilute to 2 L.
6.3 Cesium chloride solution ($CsCl_2$): 10%(w/v)
Weigh 12.7±0.1g CsCl (99.6% purity) and dilute to 100 mL volumetric flask with DI water.
6.4 Stock standard sodium and potassium, 1mg/mL (AAS grade), certified standards
6.4.1 Working standard, 10 μg/mL
Pipette accurately 1 mL stock standard into a 50 mL volumetric flask and dilute to volume with 1N HNO_3. Mix the solution by slowly inverting the flask 10 times.

6.4.2 Calibration standards
A set of standard is prepared from the working solution. The range of its concentration is prepared depending on the instrument capability and the level of minerals in the sample solution. Add 10% (w/v) CsCl to each Na and K standard solution to make the final dilution of 1% w/v CsCl.

7. APPARATUS

7.1 Volumetric flask, 100 mL
7.2 Volumetric pipette, 1, 2, 3, 4, 5, and 10 mL
7.3 Analytical balance, capable of weighing to the nearest of 0.1 mg
7.4 Water bath
7.5 Hot plate
7.6 Filter paper
7.7 Atomic Absorption Spectrophotometer (AAS)

8. PROCEDURE

8.1 Sample may be prepared by dry ashing or wet digestion as described in “Sample preparation for mineral analysis”.
8.2 Add $CsCl_2$ solution to an aliquot portion to make a final dilution of 1% w/v $CsCl_2$ prior to the analysis. Dilute to an appropriate volume.
8.3 Determination
8.3.1 AAS or ICP-OES may be used and the wavelength is as follow:

Element	*Wavelength (nm)*
Na	589.0 (for 0.5-1.5 ppm)
	330.4 (for 10-100 ppm)
K	766.5 (for 1-4 ppm)

8.3.2 Measure absorbance or intensity and their concentrations of reagent blank, series of standards, sample blank and sample test solutions in the given order.
8.3.3 Wash the system with water after each sample reading.
8.3.4 Do the Intermediate check of the instrument with one standard at every 10 to 20 measurements.

9. CALCULATION

$$\text{Na or K (mg of /100 g)} = \frac{C_0 \; x \; V \; x \; D \; x \; 100}{W \; x \; 1000}$$

where: C = concentration of sample from the calibration curve (mg/L)
V = total volume, mL
D = dilution factor

W = weight sample, g
1000 = conversion of mL to L

Report test results, in mg Na or K per 100 g sample, as round number.

10. ACCEPTANCE OF RESULTS

Duplicate results should not differ by more than 10% of the mean value.

11. METHOD VALIDATION

Follow the methods for calcium and magnesium analysis

12. SUPPLEMENTARY NOTE

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13. QUALITY CONTROL

13.1 Use in-house reference material, e.g., rice flour (IFRM-1), milk powder (IFRM-5) for checking precision.

13.2 Monitor with quality control chart and/or use certified reference material (CRM), e.g., infant formula (NIST 1846), or reference material with consensus values of nutrients, e.g., weaning food (ASFRM-5) for checking accuracy.

13.2 Accepted per cent recovery of spiked sample is 90-110%

13.3 Participate regularly in the laboratory performance study.

DETERMINATION OF IRON, COPPER, AND ZINC

1. PURPOSE/SCOPE:

The method is used for the quantitative determination of Fe, Cu and Zn in foods.

2. SAFETY

For wet digestion using perchloric acid, refer to safety in handling acid, which is described in “Sample preparation for mineral analysis – wet digestion”.

3. REFERENCE

3.1 Horwitz W (2000) (editor). Official Method of Analysis of AOAC International. 17th Edition. AOAC International, Maryland, USA (9.1.09: Determination of lead, cadmium, copper, iron, magnesium, zinc in foods and 50.1.14: Minerals in infant formula, enteral products and pet foods)

3.2 Prapaisri P. Sirichakwal, Rachanee Kongkachuichai and Prapasri Puwastien (1988). Acid digestion versus dry ashing for mineral analysis of foods. J Nutrition Assoc Thailand, 22:279-96.

4. DEFINITION

Trace elements in this procedure refer to Fe, Cu and Zn

5. PRINCIPLE

Organic matter in the sample is destroyed by wet digestion. The trace elements in the sample are quantitatively measured by atomic absorption spectrophotometer (AAS) at a specific wavelength.

6. REAGENTS

6.1 For complete list of reagents please refer to “Sample Preparation for Mineral Analysis”.

6.2 Standards Fe, Cu and Zn, 1 mg/mL (AAS grade), certified AA standards

6.2.1 Working standard, 10 μg/mL

6.2.1.1 Pipette accurately 2 mL certified standard (6.2) into a 100 mL volumetric flask and dilute to volume with 1N HNO_3.

6.2.1.2 Stopper the flask and mix the solution by slowly inverting the flask 10 times.

6.2.2 Calibration standards

Concentrations of standards depend on the instrument and level of detection of Fe, Cu and Zn in test solution. The standard solutions are prepared and diluted with 1N HNO_3.

7. APPARATUS

7.1 All necessary glassware and instruments for wet digestion are shown in “Sample preparation for minerals analysis”.

7.2 All glasswares must be soaked overnight in 20% HNO3 (v/v), cleaned and rinsed with deionised water.

8. PROCEDURE

8.1 Sample preparation: organic matter in the sample is destroyed by wet digestion as described in “Sample preparation for mineral analysis”.

8.2 Take an aliquot portion of the acidified sample, dilute to an appropriate volume and read at AAS.

8.3 Reagent blank preparation
Replace the sample with DI water and perform the analysis under the same condition as the sample.

8.4 Determination

8.4.1 AAS and ICP-OES may be used at the wavelength below.

Element	*Wavelength (nm)*
Fe	248.3
Zn	213.9
Cu	324.7

8.4.2 The order of measurement is as follows: water (set to zero), reagent blank (0 ppm, autozero), standard set, sample blank and test solution.

8.4.3 Read reagent blank and a standard every 5 measurements to check the stability of the instrument.

9. CALCULATIONS

Using the following formula

$$\text{Trace element (mg /100g sample)} = \frac{C_0 \; x \; V \; x \; D \; x \; 100}{W \; x \; P \; x \; 1000}$$

where: C_0 = concentration of the sample in mg/L

V = total volume, mL

D = Dilution factor

W = weight sample, g

P = sample solution taken, ml

1000 = conversion of mL to L

Report test results, in mg Fe or Cu or Zn per 100 g sample, as round number.

10. ACCEPTANCE OF RESULTS

10.1 Test sample is measured in duplicate and the values within the sample should not differ by more than 10% of the mean value.

10.2 Standard calibration curve for each element should have a correlation coefficient (r) of at least 0.9900.

10.3 If the correlation coefficient is less than 0.99, review the standards and exclude any standard(s) that are obvious outliers.

10.4 The in-house reference materials (or SRM/AS-FRM depending on the reference material used) should give concentrations within the three standard deviation (SD) in the control chart as per established acceptance criteria prepared for the particular mineral.

11. METHOD VALIDATION

11.1 Iron Analysis, dry ashing at 450^{o}C

11.1.1 Precision as repeatability (*r*), analysis was carried out within the same day

Parameter	Rice flour IFRM-1	Milk IFRM-5
Number of analyses (n)	9	5
Mean Fe (mg/100g)	0.69	11.81
Standard deviation (s.d.)	0.09	0.24
Relative standard deviation	13.38	2.03
Repeatability at 95% confidence limit	0.30	0.94

11.1.2 Precision as reproducibility (R), analysis was carried out on different days

Parameter	Rice flour IFRM-1	Milk IFRM-5
Number of analyses (n)	6	11
Mean Fe (mg/100g)	0.67	11.65
Standard deviation (s.d.)	0.08	0.73
Relative standard deviation	11.91	6.23
Horwitz ratio (Horrat)	0.99	0.79

11.1.3 Checking of accuracy using SRM

Parameter	SRM 1568a Rice flour	SRM 1846 Infant Formula
Number of analyses (n)	5	4
Mean Fe (mg/100g)	0.76	6.10
Standard deviation (s.d.)	0.04	0.37
Relative standard deviation,%RSD	6.17	6.23
Certified value, mg/100g	0.74± 0.09	6.31± 0.4

11.1.4 Linearity range (using a set of standards)

Parameter	Standard
Conc. Fe Ranges, μg/mL	0.04-2.0
Correlation, (r) (mean)	0.9988
Slope, (mean)	0.1006
Line intercept (mean), ± SD	0.0022 ± 0.007

11.1.5 Proficiency Test for copper analysis by Asia Pacific Accreditation Cooperation (dry ashing, 525°C, AAS)

Sample	**Median Cu (ppm)***	**Measured Cu (ppm)**
APLAC#1	1.12	1.16
APLAC#2	1.10	1.09

Note: Z score is +1.35

*The median results from 77 labs

12. SUPPLEMENTARY NOTE

12.1 UV-Spectrophotometric method can be used for iron analysis.

12.2 Graphite Atomic Absorption Spectrophotometer (GFAAS) or Inductively Coupled Plasma Optical Emission Spectrophotometer (ICP-OES) may be used for the determination of trace elements.

12.3 Recommended weight for solid sample is not more than 1.5 g and for liquid sample is 5 mL.

12.4 All activities should be done in a clean, dust free room to avoid contamination.

13. QUALITY CONTROL

13.1 Prepare quality control chart and/or use certified reference material (CRM), e.g., infant formula (NIST 1846), or reference material with consensus values of nutrients, e.g., weaning food (ASFRM-5) and/or in house reference materials for checking accuracy.

13.2 Accepted percent recovery of spiked sample is 90-110%.

DETERMINATION OF IODIDE BY SPECTROPHOTOMETRIC METHOD

1. PURPOSE/SCOPE

The method applies to the determination of iodide in foods.

2. SAFETY

2.1 Always wear protective devices, i.e. face mask, goggles, and gloves when handling toxic and corrosive chemicals.

2.2 Prepare solutions with toxic and corrosive chemicals under a hood.

2.3 Always wear laboratory ground inside the laboratory.

2.4 Consult the MSDS of chemicals before use.

3. REFERENCES

3.1 Moxon RED and Dixon EJ. Semi-automatic method for the determination of total iodine in food. Analyst 1980, 105: 344-352.

3.2 Kamlert W.,Euotrongchitt Y.and Sawangkapat R. The determination of total iodine in seaweed snack food. Bulletin of the Department of Medical sciences. 1995, 37(1): 41-46.

4. DEFINITION

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5. PRINCIPLE

Sample is prepared by ashing and the residue is dissolved in deionized water. The test solution is determined for total iodide (as iodine) by spectrophotometer, based on the catalytic reaction of the thiocyanate with nitrite or ceric ion with arsenious acid in the presence of iodide. The amount of iodine in the sample is directly proportional to the rate of reaction of thiocyanate/ceric ions.

6. REAGENTS

6.1 Potassium Iodide, A.R.

6.1.1 Potassium iodide stock standard solution (A), 4 g/L

Accurately weigh 0.5232 g of potassium iodide and dissolve in about 80 mL deionised water. Dilute to 100mL with deionised water and mix.

6.1.2 Potassium iodide standard solution (B), 40 mg/L

Pipette 1 mL of stock standard solution A into a 100 mL volumetric flask. Dilute to volume with deionised water and mix. Prepare fresh daily.

6.1.3 Potassium iodide standard solution (C), 200 ng/mL

Pipette 1 mL of Standard B into a 200 mL volumetric flask. Dilute to volume with deionised water and mix. Prepare fresh daily.

6.1.4 Working standard solution

To prepare working standards of concentrations 4, 8, 12, 16 and 20 ng/mL, pipette 1, 2, 3, 4 and 5 mL standard C (6.1.3) into 50 mL volumetric flask, respectively. Add 1 mL 30%w/v K_2CO_3 and dilute with H_2O.

6.2 10% (w/v) Zinc Sulfate ($ZnSO_4$)

Dissolve 10 g of zinc sulfate ($ZnSO_4.7\ H_2O$) in 100 mL deionised water.

6.3 0.023% (w/v) Potassium thiocyanate (KSCN)

Dissolve 0.023 g of KSCN in 100mL deionised water.

6.4 Ammonium iron sulfate reagent ($NH_4Fe(SO_4)_2$)

Dissolve 77 g $NH_4Fe(SO_4)_2.12H_2O$ in 400 mL deionised water. Add 167 mL 37% HNO_3 and dilute to 1000 mL and leave overnight prior use.

6.5 2.07% (w/v) Sodium nitrite ($NaNO_2$)

Dissolve 2.07 g $NaNO_2$ and dilute to 100 mL deionised water. Prepare fresh every use.

6.6 30% (W/V) Potassium carbonate (K_2CO_3)

Dissolve 30 g K_2CO_3 and dilute to 100 ml with deionised water

6.7 Arsenious acid solution (0.2N arsenic acid in 1N H_2SO_4)

In a 200 mL volumetric flask, dissolve 1.96 g arsenic trioxide and 1.4 g sodium hydroxide in 20 mL deionised water. When completely dissolved, add 80 mL deionised water and a few drops of phenolphthalein indicator. If the solution turns pink, add sulfuric acid to neutralize the solution until the pink color disappears. Then, add 5.6 mL sulfuric acid. Dissolve 5.0 g sodium chloride and dilute to mark with deionised water. Transfer to a polyethylene bottle.

6.8 Ceric ammonium sulfate solution

Dissolve 0.63 g ceric ammonium sulfate and add 10.4 mL conc. sulfuric acid in about 150 mL deionised water. Stand overnight and dilute to 200 mL deionised water. Transfer to a polyethylene bottle.

7. APPARATUS

7.1 Instruments

7.1.1 Analytical balance, capacity 200 g, 0.1 mg sensitivity

7.1.2 Top loading balance, capacity 100 g, 0.1g sensitivity

7.1.3 Hot air oven, can be maintained at 100°C

7.1.4 Furnace, can be maintained at 550°C

7.1.5 Steam bath

7.1.6 Centrifuge

7.1.7 Hot plate

7.2 Glasswares

7.2.1 Graduated cylinder, 50, 100 mL

7.2.2 Beaker, 250 mL

7.2.3 Erlenmeyer flask, 125 mL

7.2.4 Volumetric pipet, 1, 2, 3, 4, 5, 10 mL

7.2.5 Measuring pipet, 10 mL, 0.1 mL graduation

7.2.6 Volumetric flask, 100 mL

7.2.7 Nickel crucible or porcelain crucible, 50, 100 mL

7.2.8 Glass rod

7.2.9 Centrifuge tube, 50 mL

8 PROCEDURES

8.1 Test sample

8.1.1 Thaw out finely homogenized sample to room temperature.

8.1.2 Weigh, in duplicate, 2-10 g depending on the concentration of iodine in the sample estimated to contain at least 2 μg I_2 into a nickel crucible or porcelain crucible.

8.2 Preparation of recovery test sample

8.2.1 Weigh, in duplicate, a well-mixed sample (2 to 10g) depending on the concentration of iodine in the sample estimated to contain at least 2 μg I_2 into a nickel crucible or porcelain crucible.

8.2.2 Add 50-200 ng/mL potassium iodide standard (6.1.3) depending on the iodine content in the sample.

8.3 Blank test

Run one blank test (reagent without sample) periodically per new batch of reagent along with samples to measure any contamination from reagents.

8.4 Sample preparation before dry ashing

8.4.1 Add 1 mL 10% zinc sulfate, 1 mL deionized water and 1 mL 30%w/v K_2CO_3 to the sample. Mix, using glass-stirring rod.

8.4.2 Place crucible with samples on a steam bath or oven and heat slowly until dry.

8.4.3 Place crucible with samples in a hot air oven overnight at 100^{o}C. Heat on hot plate at 300^{o} C until the fume ceases.

8.5 Ashing

8.5.1 Place the crucible with the prepared sample in a muffle furnace and ash at 550^{o} C for 90-120 min or until a nearly white ash is obtained.

8.5.2 Turn off the furnace and allow the temperature to lower to 180°C. Remove samples from the furnace and cool to room temperature.

8.5.3 Add 1 mL 10% zinc sulfate solution and break the charred residue with a glass rod to disperse the solution. Rinse the glass rod with deionised water adding the rinsing in the crucible.

8.5.4 Heat samples in a hot air oven or steam bath at 100°C until dry.

8.5.5 Repeat ashing (8.5.3 to 8.5.6) until white ash is obtained.

8.5.6 Dissolve the ash with deionised water and dilute to 50 mL, centrifuge the solution at 3000 rpm for 5 min, and transfer the supernatant into a polyethylene bottle.

8.6 Determination

8.6.1 For thiocyanate reaction

Pipette 5 ml test solution into a test tube, add 1 mL 0.23% KSCN and 2 mL 7.7% ferric ammonium sulfate solution. Mix and add 1 mL 2.07% sodium nitrite. Mix thoroughly using a vortex mixer for 2-3 min. Stand to develop the color for exactly 20 min. Measure the absorbance of each solution at 450 nm. Keep a constant 30 seconds interval time between readings.

8.6.2 For ceric-arsenic reaction

Pipette 1mL test solution into a test tube, add 1 mL arsenious acid solution and 1 mL cerric ammonium sulfate solution. Mix, transfer immediately into cuvette and measure the absorbance of each solution at 410 nm. Use a stopwatch or automatic reading to keep a 30 and 60 seconds constant interval time between readings.

8.6.3 Standard curve preparation

Each working standard solution (6.1.4) is treated as a test solution as mentioned above (8.6.1 or 8.6.2). Measure the absorbance of each standard solution against concentration (0, 4, 8, 12, 16 and 20 ng/mL).

9. CALCULATION

Calculate the iodine content using linear regression, as follow:

X axis is iodine concentration (ng/mL)

Y axis is absorbance reading (for thiocyanate reaction) or differential absorbance between interval readings (for ceric-arsenic reaction).

$$\text{I } (\mu\text{g}/100\text{g}) = \frac{C_0 \; x \; D \; x \; 100}{W \; x \; 1000}$$

C_0 = iodine content read from the calibration curve (ng/mL)

D = dilution factor

W = weight sample (g)

1000 = conversion of ng to μg

The amount of iodine is reported in μg/100g sample, as round number.

10. ACCEPTANCE OF RESULTS

10.1 Test sample is measured in duplicate and the values within the sample should not differ by more than 10% of the mean value.

10.2 Recovery is performed by spiking a sample in every batch of analysis and the acceptance level is 80-120%.

10.3 In-house quality control should be within $\pm$ 2 SD. Fish meal is suggested as one of an in-house quality control sample.

11. METHOD VALIDATION

Standard reference materials (NIST 1589, non-fat milk powder)

12. SUPPLEMENTARY NOTE:

Discard the whole batch if blank test is positive as a periodical check to contamination.

DETERMINATION OF IODIDE IN FOODS BY INVERSE COLORIMETRIC METHOD USING TECHNICON AUTOANALYZER

1. PURPOSE/SCOPE

The method applies to the determination of iodide in food.

2. SAFETY

2.1 Always wear protective devices, i.e. face mask, goggles, and gloves when handling toxic and corrosive chemicals.

2.2 Prepare solutions with toxic and corrosive chemicals under a hood.

2.3 Always wear laboratory gown inside the laboratory.

2.4 Consult MSDS of chemicals before using

3. REFERENCES

3.1 Technicon Autoanalyzer II Industrial Method No. 530-77

3.2 A Semi-Automated Method for the Determination of Iodide in Total Diet Market Basket, Ronald G. Luchtefeld, FDA, Kansas City Field Office.

4. DEFINITION

Iodide in this method is the amount measured by the decrease in absorbance of the yellow color of the ceric ion, which is inversely proportional to the iodide concentration in the sample.

5. PRINCIPLE

The determination of iodide is based on the inverse colorimetric method in which iodide catalyses the reduction of ceric ions using arsenious acid. The decrease in absorbance of the yellow ceric ion at 420 nm is inversely proportional to iodide concentration in the sample.

6. REAGENTS AND MATERIALS

6.1 Potassium Iodide, A.R., Univar/AJAX

6.1.1 Potassium iodide stock standard solution (A), 100 mg/L

Accurately weigh 130.8 mg potassium iodide and dissolve in about 800 mL deionised water. Dilute to 1 L with deionised water and mix.

6.1.2 Potassium iodide standard solution (B), 2 mg/L

Pipette 2 mL Stock Standard solution A (6.1.1) into 100 mL volumetric flask. Dilute to volume with deionised water and mix. Prepare fresh daily.

6.1.3 Potassium iodide standard solution (C), 200 ng/mL

Pipette 10 mL Standard B (6.1.2) into a 100 mL volumetric flask. Dilute to volume with deionised water and mix. Prepare fresh daily.

6.2 Brij 35, 30%, Sigma

Add 2 ml of Brij-35 to one liter of deionized water and mix.

6.3 Sodium hydroxide (NaOH), A.R., Baker

Sodium hydroxide, 1N: Carefully add 40 g NaOH to about 800 mL deionized water. Dilute to 1 L with deionized water and mix.

6.4 Arsenic Trioxide (As_2O_3), A.R., Baker

Arsenious acid, 1%: Add 10 g As_2O_3 and 100 mL 1N NaOH to about 500 mL deionized water. Heat solution until As_2O_3 is completely dissolved. Allow to cool and carefully add 44 mL conc. sulfuric acid. Dilute to 1 L with deionized water, mix and filter before use.

6.5 Ceric ammonium sulfate ($(NH_4)_4(CeSO_4)_4 \cdot 2H_2O$), A.R., Sigma

Ceric ammonium sulfate, 1%: Carefully add 44 mL conc sulfuric acid to about 800 mL deionized water. Add 10 g ceric ammonium sulfate and dissolve. Dilute to 1 L with deionized water, mix and filter before use.

6.6 Sulfuric acid, A.R., Merck

6.6.1 *Sulfuric acid, 2N*

Carefully add 28 mL conc. sulfuric acid to about 400 mL deionized water. Dilute to 500 mL with deionized water and mix.

6.6.2 *Sulfuric acid, 20N*

Carefully add 280 mL conc. sulfuric acid to about 200 mL deionized water. Dilute to 500 mL with deionized water and mix.

6.7 Potassium Hydroxide, A.R., Baker

6.7.1 Sample diluent

Dissolve 60 g KOH in about 1 L deionized water. Carefully add 500 mL 2N H_2SO_4. Dilute to 2 L with deionized water and mix.

6.7.2 Potassium hydroxide : Water, (1:1, w/v)

Dissolve 50 g of KOH in 50 mL deionized water and mix.

6.8 EthanoL, 95%, A.R., Baker or Absolute ethanol, A.R., Baker

If absolute ethanol is used, mix 10 mL deionized water & 190 mL absolute ethanol**.**

6.9 Zinc sulfate, A.R., J.T. Baker

Zinc Sulfate, 10%: Weigh 10g zinc sulfate, dissolve in deionized water and dilute to 100mL with water.

7. APPARATUS

7.1 Instruments

7.1.1 Analytical balance, capacity 200 g, 0.1 mg sensitivity

7.1.2 Top loading balance, capacity 100 g, 0.1 g sensitivity

7.1.3 Forced draft oven, can be maintained at 220°C

7.1.4 Furnace, capable of being maintained at 550°C

7.1.5 Steam bath

7.1.6 Hot plate

7.2 Glasswares

7.2.1 Graduated cylinder, 50, 100 mL with 1.0 mL graduation

7.2.2 Beaker, 250 mL

7.2.3 Erlenmeyer flask, 125 mL

7.2.4 Volumetric pipet, 1, 2, 4, 5, and 10 mL

7.2.5 Measuring pipet, 10 mL, 0 .1 mL graduation

7.2.6 Volumetric flask, 100 mL

7.2.7 Nickel crucible, 50, 100 mLGlass rod

8 PROCEDURES/ACTIONS

8.1 **Test sample**

8.1.1 Thaw out finely ground/blended sample to room temperature

8.1.2 Weigh in duplicate a well-mixed sample (2 to 10g) depending on the concentration of iodine in the sample estimated to contain at least 2 μg I_2 into a nickel crucible.

8.2 **Preparation of recovery test sample**

8.2.1 Weigh in duplicate a well-mixed sample (2 to 10 g sample) depending on concentration of iodine in the sample estimated to contain at least 2 μg iodine into a nickel or porcelain crucible.

8.2.2 Add 50-200 ng potassium iodide stock standard solution C to each crucible.

8.3 Blank test

Run one blank test (replace sample with water) per new batch of reagent along with samples to determine contaminations.

8.4 Digestion

8.4.1 Add 10 mL 95% EtOH, 10 mL deionized water and 6 mL KOH:deionized water (1:1) to each crucible with sample. Mix using glass stirring rod.

8.4.2 Place crucible on a steam bath and heat slowly until frothing ceases. Heat for approximately 15 min or more.

8.4.3 Place crucible in a forced draft oven and gradually increase temperature from 95^{o}C to 150^{o}C. Heat overnight at 150^{o}C. Raise the temperature of the oven to 220^{o}C and heat for 2h.

8.5 Ashing

8.5.1 Place the crucible with samples in a muffle furnace and ash at 550^{o} C for 45 min or until a nearly white ash is obtained.

8.5.2 Turn off the furnace and allow the temperature to lower to 180°C. Remove samples from the furnace and cool to room temperature.

8.5.3 Add 1 mL 10% zinc sulfate solution and break the charred residue with a glass rod to disperse residue in the solution. Rinse the glass rod with deionized water adding the rinsing in the crucible.

8.5.4 Dry samples in a force draft oven at 100°C.

8.5.5 Repeat ashing (8.5.2-8.5.4) until white ash is obtained.

8.5.6 Add 15 mL deionized water to the samples and heat to boiling on a hot plate.

8.5.7 Filter samples using Whatman® filter paper No. 41 or S&S® filter paper No. 589 or equivalent into a 125 mL Erlenmeyer flask. Repeat steps 8.5.6 and 8.5.7 (3x), wash filter paper and combine the washings in the 125 mL flask.

8.5.8 To make the filtrate acidic, add 5 mL 20N sulfuric acid drop wise. Swirl carefully.

8.5.9 Transfer the filtrate to 100 mL volumetric flask and dilute to volume with deionized water. Transfer solution to polyethylene bottles for iodine detection.

8.6 Detection

Prepare calibration standard using the table below. Dilute to volume using sample diluent.

Calibration standards

Volume KI Standard Soln. (C) (mL)	Total dilution volume (mL)	μg I / L
1	50	4
2	50	8
3	50	12
4	50	16
5	50	20

8.6.2 Run KI calibration standards in a Technicon® Autoanalyzer (refer to SOP NUT-EO-016). Make appropriate dilutions if necessary using the sample diluent.

9 CALCULATION OF RESULTS

9.1 From the chart/chromatogram obtained from the Tecnicon® Continuous Flow Autoanalyzer, record the standard and sample peak height. Subtract baseline reading.

9.2 Compute for iodine concentration using linear regression

Where: x axis = concentration of the standard in ng/ml

y axis = peak height of the standard minus the baseline reading

9.3 Compute ug I/100g sample using the formula:

$$\text{I } (\mu g/100g) = \frac{C_0 \; x \; V \; x \; 100}{W \; x \; 1000}$$

C_0 = iodine content read from the calibration curve (ng/mL)

V = final dilution volume of solution (mL)

W = weight sample (g)

1000 = conversion of ng to μg

The amount of iodine is reported in μg/100g sample, as round number.

10. ACCEPTANCE OF TEST RESULTS

10.1 Recovery test should be between 80 to 120%.

10.2 Mean results of duplicate analysis should not vary by more than 10%.

10.3 The coefficient of linear regression (*r*) should be> 0.9.

10.4 In–house control sample should not be more than $\pm$ 2SD.

11. METHOD VALIDATION

Use of Standard Reference Material from NIST 1589 (non-fat milk powder)

12. SUPPLEMENTARY NOTES:

Discard the whole batch if blank test is positive as a periodical check to contamination.

13. METHOD VALIDATION:

13.1 Linear Range, Slope and Intercept, Limit of Detection and Quantitation

Confidence Limit of Slope & Intercept for Iodine Analysis

Date	**X**	**8**	**12**	**16**	**20**	**24**	**16**			
	$(x-x)^2$	64	16	0	16	64	160			
	X^2	64	144	256	400	576	1440	r	a	b
9/25/01	y1	22.625	31.875	40	47.75	56.25		0.99563	6.45	2.0781
8/21/01	y2	19.25	27.625	35.625	43.375	51.25		0.99989	3.525	1.9937
1/18/02	y3	18.125	27	35.88	44.25	51.88		0.99951	1.523	2.119
1/31/01	y4	20.125	29.875	39.25	47.625	55.875		0.99925	2.8518	2.2311
4/18/02	y5	19.375	28.875	37.625	46.25	54.375		0.9996	2.35	2.1843
5/15/02	y6	18	27	35.5	43.75	51		0.99923	1.95	2.0687
6/19/02	y7	17.5	25.5	34	41.375	48.625		0.9995	2.15	1.9531
	Mean	19.286	28.25	36.84	44.911	52.751		0.9996	2.9714	2.0898
	$y\pi$	19.689	28.049	36.408	44.767	53.126		1	2.9714	2.0898
	$\lvert Y- Y\pi \rvert 1$	0.4041	0.2014	0.4318	0.1433	0.3759				
	$(Y-Y\pi)^2$	0.1632	0.0405	0.1412	0.0205	0.1412				

Sum - 0.0034 0.55214

MEAN - 0.0007 0.11043

$Sy/x = sqrt(sum(y- y\pi)^2 /(n-2)$

Sy/x= 0.42901

$Sb= Sy/x/sqrt (sum(x-x)^2$

Sb=0.0339

Sb= 0.5756

Slope confidence limit= 2.0898 $\pm$ (3.18*.034) = 1.982 to 2.198

Intercept confidence limit= 2.9714 $\pm$ (3.18*0.576) = 1.141 to 4.8018

LIMIT OF DETECTION AND QUANTIFICATION

Average of 7 intercepts = 2.9714 SD = 1.664502

LIMIT OF DETECTION = 2.9714 + 3(1.7) = 4.9935 $\approx$ 2.4 $\mu g\ I_2/ml$

LIMIT OF QUANTIFICATION = 2.97 +(10*1.7) 16.645 $\approx$ 8.0 $\mu g\ I_2/ml$

DETERMINATION OF MINERALS BY INDUCTIVELY COUPLED PLASMA ATOMIC EMISSION SPECTROPHOTOMETRIC METHOD

(Applicable to determination of Al, Sb, As, Ba, Be, Cd, Ca, Cr, Co, Cu, Fe, P, Pb, Mg, Mn, Mo, Ni, K, Se, Ag, Na, Tl, V, and Zn)

The method has been imported from EPA and inserted under Food Section of AOAC. Currently, undergoing final validation

1 PURPOSE/SCOPE

This method is applicable to the simultaneous determination of elements in a variety of foods by using ICP-AES.

2 SAFETY

Sample preparation using hydrogen peroxide gives rise to a vigorous reaction and should be carried out in fume hood.

3 REFERENCES

3.1 An Interlaboratory Study of Inductively Coupled Plasma Atomic Emission Spectroscopy Method 6010 and Digest Method 3050, NTIS No. PB88-124318.

3.2 National Technical Information Service, 5285 Port Royal Road, Springfield, VA22161. JAOAC 73, 404 (1990).

4 DEFINITIONS

4.1 *Test sample* refers to the laboratory sample before sample separation

4.2 *Test solution* refers to a solution of analytical sample taken for analysis

4.3 *Blank sample* refers to a sample which contains no detectable level of the analyte or a complete analysis without the analyte

5 PRINCIPLE

Multielement can be measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES). The sample is aspirated into argon plasma whose temperature is about 8000oK. At this temperature, electrons in the atoms are excited to higher energy levels. As these electrons drop back to their ground states, they emit light at specific wavelength is proportional to the concentration of that metal in the sample.

6 REAGENTS

6.1 Deionised water

6.2 Nitric Acid, 65%, AR

Nitric acid, 50% (v/v): Add 500 mL conc. HNO_3 to 400 mL water and dilute to 1 L.

6.3 Hydrochloric acid, AR

Hydrochloric acid, 50% (v/v): Add 500 mL conc. HCL to 400 mL water and dilute to 1 L.

6.4 Hydrogen peroxide, 30%

6.5 Argon gas, Welding grade

6.6 Calibration blank

Add 20 mL 50% HNO_3, (6.2), and 100 mL 50% HCl, (6.3) and dilute to 1 L.

6.7 Reagent blank

Prepare and process the same as for samples, except exclude food material

6.8 Standard reference solutions, certified

NOTE**:** Certified mixed element standards can be purchased from National Institute of Standards and Technology (NIST) or other sources. If mixed element standards are prepared from single element solutions, the latter should be analyzed separately to determine contribution of any relevant contaminants or interferences. When mixed standards are prepared, compatibility of cations and anions must be considered in order to produce stable solutions.

6.8.1 Prepare concentrations in calibration ranges by diluting portions of mixed standards to 100 mL calibration blank solution (6.6).

6.8.2 For Ag of concentrations above 2 mg/L, use more than 10 mL 50% HCl per 100 mL.

6.8.3 Store diluted standard solutions in acid-cleaned FEP fluorocarbon bottles, acid-cleaned polyethylene bottles, or acid-cleaned polypropylene bottles.

6.9 Instrument check standard solution

Prepare by diluting mixed standards near midpoint of calibrated ranges to 100 mL solution 6.6.

6.10 Interference check standard solution

Prepare 100 mL aqueous solution of 2 mL 50% HNO_3 and 10 mL 50% HCl, and (per liter) 500 mg Al, Ca, and Mg, 200 mg Fe, 5mg Pb, and 1 mg Ba, Be, Cd, Cr, Co, Cu, Mn, Ni, Ag, T1, V and Zn.

NOTE: This solution is used to test if interference correction factors yield data within ±3 standard deviations of calibration means.

6.11 Quality control standard solutions

Prepare from reference sources with analyte concentrations near 10x instrument detection limits (Table 990.08 B), and dilute to 100mL solution 6.6

7 Instrument and glassware

NOTE: Glassware can be acid-cleaned by soaking detergent-cleaned ware for 2h in 50% HNO_3, rinsing in water, soaking for 2h in 50% HCl, and rinsing in water. Any alternative procedure shown to minimize blank levels is acceptable. Avoid use of chromic acid for cleaning plastic and when Cr is an analyte.

7.1 Apparatus

7.1.1 Inductively coupled plasma atomic emission spectrometer, Computer-controlled with background correction capacity and radio frequency generator.

7.1.2 Conical Beakers, 250 ml

7.1.3 Watch glass

7.1.4 Filter paper, Whatman® No. 41
7.1.5 Hot plate or Steam bath
7.1.6 Centrifuge, capable of 200rpm, *(optional)*

8 PROCEDURE

8.1 Sample Preparation

8.1.1 Weigh 1-2 g test portion of homogenized material to nearest 0.01 g (wet basis), and transfer to 250 mL beaker.

8.1.2 To express results in dry weight basis, dry another portion of material to constant weight to determine wet/dry weight ratio. NOTE: Do not digest and analyse this portion because dried portion could be altered.

8.1.2.1 Add 10 mL 50 % HNO_3 to undried test portion and mix. Cover beaker with watch glass, heat to 95^{o}C. Let solution reflux for 10-15 minutes.

8.1.2.2 Let digest cool, add 5 mL conc. HNO_3, cover, and let solution reflux for another 30 minutes at 95^{o}C.

8.1.2.3 Let digest cool, add 50 ml concentrated HNO_3, cover, and let solution reflux for another 30 minutes at 95^{o}C.

8.1.3 Evaporate solution to ca 5 mL and avoid drying of sample (ribbed watch glass allows evaporation and protects beaker contents from dust).

8.1.4 Let solution cool, add 2 mL water and 3 mL 30% H_2O_2, cover with watch glass, and heat slowly (to avoid losses by excessive reaction) to initiate peroxide reaction. Continue heating beaker until effervescence subsides. Let solution cool, and add 7 mL 30% H_2O_2, 1 mL at a time, while warming (so that all samples will receive 30% H_2O_2 and reagent blank contribution from H_2O_2 will be constant).

8.1.5 Let solution cool, add 5 mL conc. HCl, and 10 mL water, cover with watch glass and let solution reflux for another 15 minutes without boiling. Let solution cool, dilute to 100mL water, and mix.

8.1.6 Remove any particulate matter in digest by filtration, centrifugation or settling. If any analyte level exceeds linear range, dilute digest further with calibration blank solution.

8.2 Determination

8.2.1 Follow instrument's manufacturer instructions for general guidance in setting operating conditions.

8.2.2 Establish instrument sensitivity, detection limit, precision, linear range, and interference effects for each wavelength used in analysis. Verify that operating conditions satisfy analytical requirements. Record quality control data to confirm instrument performance.

8.2.3 After instrument response is stable (usually 30 minutes after plasma is started), determine profile and calibrate instrument according to manufacturer's instructions.

8.2.4 Flush system for ≥1 min with calibration blank solution before introducing standards or sample solutions.

8.2.5 System

8.2.5.1 Verify calibration accuracy (±10%) by analysis of certified reference solutions as quality control standards before any test samples are analysed.

8.2.5.2 Analyze instrument check standards and calibration blank before each 10 test samples to confirm that calibration is still acceptable.

8.2.5.3 Verify inter-element and background corrections (±3 standard deviations of mean) by analysis of interference check standard at beginning and end of analysis session and at least twice in each 8 h period.

8.2.5.4 Recommended quality control includes at least one reagent blank for each batch of test samples, and one duplicate digest for each 20 test samples.

8.2.5.5 For new or unusual matrixes, absence of significant interference is indicated by 5-fold dilution that is within 10% of expected value (provided concentrations are not near detection limit) and/or by spike added to digest that is recovered with 10% of expected value (spike level should be 2-4 times unspiked concentration). When matrix interference is indicated, dilution or the method of standard additions can usually compensate for the effect.

8.2.5.6 Recommended wavelengths and typical instrumental detection limits are given in Table 990.08B. Other wavelengths may be used if they provide the needed sensitivity and are treated with the same correction techniques for spectral interferences.

8.2.6 The method is suitable for determining many elements but only those elements for which interlaboratory performance data are available are listed.

9 CALCULATION

Calculation involves subtraction of reagent blank values and adjustment for wet weight of test portions (multiply by wet/dry weight fraction), digest volume and any additional dilutions.

$$C_s = (I_c - B) \times D \times R$$

Where B = reagent blank concentration, mg/L

Cs = concentration of element in test portion, mg/kg

D = dilution factor (= 50 when preparation of 2 g test portion results in 100 mL solution and no extra dilution is required)

Ic = test solution digest concentration from instrument, mg/L; and R = wet weight/dry weight ratio for test portion.

Table 990.08B
Recommended Wavelengths and Estimated Instrumental Detection Limits

Analyte	Chemical Abstracts Registry No.	Interfering Elements	Recommended Wavelength, nm	Estimated Instrumental Detection Limit, μg/L
Aluminum	7429-90-5	Mn, V	308.215	45
Antimony	7440-36-0	Al, Cr, FE, Tl, V	206.833	32
Arsenic	7440-38-2	Al, Cr, V	193.696	53
Barium	7440-39-3		455.403	2
Beryllium	7440-41-7	Tl, V	313.042	0.3
Cadmium	7440-43-9	Fe, Ni	226.502	4
Calcium	7440-70-2	Cr, Fe, Mg, Mn, Tl, V	317.933	10
Chromium	7440-47-3	Fe, Mn, V	267.716	7
Cobalt	7440-48-4	Cr, Fe, Ni, Tl	228.616	7
Copper	7440-50-8	Fe, Tl,V	324.754	6
Iron	7439-89-6	Mn	259.940	7
Lead	7439-92-1	Al	220.353	42
Magnesium	7439-95-4	Ca, Cr, Fe, Mn, Tl, V	279.079	30
Manganese	7439-96-5	Al, Cr, Fe, Mg	257.610	2
Molybdenum	7439-98-7	Al, Fe	202.030	8
Nickel	7440-02-0		231.604	15
Phosphorus			177.499	18
Potassium	7440-09-7	Mg	766.491	599
Selenium	7782-49-2	Al, Fe	196.026	75
Silver	7440-224		328.068	7
Sodium	7440-23-5	Tl	588.995	29
Thallium	7440-28-0	Al	190.864	40
Vanadium	7440-62-2	Cr, Fe, Tl	292.402	8
Zinc	7440-66-6	Cu, Ni	213.856	2

a Underlined elements indicate apparent (false) analyte concentrations exceeding μg/L caused by 100 mg/L of the interfering element.

DETERMINATION OF VITAMIN A, VITAMIN E AND β-CAROTENE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

1. PURPOSE/SCOPE

1.1 Used for the quantitative determination of Vitamin A (retinol), Vitamin E (tocopherol) and β-carotene in a variety of food e.g. milk, dried milk products, cheese, eggs, dried egg products, vegetables, potatoes, meat, fish and ready-cooked meals, beverages and butter, margarine and the oil and fat products by High Performance Liquid Chromatography (HPLC)

1.2 The method is also used for determination of β-carotene in fruits and vegetables.

2. SAFETY/PRECAUTION

2.1 Vitamin A, vitamin E, β-carotene are sensitive to UV radiation and oxygen. All procedures must be done under subdued light. Use amber glassware, UV-absorbent or aluminum foil for proper protection. Solutions must be evaporated at reduced pressure and nitrogen flushing by means of a rotary evaporator at a temperature below 40^{o} C.

2.2 Pyrogallol, vitamin A acetate and methanol, petroleum benzene are toxic; avoid inhalation, skin or eye contact.

2.3 Digestion, extraction of samples should be carried out in fume hood.

3. REFERENCES

3.1 Horwitz W. 2000. Official Method of Analysis of AOAC International. 17th Edition. Vol. II AOAC International. Maryland, USA.

3.2 Brubacher G, Muller-Mulot W and Southgate DAT. 1985. Methods for the determination of vitamins in food. Elsevier Applied Science Publishers. London.

3.3 Sullivan DM, Carpenter DE. 1993. Methods of Analysis for Nutrition Labeling. AOAC International, Arlington, Virginia USA. Pp. 549-559.

3.4 Tee ES, Kuladevan R, Young SI, Khor SC and Zakiyah O. 1996. Laboratory procedures in Nutrient analysis of foods. Division of Human Nutrition, Institute for Medical Research, Kuala Lumpur.

3.5 Thaifoods. 2002. Methods of Food Analysis. Thailand.

3.6 FNRI. 2001. Analytical Methods Manual. Food Analytical Service Laboratory, Food Quality and Safety Section.

3.7 APFAN 1994. Methods Manual. APFAN Second Food Analysis Workshop, Brisbane, Australia. P 1-8.

3.8 Ball GFM. 1988. Fat-Soluble Vitamin Assays in Food Analysis A Comprehensive Review. Elsevier Applied Science. London.

4. DEFINITION

4.1 Vitamin A content in this method refers to all-trans retinol (vitamin A alcohol) as determined by the method described here, expressed as μg per 100 g of sample. 1 μg of all-trans-retinol or 1.1467 μg retinyl acetate corresponds to 3.33 IU; 1 μg retinyl acetate corresponds to 2.90 IU.

4.2 The content of vitamin E is refers to α-tocopherol content determined by the procedure described here. It is given in mg of α-tocopherol/ 100 g of sample. 1 mg of d-α-tocopherol (2R, 4'R, 8'R-α-tocopherol or RRR-α-tocopherol) is equivalent to 1.49 IU; 1 mg of dl-α-tocopherol (all-rac-α-tocopherol, fully synthetic) is equivalent to 1.1 IU; 1 mg of all-rac-α-tocopheryl acetate is 1.0 IU.

4.3 β-carotene content determined by the method described here is expressed as μg/ 100 g of sample.

5. PRINCIPLE

After homogenisation and saponification of the material under investigation in a solution of ethanolic potassium hydroxide, the retinol (vitamin A alcohol), tocopherol (vitamin E) and β-carotene released is totally extracted with organic solvents. Separation of the retinol, tocopherol and β-carotene content is done with part of the extract by reversed-phase HPLC. Quantitation is carried out against an external vitamin A, vitamin E and β-carotene standard that has undergone the same procedure as the sample.

6. REAGENTS

6.1 Ethanol, (99.98% v/v) Analytical grade

6.2 Potassium hydroxide (KOH) Analytical grade, 100% (w/v): dissolve 70 g KOH pellets in 70 ml deionized water and dilute to 50 ml with 95% ethanol, freshly prepared.

6.3 Potassium hydroxide (KOH), Analytical grade, 5% (w/v): dissolve 5 g KOH in 100 ml deionized water.

6.4 Antioxidant: Pyrogallol or Hydroquinone or BHT or Sodium ascorbate, or Ascorbic acid: dissolve 10 g ascorbic acid in 100 ml deionized water, freshly prepared.

6.5 Extraction solvent : Hexane or Petroleum ether (petroleum benzene) and Hexane mixture (2 :1, petroleum ether: Hexane) Analytical grade

6.6 Phenolphthalein indicator 1% in alcohol

6.7 Sodium sulphate, anhydrous, Analytical grade

6.8 HPLC mobile phase: Methanol (90 to 100% in water) or Acetonitrile/Methanol/Ethyl acetate (88:10:2), HPLC grade

6.9 Nitrogen gas, Oxygen Free Nitrogen grade

6.10 Standards

6.10.1 all-trans retinol or retinyl acetate, or all trans retinol palmitate

6.10.2 dl-α-Tocopherol

6.10.3 all-trans β-carotene

7. APPARATUS

7.1 UV-VIS Spectrophotometer

7.2 High Performance Liquid Chromatography (HPLC)

7.3 Column
- 7.3.1 for vitamin A and tocopherol: C18 Hypersil® H5ODS, 25 cm x 4.6 mm with guard column or Ultracarb 5ODS, 150x46 mm. with guard Hypersil BDS C-18 5 um 10 X 4 mm or Pinnacle Amine ODS C18 (5 μm) 250 x 4.6 mm or μ-Bondapack C18, 3.9 x 300 mm.
- 7.3.2 for β-carotene: Vydac 201TP54, 250x46 mm. with guard Hypersil BDS C-18 5 μm 10 X 4 mm or Purosphere RP –18e, 5 μm, 4 x 125 mm. or 250 x 4.6 mm or μ-Bondapack® C18, 3.9 x 300 mm.

7.4 Membrane filter, PTFE diameter 1.30 mm pore size 0.45 μm
7.5 Digestion apparatus: flat bottom flask 100 and 250 ml, heating mantle, condenser
7.6 Vacuum rotary evaporator
7.7 Solvent filtration apparatus
7.8 Syringe 10 ml for sample filtration
7.9 Analytical balance
7.10 Round bottom flask 250 ml
7.11 Volumetric flask, 10, 25, 50, 100 ml
7.12 Separating funnel, 250 ml
7.13 Beaker, 5, 25 ml
7.14 Volumetric pipette, 1, 2, 3, 4, 5, 10 ml
7.15 Spatula
7.16 Funnel
7.17 Cylinder, 100 ml
7.18 Stirring rod
7.19 Washing bottle
7.20 Glass bead
7.21 Whatman® No. 1

8. PROCEDURE

8.1 Standard preparation
- 8.1.1 Standard Vitamin A: weigh 1 mg of retinol into a 100 ml volumetric flask, dissolve with small amount of chloroform until all retinol is dissolved, then dilute to mark with ethanol and mix. Dilute this solution ten times in another volumetric flask. Read absorbency of this final solution with UV-spectrophotometer at 325 nm and calculate concentration of retinol by extinction coefficient E (1% in ethanol at 325 nm) = 1850 (1835). Dilute this final solution further to a concentration of about 0.2-1.0 μg/ml.
- 8.1.2 Standard Vitamin A: weigh about 25 mg of retinyl acetate into a 5 ml beaker; dissolve to concentration about 4 μg/ml with 99.8% ethanol. Dilute 1 ml of this standard to 50 ml with 99.8% ethanol. Read absorbency of this final solution with UV-spectrophotometer at 325 nm and calculate concentration of retinol acetate by extinction coefficient E (1% in ethanol at 325 nm) = 1550.
- 8.1.3 Standard Tocopherol: weigh 25 mg of *alpha*- tocopherol into a 25 ml volumetric flask, dissolve with small amount of $CHCl_3$ until all crystal is dissolved, then dilute to mark with ethanol and mix. Dilute this solution ten times in another volumetric flask (10 ml to 100 ml).

Read absorbency of this final solution with UV-spectrophotometer at 292 nm and calculate concentration of tocopherol by extinction coefficient E (1% in ethanol at 292 nm) = 71 Dilute this solution further to concentration about 2-50 μg/ml.

8.1.4 Standard β-carotene: weigh 1.0 mg of β-carotene into a 25 ml volumetric flask, dissolve with hexane until all β-carotene is dissolve, and then dilutes to mark with hexane. Read absorbance at 450 nm and calculate concentration of β-carotene by extinction coefficient E (1% in hexane at 450 nm) = 2592. Dilute this solution further to concentration about 1-50 μg/ml. Purity of standard β-carotene was checked by inject the standard to the HPLC. If there is more than one peak, it should be repurified by crystallization.

8.2 Preparation of standard calibration curve

If desired, prepared calibration curve for vitamin A, Vitamin E and β-carotene by injecting different concentration of each vitamins into the HPLC and plotting a curve of amount of vitamin versus peak area (or peak height).

8.3 Preparation of sample

The sample must be comminuted as finely as possible (e.g. using chopper, mincer, etc.) and homogenised. It must be analysed immediately and quickly. If this can not be done, place homogenized sample in brown bottle, flush with nitrogen, seal and store in freezer until use for analysis.

8.4 Preparation of test portion

Weigh accurately in duplicate 0.5-1 g (milk powder) or 5-10 g (liquid sample) (depending on vitamin A, vitamin E and β-carotene concentration) in a brown 250 ml round bottom flask. For powder sample, rehydrate by adding an equal amount of water; mix thoroughly to disperse any lumps.

8.5 Saponification

8.5.1 Add 1 g sodium ascorbate or 10 ml ascorbic acid solution or 0.1 g pyrogallol or 100 mg hydroquinone. Add a volume (in ml) of 95% ethanol equal to 4 times the weight (in g) of sample ($\geq$ 40 ml) and a volume of 100% KOH equal to the weight of the sample. Mix well after adding each reagent, and add 3 glass beads.

8.5.2 Reflux on a boiling water bath (80^{o}C) or digestion apparatus for 30 minutes. Stir continuously (using magnetic stirrer) or swirl several times.

8.6 Extraction

8.6.1 Cool the flask rapidly to room temperature. Add 70 ml petroleum ether (or 50 ml hexane or 70 ml petroleum ether: hexane (2:1)) or 100 ml hexane/acetone (60/40) and shake vigorously.

8.6.2 After separation of the two layers, transfer the upper layer into a brown 250 ml separating funnel. For high fat sample place 50 ml 5% KOH solution in to the separating funnel.

8.6.3 Re-extract the sample in saponification flask 2 times with 40 ml each of extraction solvent and combine the upper layers into the separating funnel. Shake the separating funnel and let the layers separate. Discard the lower layer.

8.6.4 Wash the extract with 80-100 ml water until the discarded water is alkaline free (about 3 times).

8.6.5 Dry the extract with sodium sulphate or strips of filter paper. Transfer all extract into a 250 ml brown round bottom flask.

8.7 Evaporation

8.7.1 Dry extraction solvent by means of a rotary vacuum evaporator (<40^{o}C) and flush with nitrogen gas.

8.7.2 Dissolve the residue immediately with mobile phase and dilute to a known volume (V). Filter through a 0.45 μm membrane using solvent filtration apparatus.

8.8 Recovery study

Recovery study can be carried out by simultaneous analysis of sample spike with a known amount of standard (equal to the amount of the vitamin expected to be present in the test aliquot). The difference in the vitamin level for the spiked and the unspiked sample, express as percentage of the amount of the vitamin added, gives the percent recovery.

8.9 Extraction of Standard (if retinyl acetate is used)

Pipette 25 ml of standard retinyl acetate into a round bottom flask, add 0.1 g pyrogallol, 5 ml 50% KOH, 3 glass beads, bring to reflux and extract as in 8.3 – 8.7.

8.10 HPLC condition

HPLC condition for vitamin A

Column/Mobile phase	RP ODS C18/ CH_3OH: water (90-100: 10-0) or μ Bondapack/ Acetonitrile/Methanol/Ethyl acetate (88:10:2) or μ Bondapack/ CH_3OH: water (90: 10) or Novapak C18/ CH_3OH: water (90: 10) or Inertsil ODS-3 /CH_3OH: THF (96.5:3.5)
Injector volume	20 μl
Flow rate	1 - 1.3 ml/min
Detector	UV at 325 nm

HPLC condition for vitamin E

Column/ Mobile phase	RP ODS C18 /CH_3OH: water (93-100: 7-0) or μ Bondapack/ Acetonitrile/Methanol/Ethyl cetate (88:10:2)
Injector volume	20 μl
Flow rate	1 - 1.5 ml/min
Detector	UV at 292 nm

HPLC condition for β-carotene

Column/ Mobile phase	Vydac 201TP54, 250x46 mm./ CH_3OH : THF (99.5: 0.5) or Purospher RP-18e, 4 x 125 mm/ Acetonitrile: isopropanol: dichloroethane (92.5: 5: 2.5) or Acetonitrile: methanol: THF = (40: 55: 5) or μ Bondapack/ Acetonitrile/Methanol/Ethyl acetate (88:10:2) or CH_3CN: CH_3OH: $CHCl_3$ (49: 49: 2) or Acetonitrile: Methanol: Chloroform (89:9:2)
Injector volume	20 μl
Flow rate	1.0 - 1.5 ml/min
Detector	VIS at 450 nm

9. CALCULATIONS

The content of total vitamin A in the material being analysed is calculated from the peak height (or areas) of retinol in the injected volume (20 μl) of sample solution and standard solution:

$$\text{Retinol (μg/100g)} = \frac{100 \times V \times D}{W}$$

$$\text{Tocopherol (mg/100g)} = \frac{100 \times V \times D}{W}$$

$$\text{β-carotene (μg/100g)} = \frac{100 \times V \times D}{W}$$

where V = total volume (ml)
W = weight of sample (g)
D = dilution factor (if any)

If calibration curve is prepared, read the amount of retinol (μg/ml) or tocopherol (μg/ml) and β-carotene (μg/ml) from calibration curve and calculate to μg or mg per 100 g of sample.

If results are to be reported in IU the following conversion factors can be used:

Vitamin A (IU/100 g) = Retinol (μg/100 g) / 0.3
Vitamin E (IU/100 g) = Tocopherol (mg/100 g) / 0.909

10. ACCEPTANCE OF RESULTS

10.1 HPLC Instruments should be check for accuracy e.g., flow rate, injection volume, detector wavelength
10.2 Duplicate results should have a difference of less than 10%
10.3 Accuracy & Precision Control:

10.3.1 Standard Reference Material such as 1846 'Infant Formula' from National Institute of Standards & Technology, Gaithersburg, MD 20899, USA. 1996, or CRM white bread.

10.3.2 In-house control sample such as 'powder milk' prepared in the laboratory. Control chart should be within ± 2 SD of the established mean value.

10.4 % recovery should be 100 ± 20%

10.5 % CV should be less than 20%

11. APPENDIX/ SUPPLEMENTARY NOTE

11.1 If it is desired to analyse for other carotenoid such as α-carotene and lycopene, find the standard for that carotenoid and prepared standard solution for comparing with test sample. These additional standards can be mixed with the β-carotene standard and injected together in to the HPLC.

11.2 Plant food may also be blended with about 3 g hyflosupercel, 0.1 g $MgCO_3$, 80 ml ethanol and 60 ml hexanes for 2 minutes. Filter the mixture under suction using Buchner funnel with Whatman filter paper # 41, collecting the extract. Re-extract using another 80 ml ethanol and 60 ml hexane, combine the extract and use this for saponification (8.5 – 8.7).

11.3 For plant sample, cold saponification can also be used. Use 10% methanolic KOH and a pinch of BHT for saponification in the dark at room temperature for 16-18 hr.

11.4 Alternative method for analysis of β-carotene in food products is separately provided.

DETERMINATION OF BETA-CAROTENE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (ALTERNATIVE METHOD)

1. PURPOSE/ SCOPE

This method is used for the quantitative determination of β-carotene in a variety of food products.

2. SAFETY/ PRECAUTION

2.1 β-carotene is sensitive to UV radiation and oxygen. All procedures must be done under subdued light. Use amber glassware, UV-absorbent or aluminum foil for proper protection. Solutions must be evaporated at reduced pressure and nitrogen flushing by means of a rotary evaporator at a temperature below 40^{o} C.

2.2 Digestion, extraction of samples should be carried out in fume hood

2.3 THF is toxic; handle with extreme caution

2.4 Upon standing for sometime, THF may contain substances which may oxidise carotene; use freshly open bottle.

3. REFERENCE

Shof PJM, Xu C, van de Bovenkamp P, Muhilal and West CE. 1997. Application of a Validated Method for the Determination of Provitamin A Carotenoids in Indonesian Foods of Different Maturity and Origin. J Agric Food Chem. 45: 1174-1179.

4. DENITION

β-carotene content determined by the method described here is expressed as μg per 100 g of sample.

5. PRINCIPLE

The carotenoids of the ground food sample are first extracted repeatedly using tetrahydrofuran containing BHT in the present of sodium sulfate, calcium carbonate and analysed by HPLC.

6. REAGENTS

6.1 Sodium sulfate
6.2 Calcium carbonate
6.3 Tetrahydrofuran (THF)
6.4 THF/ methanol (1:1)
6.5 BHT 0.01%
6.6 Mobile phase: Methanol:THF (96.5:3.5) or (98: 2)
6.7 Standard: all-trans β-carotene

7. APPARATUS

7.1 UV-VIS Spectrophotometer
7.2 High Performance Liquid Chromatography (HPLC)

7.2.1 Column
Vydac® 218TP54, 250x46 mm. with guard 10 X 4.6 mm pack with the same material as analytical column or Inertsil® ODS-3; 5um, TQ5 – 1556, 4.6 ID x 150 mm
7.2.2 Membrane filter, PTFE diameter 1.30 mm pore size 0.45 μm
7.3 Vacuum rotary evaporator
7.4 Solvent filtration apparatus
7.5 Syringe 10 ml for sample filtration
7.6 Analytical balance
7.7 Volumetric flask, 10, 25, 50, 100 ml
7.8 Beaker, 5, 25 ml
7.9 Volumetric pipette, 1, 2, 3, 4, 5, 10 ml
7.10 Spatula
7.11 Glass funnel
7.12 Cylinder, 100 ml
7.13 Whatman® No. 54

8. PROCEDURE

8.1 Homogenise the sample using a mixer
8.2 Extract the sample (2 g) in duplicate with Na_2SO_4 (4.0 g), $CaCO_3$ (0.3 g), and tetrahydrofuran containing 0.01%BHT (30 ml) in a 100ml measuring cylinder, using a rod mixer at moderate speed for 1 min.
8.3 Filter the extract through a glass funnel fitted with Whatman® filter paper No. 54 (diameter 11 cm).
8.4 Re-extract the extract until colourless (usually two additional extractions with 30 ml of THF were sufficient)
8.5 Reduce to near dryness of the volume of the combined filtrate under nitrogen in a rotary evaporator.
8.6 Transfer the concentrated filtrate to a 25 ml volumetric flask
8.7 Bring to volume with THF/methanol (1:1 v/v)
8.8 Filter the sample solution through an HPLC filter
8.9 Inject 20 μl of the filtered solution into the HPLC system.
Set up the instrument's operational conditions:

Column/ Mobile phase	GL Sciences Inc. – Inertsil ODS-3; 5um TQ5 – 1556, 4.6 ID x 150 mm/ 96.5:3.5 Methanol: THF or Vydac 218TP54, 250x46 mm. / 98:2 Methanol: THF
Column temp	25°C
Flow Rate	1.00 mL/min
Injection Volume	20 μl
Wavelength	450 nm

8.10 Freshly prepare stock standard (1000 ppm)
8.11 Standard check of beta carotene standard – 6 ppm (Routine)
8.12 Perform a standard one point check using working standard 6 ppm on routine sample analysis for every batch of sample.

8.13 Perform beta carotene standard curve calibration on a non-routine basis i.e. when the check standard is found to be out of the acceptable limit of ± 5% or the standard check solution had expired.

9. CALCULATIONS

9.1 Integrate the areas under the peak for the calibration standards.
9.2 Plot the areas under the peaks against the corresponding concentrations.
9.3 Determine the linear equation of peak area (y-axis) vs. concentration of β-carotene (x-axis) and its coefficient of linearity, R^2, which is to be 0.99 or higher.
9.4 Re-calibrate if linear regression is less than 0.99
9.5 Determine the slope of the calibration line.
9.6 Divide the peak area of the unknowns by the value of the slope to give the concentration of β-carotene for the unknown test solutions.
9.7 Multiply the concentration obtained by volume of test solution.
9.8 Divide the weight of β-carotene (in μg) by the weight of sample (g) to give the value in μg/g or mg/kg as required.
9.9 Express the test result in μg β-carotene/100 g sample
9.10 Calculate the concentration of β-carotene in the samples as follows:

$$C_m = \frac{C_s \times V \times 100}{W}$$

where, C_m = concentration of β-carotene in sample, μg/100 g
C_s = test solution concentration from instrument, μg/mL
V = volume of sample solution, mL
W = weight of sample taken, g

10. ACCEPTANCE OF RESULTS

10.1 HPLC Instruments should be check for accuracy e.g., flow rate, injection volume, detector wavelength
10.2 Duplicate results should have a difference of less than 10%
10.3 Accuracy & Precision Control:
  10.3.1 Standard Reference Material such as 1846 'Infant Formula' from National Institute of Standards & Technology, Gaithersburg, MD 20899, USA. 1996, or CRM white bread.
  10.3.2 In-house control sample such as 'powder milk' prepared in the laboratory. Control chart should be within $\pm$ 2 SD of the established mean value.
10.4 % recovery should be 100 $\pm$ 20%
10.5 % CV should be less than 20%

DETERMINATION OF THIAMIN BY FLUOROMETRIC METHOD

1. PURPOSE/SCOPE

To determine Thiamin (vitamin B_1) content by spectrofluorometric method (Thiochrome method) in foodstuffs. Results are expressed as milligram per 100 g food sample.

2. SAFETY

When using a hazardous reagent such as concentrated acid or alkali, gloves should be worn, eye protected, and the work done in a fume hood.

3. REFERENCES

3.1 Horwitz, W. (editor) Official Methods of Analysis of AOAC International, 17th ed., Vol. 2., Thiamin Fluorometric Methods 942.23 Ch. 45 pp. 6-7, 957.17 Ch. 45 pp. 8-9, 2000.

3.2 Ellefson, W.C., Thiamin, In : Augustin, J., Klein, B.P., Beker, D.A., and enugopal, P.B. (editor) Methods of Vitamin Assay, 4th ed., John Wiley & Sons, Inc., New York, U.S.A., 349-363, 1985

4. DEFINITION

The names thiamin, aneurine and vitamin B_1, and the obsolete terms polyneuramin, antiberiberi vitamin and antineuric vitamin refer to a single compound. Thiamin consists of a substituted pyrimidine and thiazole ring connected by a CH_2 bridge and has the empirical formula $C_{12}H_{17}N_4O_2$. Thiamin can occur in a variety of forms: as free thiamin, as a protein complex, as mono-, di-, or triphosphate esters, or as a phosphorus protein complex. Products such as nuts, pork, yeast, and cereal germs are especially rich in this vitamin.

5. PRINCIPLE

Thiamin in the foodstuff is first extracted using dilute acid; the acid medium also serves to stabilise the vitamin in the extract. An enzyme preparation is then added to aid in the conversion of bound thiamin to its free form. The phosphatase will hydrolyse any phosphate esters of thiamin that may be present, while the diastase facilitates the liberation of the vitamin from starchy products. The extract is next purified by passing through a cation exchange column (Biorex or Decalso). Thiamin will first be adsorbed onto the column, thereby separating it from any substances which might interfere with subsequent steps in the determination. Acid potassium chloride is then passed through the column to elute the adsorbed thiamin. Thiamin in the potassium chloride solution is then treated with alkaline potassium ferricyanide to oxidise it to thiochrome. The so-formed thiochrome is extracted into isobutyl alcohol and its fluorescence measured in a fluorometer.

6. REAGENTS

6.1 2N sodium acetale

Dissolve 272 g $CH_3COONa.3H_2O$ in enough H_2O to make 1 L.

6.2 Bromocresol green pH indicator
Dissolve 0.1 g indicator by triturating in agate mortar with 2.8 mL 0.05M NaOH, and dilute to 200 ml with H_2O. Transition range: 4.0 (green)-5.8 (blue)

6.3 Bromophenol blue indicator
Dissolve 0.1 g indicator by triturating in agate mortar with 3.0 mL 0.05M NaOH, and dilute to 250 mL with H_2O. Transition range: 3.0 (yellow)-4.6 (blue)

6.4 Enzyme solution
Prepare on day on which it is to be used, 10% aqueous solution of enzyme preparation potent in diastatic and phosphorolytic activity. (Among enzymes available for this purpose are Mylase 100 (U.S. Biochemical Corp) Takadiastase (Pfalz and Bauer) and α-amylase (Miles Laboratories, Inc.,) Check each batch for performance against thiamin mono-,di- and triphosphate. Evaluate % recovery compared with USP Reference Standard Thiamin. If recovery is >85%, enzyme preparation is suitable.

6.5 Bio-Rex 70 (hydrogen form)–50-100 mesh. (when use Decalso, see appendix)
Add 300 mL 2M HCI to 50 g Bio-Rex 70 (Bio-Rad Laboratories), stir 15 min, decant, and repeat. Add 300 mL H_2O, stir 1 min decant, and repeat until pH of H_2O is 4.5-7.0. H_2O should be free of suspended resin when allowed to settle 15 seconds. If not, repeat H_2O washing until clear.

6.6 Chromatographic columns (when use Decalso, see appendix)

6.6.1 Use glass chromatographic tubes (ca 275 mm overall length, with reservoir capacity ca 60 mL) consisting of 3 parts fused together with following approximate dimensions (O.D. x length, mm):
- reservoir at top, 35 x 95,
- adsorption tube in middle, 8 x 145
- Capillary at bottom 35mm long and of such diameter that when tube is filled, rate of flow will be 1mL/min.

6.6.2 Prepare tubes for use as follows:
- Over upper end of capillary, with aid of glass rod, place fine glass wool.
- To adsorption tube, add H_2O suspension resin to height of approximately 100mm, taking care to wash down all silicate form walls of reservoir.
- To keep air out of adsorption column, keep layer of liquid above surface of silicate during adsorption process. (Prevent tube from draining by placing rubber cap, filled with H_2O to avoid inclusion of air, over lower end of capillary.)

6.7 Neutral potassium chloride solution
Dissolve 250 g KCI in H_2O to make 1 L

6.8 Acid potassium chloride solution
Add 8.5 mL HCI to 1 L of the neutral KCI solution.

6.9 Sodium hydroxide solution 15%
Dissolve 15 g NaOH in H2O to make 100ml.

6.11 Potassium ferricyanide solution, 1%
Dissolve 1 g $K_3Fe(CN)_6$ in H_2O to make 100mL. Prepare solution on day it is used

6.12 Oxidizing reagent
Mix 4.0 mL of the 1% $K_3Fe(CN)_6$ solution with the 15% NaOH solution to make 100 mL. Use solution within 4h.

6.12 Isobutyl alcohol

6.13 Quinine sulfate stock solution
Use quinine sulfate solution to govern reproducibility of fluorometer. Prepare stock solution by dissolving 10mg quinine sulfate in 0.05M H_2SO_4 to make 1 L. Store solution in light-resistant containers.

6.14 Quinine sulfate standard solution
Dilute 1 volume quinine sulfate stock solution with 39 volumes 0.05M H_2SO_4 (Solution fluoresces to ca same degree as does isobutanol extract of thiochrome obtained from 1 ug thiamin HCl). Store solution in light-resistant containers.

6.15 Thiamin hydrochloride standard solutions –

(1) Stock solution - 100 μg/mL.
Accurately weigh 50-60 mg USP Thiamin Hydrocloride Reference standard that has been dried to constant weight over P_2O_5 in a desiccator. (Reference standard is hygroscopic; avoid absorption of moisture). Dissolve in 20% alcohol adjusted to pH 3.5-4.3 with HCl and dilute to 500 mL with the acidified alcohol. Add enough additional acidified alcohol to make concentration exactly 100 μg thiamin HCl/mL. Store at ca 10°C in glass-stoppered, light-resistant bottle.

(2) Intermediate solution – 10 μg/mL.
Dilute 100 mL stock solution to 1 L with 20% alcohol adjusted to pH 3.5-4.3 with HCl. Store at ca 10°C in glass-stoppered, light-resistant bottle.

(3) Working solution – 0.2 μg/mL
Add ca 50 mL ca 0.1M HCl digest or autoclave as in 8.1(5) cool and dilute to 100ml with the 0.1M HCl. Prepare fresh solution for each assay. Take 20 mL, proceed as in 8.2(2) and chromatography as in 8.3.

7. APPARATUS

7.1 Spectrofluorometer
7.2 Autoclave
7.3 pH-meter
7.4 Incubator or water-bath
7.5 Erlenmeyer flask, 250ml
7.6 Volumetric flask 10, 25, 50, 250, 500 and 1000ml
7.7 Beaker 25ml
7.8 Pipette

8. PROCEDURE

8.1 Sample Extraction

8.1.1 Weigh 1-5 g of the well-homogenised foodstuff into 100 mL conical flasks. Each aliquot should contain about 10-30 μg thiamin.

8.1.2 Add about 65 mL 0.1N HCl to the flask.

8.1.3 Heat for 30 minutes in a boiling water bath or in an autoclave at 121-123°C for 30 minutes.

8.1.4 Mix contents of flasks frequently, making sure that all particles are evenly dispersed in the liquid.

8.2 Enzyme hydrolysis

8.2.1 Cool the acid extract to 50°C or lower.

8.2.2 Adjust pH to between 4.0 and 4.5 with 2.5M sodium acetate, using pH meter or bromocresol green indicator. End point should be definitely on the blue side of colour change.

8.2.3 Add 5 ml of the enzyme solution. Mix well and incubate 3 hours at 45-50°C or overnight at 35-37°C.

8.2.4 Cool to room temperature.

8.2.5 Adjust pH to approximately 3.5 using 1N HCl, using pH meter or bromophenol blue indicator. End point should be definitely on the blue side of colour change.

8.2.6 Transfer quantitatively into a 100 ml volumetric flask and make up to the mark with water.

8.2.7 Filter.

8.3 Chromatography (when use Decalso column, see appendix)

8.3.1 Pass an aliquot (25 ml) of filtered solution (containing ca 5 μg thiamin) through prepared chromatorgraphic column

8.3.2 Wash column with three 5 mL portions of almost boiling H_2O. Do not permit surface of liquid to fall below surface of resin.

8.3.3 Elute thiamin from resin by passing five 4.0-4.5 mL portions almost boiling (>60°C) acid-KCl solution through column. Do not permit surface of liquid to fall below surface of resin until final portion of acid-KCl solution has been added.

8.3.4 Collect eluate in 25 mL volumetric flask, cool and dilute to volume with acid-KCl solution. Designate this as Test Solution.

8.4 Oxidation of thiamin to thiochrome

In this and subsequent stages, undue exposure of the solution to direct sunlight or other sources of UV light must be avoided.

Pipette the test solution, standard solution, and other reagents as follows:

Tube No.	Std	Bl-Std	S	Bl-S
KCl or NaCl (g)	1.5	1.5	1.5	1.5
Sample solution (ml)	-	-	5	5
Std. Solution conc. 0.2 ug/ml (ml)	5	5	-	-
Oxidizing reagent (ml)	3	-	3	-
15% NaOH	-	3	-	3
	Swirl the tubes			
Isobutyl alcohol (ml)	13	13	13	13
	After add each tube shake vigorously at least 15 sec.			
	After add all tubes, shake. 2 min.			
	Centrifuge at low speed			
Pipette or decant isobutyl alcohol to Measure fluorescence	S	SB	U	UB

Note:
1. Use pipette that delivers 3 mL in 1-2 sec for addition of oxidising agent.
2. Set fluorometer emission at 365 nm and excitation at 435 nm.
3. Zero instruments with distilled water.
4. Check the fluorometer between readings with the standard solution to ensure that it gives the reading that was initially obtained.
5. If the decant isobutyl alcohol is not clear, do as follow.
   - Siphon out the lower layer
   - Add ca 2 g anhydrous Na_2SO_4 to each tube
   - Shake tube for a few seconds
   - Pipette upper layer to measure fluorescence.

9. CALCULATIONS

$$\text{Thiamin (mg per 100 g sample)} = \frac{U - UB}{S - SB} \times \frac{C}{A} \times \frac{25}{V} \times \frac{100}{wt.} \times \frac{100}{1000}$$

U = fluorescence reading of sample (tube S)
UB = fluorescence reading of sample blank (tube Bl-S)
S = fluorescence reading pf standard (tube Std)
SB = fluorescence reading of sample (tube Bl-Std)
C = concentration of the standard (μg)
A = test solution taken (mL)
25 = final volume of elute (mL)
V = volume used in purification step (mL)
100 = volume of original sample was made up to (mL)
wt. = sample weight (g)

10. ACCEPTANCE OF RESULTS

10.1 The sample results are acceptable if the control sample result is within the level of two standard deviations of the mean on QC chart.

10.2 Sample duplicate
where, d1 = duplicate 1, d2 = duplicate 2, and d1 $\geq$ d2;
then, $\frac{(d1-d2)}{d1} \times 100 \leq 5\%$

10.3 The standard addition or spiked recovery sample shall be between 90-110%.

11. APPENDIX/SUPPLEMENTARY NOTES

11.1 Purification by Decalso column

11.1.1 Preparation of the Decalso
Thiochrome Decalso (Fisher Scientific Co., Cat.no. T-97).
Activate the decalso as follows;

1) Place 100 g Decalso (60-80 mesh) in a beaker.
2) Add 100 mL hot 3% acetic acid and place on boiling water bath for 20 minutes, with frequent stirring. Pour off the acid
3) Repeat this washing procedure three times.
4) Next wash three times with hot 100 ml 25% KCl solution and placing the mixture on a boiling water bath for 20 minutes each time.
5) Finally wash with boiling distilled water on a Buchner funnel until the washings are free from chlorides.
6) Dry at 100^{o}C and keep in a stopper bottle.
7) A recovery test should be done on each fresh batch of Decalso.

11.1.2 Preparation of column for chromatography using Decalso
Use glass chromatographic tubes consisting of three parts fused together with the following approximate dimensions (O.D. x length, mm):

1) Reservoir at top, 35 x 95;
2) Adsorption tube in middle, 8 x 145;
3) Capillary at bottom, 35 mm long, and of such diameter that when tube is filled, rate of flow will be less than 1 ml/min.

Prepare tube for use as follows:

1) Place small glass wool or cotton plug over upper end of the capillary.
2) Prepare a suspension of Decalso in water.
3) Pour this suspension into the column so that the height of the adsorbent is about 10 cm in the adsorption tube.
4) Keep air out of the adsorption column by having a layer of *liquid above surface of Decalso at all times.*
5) Before performing chromatography of the test solution, pass 10 ml of 3% acetic acid through the column.

11.1.3 Chromatography

1) Pass an aliquot 5 - 25 mL (containing about 5 μg thiamin) of the filtered extract through the prepared column.
2) Discard the filtrate that percolates through the column.
3) Wash the column with three successive 10 mL portions of almost boiling water, discarding all washings.
4) Elute the adsorbed thiamin from the Decalso by passing five 4.5 mL portions of almost boiling acid potassium chloride solution through the column.
5) Collect eluate in a 25 mL volumetric flask.
6) Cool, and dilute to volume with 25% acid potassium chloride solution.
7) Similarly chromatograph 25 mL (containing 5.0 μg thiamin hydrochloride) of the working thiamin standard solution [and making up the volume of the eluate to 25 mL.

DETERMINATION OF RIBOFLAVIN BY FLUOROMETRIC METHOD

1. PURPOSE/SCOPE

This method is specified for the determination of vitamin B_2 content in all foods by fluorometric method.

2. SAFETY

When using a hazardous reagent such as concentrated acid or alkali, gloves should be worn, eye protected, and the work done in a fume hood.

3. REFERENCES

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4. DEFINITION

Riboflavin is 7,8-dimethyl-10-(1'-D-ribityl) isoalloxazine, a yellow-green, fluorescence and has the empirical formula $C_{12}H_{17}N_4O_2$. Riboflavin is the official name. The name vitamin B_2 is frequently used, and vitamin G, lyochrome, ovoflavin, lactoflavin, uroflavin and hepatoflavin are historical names. Riboflavin can occur in food as free riboflavin and as two of its derivatives, flavin mononucleotide (FMN, riboflavin 5'-monophosphate) and flavin adenine dinucleotide (FAD). The richest natural sources of vitamin B_2 are yeast, milk and milk products, meat, egg, legumes, fruits, fresh vegetables, and cereal grains.

5. PRINCIPLE

Since riboflavin may occur combined with proteins, the foodstuff has to be treated with enzyme or dilute acid to liberate the vitamin. Protein and other interfering substances are then precipitated at a pH of 6.0. After filtering, any protein remaining in solution may be further precipitated at a pH of 4.5. Potassium permanganate is then added to the extract to remove interfering fluorescent substances, followed by the addition of hydrogen peroxide to remove excess permanganate. Fluorescence of the extract may then be measured in a fluorometer. A little sodium hydrosulphite is added to quench the fluorescence due to riboflavin and the fluorescence reading again taken. The remaining fluorescence is due to other interfering substances and should be subtracted from the initial fluorescent reading.

6. REAGENTS

6.1 Hydrochloric acid, 0.1N

Add 8.3 mL concentrated hydrochloric acid to 500 mL of deionized water and dilute to 1L with deionized water.

6.2 0.02 N CH_3COOH

Add 1.14 mL glacial acetic acid to 500 mL of deionized water and dilute to 1 L with deionized water.

6.3 1 N sodium hydroxide
6.4 Glacial acetic acid
6.5 Potassium permanganate solution, 4%
6.6 Hydrogen peroxide solution, 3%
Dilute 30% H_20_2 1:10 with water. Prepare just before use.
6.7 Sodium hydrosulphite (sodium dithionite).
Check suitability for use as follows:
a. To each of two tubes, add 10 ml water and 1 ml standard riboflavin solution containing 20 μg per ml.
b. Add 1 ml of glacial acetic acid to each tube and mix.
c. Add 0.5 ml of 4% potassium permanganate, mix, and allow to stand for 2 minutes. D
d. Add 0.5 ml 3% hydrogen peroxide; mix thoroughly. Red colour should disappear within 10 seconds.
e. Measure fluorescence in a fluorometer.
f. Add 8 mg of sodium hydrosulphite and mix.
g. Read solution again in fluorometer; riboflavin should be completely reduced in 5 seconds.
6.8 Riboflavin standard solutions.
a. Stock solution: dissolve, with heating, 100 mg pure riboflavin in 1 litre of 0.02N acetic acid. Store in amber bottle under toluene in refrigerator (5-10^oC). Solution contains 100 μg riboflavin per ml.
b. Intermediate solution: dilute 10 ml stock solution to 100 ml with 0.02N acetic acid to give a concentration of 10 μg per ml. Store in amber bottle under toluene in refrigerator (5-10^oC).
c. Working solution: dilute 10 ml intermediate solution to 100 ml with water. Prepare fresh for each assay. Solution contains 1 μg riboflavin per ml.
6.9 Extraction solution
Mix 300 mL methanol, 100 mL pyridine, 100 mL H_2O, and 10 mL CH_3COOH. (Proportionate amounts may be prepared.)

7. APPARATUS

7.1 Spectrofluorometer
7.2 Autoclave
7.3 pH-meter
7.4 Analytical balance
7.5 Erlenmeyer flask, 250 ml
7.6 Volumetric flask 10, 25, 50, 250, 500 and 1000 ml
7.7 Beaker 25 ml
7.8 Pipette

8. PROCEDURE

8.1 Sample extraction
Place measured amount of test portion in suitable size flask and proceed by one of following methods:

8.1.1 For dry or semidry products containing no appreciable amount of basic substances
(1) Weigh 1-5 g the sample into the Erlenmeyer flask
(2) Add 60 ml 0.1 N HCl (equal to ≥10 times dry weight test portion in g)
(3) Mix contents of flasks well so that the solids are dispersed in the liquid.
(4) Heat mixture in autoclave 30 min at 121-123°C and cool.
(5) Mix regularly to ensure that no lumping of solids occur.

8.1.2 For dry or semidry products containing appreciable amounts of basic substances
(1) Weigh 1-5 g the sample into the Erlenmeyer flask
(2) Adjust mixture to pH 5.0-6.0 with dilute HCl.
(3) Add amount of deionized water such that total volume liquid is equal in ml to ≥10 times dry weight test portion in g. Then add equivalent of 1.0 ml 10 N HCl/100ml liquid.
(4) Heat mixture in autoclave 30 min at 121-123°C and cool.
(5) Mix regularly to ensure that no lumping of solids occur.

8.1.3 For liquid products
(1) Weigh 1-5 g the sample into the Erlenmeyer flask
(2) Adjust pH to 5.0-6.0 with dilute HCl or, with vigorous agitation, NaOH solution.
(3) Add amount of deionized water such that total volume of liquid is equal in ml to ≥10 times dry weight test portion in g. Then add equivalent of 1.0 ml 10 M HCl/100ml liquid.
(4) Heat mixture in autoclave 30 min at 121-123°C and cool.
(5) Mix regularly to ensure that no lumping of solids occurs.

8.1.4 For concentrates, premise, and multivitamin supplements.
(1) Place measured amount of test portion in flask
(2) Add volume extraction solution equal in ml to ≥10 times dry weight test portion in g. If test portion is not readily soluble, comminute so that it may be dispersed evenly in liquid. Then agitate vigorously and wash down sides of flask with extraction solution.
(3) Reflux mixture 1 hour and cool.
(4) Mix regularly to ensure that no lumping of solids occurs.

8.2 Precipitation of interfering impurities

8.2.1 Adjust pH to 6.0-6.5 with 1 N sodium hydroxide.
Swirl the extract constantly during the addition of alkali to prevent local areas of high pH.

8.2.2 Add immediately 1 N hydrochloric acid until no further precipitation occurs (pH around 4.5).

8.2.3 Transfer quantitatively into a 100 ml amber volumetric flask and make up to the mark.

8.2.4 Filter solution.

8.2.5 To a 50 ml aliquot of the filtrate, add 1 N hydrochloric acid drop-wise until no precipitation of dissolved protein occurs.

8.2.6 Add 1 N sodium hydroxide with constant shaking to pH 6.8

8.2.7 Dilute the aliquot to 100 ml with water.
8.2.8 Filter solution again if necessary.

8.3 Determination

Tube No.	S1	S2	S1+Std	S2+Std
Sample assay (ml)	10	10	10	10
Std. Solution (1 ug/ml)	-	-	1	1
Deionized water (ml)	1	1	-	-
	Mix			
Glacial acetic acid (ml)	1	1	1	1
	Mix			
4% $KMnO_4$ (ml)	0.5	0.5	0.5	0.5
	Mix and stand for 2 min.			
3% H_2O_2 (ml)	0.5	0.5	0.5	0.5
Mix thoroughly (red permanganate color should disappear in 10 sec.)				
Shake vigorously until excess oxygen is expelled				
Measure fluorescence Exc. 440 nm, Emiss. 565 nm.	[A]	[A]	[B]	[B]
Sodium dithionite (mg)	20	20	20	20
	Mix			
Measure fluorescence within 5 seconds	[C]	[C]	[C]	[C]

Note:

1. Check fluorometer between readings with the standard solution to ensure that it gives the reading that was initially obtained.
2. Quantity of $Na_2S_2O_4$ appreciably > 20 mg may reduce foreign pigments and / or foreign fluorescing substances, thereby causing erroneous results.

9. CALCULATIONS

$$\text{Riboflavin (mg/100 g)} = \frac{A - C}{B - A} \times \frac{20}{W} \times \frac{100}{1000}$$

A = Fluorescence reading of test sample
B = Fluorescence reading of standard
C = Fluorescence reading of blank
W = Sample weight (g)

10. ACCEPTANCE OF RESULTS

10.1 The sample results are acceptable if the control sample result is within the level of two standard deviations of the mean on QC chart.

10.2 Sample duplicate
where, d1 = duplicate 1, d2 = duplicate 2, and d1 $\geq$ d2;
then, $\frac{(d1-d2)}{d1} \times 100 \leq 5\%$

10.3 The standard addition or spiked recovery sample shall be between 90-110%.

11. APPENDIX

Since riboflavin is light sensitive and is most readily destroyed by light in the blue and UV regions, it is necessary to perform all operations in the absence of strong light, and to use amber glassware. Throughout all stages, pH of all solutions containing the vitamin must be below 7.0. Where directed to filter through paper, use paper known not to adsorb riboflavin [ash-free papers have been found satisfactory].

DETERMINATION OF THIAMIN AND RIBOFLAVIN BY HPLC (FLUORESCENCE DETECTOR)

1. SCOPE

A method is described for the determination of thiamin and riboflavin in foods by HPLC. Results are expressed as milligram per 100g of food.

2. SAFETY

When using a hazardous reagent such as concentrated acid or alkali, gloves should be worn, eye protected, and the work done in a fume hood.

3. REFERENCES

3.1 Horwitz, W. (editor) Official Methods of Analysis of AOAC International, 17th ed., Vol. 2, Thiamin Fluorometric Methods 942.23 Ch. 45 pp. 6-7, 957.17 Ch. 45 pp. 8-9, and Riboflavin Fluorometric Methods 970.65 ch.45 pp.9-10, 2000.

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4. DEFINITION

4.1 Thiamin

The names thiamin, aneurine and vitamin B_1, and the obsolete terms polyneuramin, antiberiberi vitamin and antineuric vitamin refer to a single compound. Thiamin consists of a substituted pyrimidine and thiazole ring connected by a CH_2 bridge and has the empirical formula $C_{12}H_{17}N_4O_2$. Thiamin can occur in a variety of forms: as free thiamin, as a protein complex, as mono-, di-, or triphosphate esters, or as a phosphorus protein complex. Products such as nuts, pork yeast, and cereal germs are especially rich in this vitamin.

4.2 Riboflavin

Riboflavin is 7, 8-dimethyl-10-(1'-D-ribityl) isoalloxazine, a yellow-green, fluorescence and has the empirical formula $C_{12}H_{17}N_4O_2$. Riboflavin is the official name. The name vitamin B_2 is frequently used, and vitamin G,

lyochrome, ovoflavin, lactoflavin, uroflavin and hepatoflavin are historical names. Riboflavin can occur in food as free riboflavin and as two of its derivatives, flavin mononucleotide (FMN, riboflavin 5'-monophosphate) and flavin adenine dinucleotide (FAD). The richest natural sources of vitamin B_2 are yeast, milk and milk products, meat, egg, legumes, fruits, fresh vegetables, and cereal grains.

4.3 Thiochrome

Thiochrome is the oxidized form of thiamin which fluorescence in ultraviolet light.

5. PRINCIPLE

The vitamins are extracted from the food by acid hydrolysis followed by enzymatic hydrolysis. The aqueous extract is injected onto a reverse phase HPLC column. The fluorescence of riboflavin is measured and thiamin is determined after post column derivatisation with alkaline potassium ferricyanide that converts the thiamin to thiochrome.

6. REAGENTS

6.1 Acetic acid, glacial (irritating and corrosive**)**

6.2 1-Hexanesulphonate sodium salt

6.3 5% Sodium hydrogen carbonate
Dissolve 5 g sodium hydrogen carbonate in 50 ml of deionized water and dilute to 100 ml with deionized water.

6.4 Hydrochloric acid, 0.1 N
Add 8.3 ml concentrated hydrochloric acid **(**highly corrosive) to 500 ml of deionized water and dilute to 1 L with deionized water.

6.5 Sodium acetate, 2.5 M
Dissolve 205 g anhydrous sodium acetate or 340 g sodium acetate trihydrate in 500 ml of deionized water and dilute to 1 L with deionized water.

6.6 Takadiastase 10%
Dissolve 10 g of the enzyme (Fluka Cat. No. 86250) in 50 ml of 2.5 M sodium acetate (8.1.5) and dilute to 100 ml with 2.5 M sodium acetate.

6.7 Potassium ferricyanide

6.8 Sodium hydroxide

6.9 Acidified 20% ethanol
20% ethanol adjusted to pH 3.5-4.3 with 0.1N HCl

6.10 0.02 N CH_3COOH
Add 1.14 ml glacial acetic acid to 500 ml of deionized water and dilute to 1 L with deionized water.

6.11 Methanol

6.12 Deionized water

6.13 50% methanol in water
Filter and degas before use.

6.14 0.005 M hexanesulphonic acid (sodium salt) in 85% methanol in water, pH 6.0

Dissolve 0.5156 g of 1-hexanesulphonic acid sodium salt monohydrate in 500 ml of 85% methanol in water. Add 1 ml of glacial acetic acid, and adjust pH of solution to 6.0 by addition of 5% aqueous $NaHCO_3$.
Filter and degas before use.

6.15 Oxidant, 0.001M potassium ferricyanide in 0.375 M sodium hydroxide.
Dissolve 0.165 g potassium ferricyanide in 50 ml of water, mix with 100 ml water containing 7.5 g NaOH and dilute the mixture to 500 ml. Store in an amber glass container. Prepare fresh before use.

6.16 Standards

6.16.1 Stock thiamin, 100 μg/ml
Accurately weigh 0.0224 g thiamin-HCl (Sigma Cat. No. T-4625) into a 200 ml volumetric flask. Dissolve and dilute to the volume with the acidified 20% ethanol.

6.16.2 Stock riboflavin, 100 μg/ml
Accurately weigh 0.0200 g riboflavin (Sigma Cat. No. R-4500) into a 200 ml volumetric flask. Add about 50 ml 0.02 N acetic acid and gently warm in a water bath until riboflavin is dissolved. Cool and dilute to the volume with 0.02 N acetic acid.

6.16.3 Composite intermediate standard I, 10 μg/ml (for %recovery test)
Pipette 10 ml of both 6.16.1 and 6.16.2 into a 100 ml volumetric flask. Dilute to the volume with water.

6.16.4 Composite intermediate standard II, 10 μg/ml (for standard curve)
Pipette 10 ml of both 6.16.1 and 6.16.2 into 100 ml volumetric flask. Dilute to the volume with water.

Note *Stock standards must be kept at 4°C and protected from light in amber glass bottles. Prepare fresh stock standards every two months.*

7. INSTRUMENT AND GLASSWARES

7.1 Erlenmeyer flasks, 125 ml
7.2 Water bath 100°C
7.3 Water bath 37°C or 45°C
7.4 Volumetric flasks, 100 ml
7.5 Filter papers (Whatman® No.42)
7.6 Solvent filtration apparatus with 0.45 μm membrane filter discs (Gelman; type Nylaflo)
7.7 Filter holder with 0.45 μm membrane filter discs (Sartorius; cellulose acetate)
7.8 Amber glass vials (sample vial)
7.8 HPLC apparatus including two pumps, fluorescence detector, mixing block (T- piece) for post-column derivatisation and recorder.

8. PROCEDURE

Thiamin and riboflavin degrade in UV (including fluorescent) light. Preparation and analysis should be carried out under tungsten lighting or subdued lighting, i.e. lights in near proximity switched off and blinds drawn.

Include a blank, recovery, and control sample (QCM) and run samples in duplicate in each batch.

Before weighing or pipetting an aliquot of frozen sample for analysis, thaw entire sample and remix.

8.1 Extraction

Weigh accurately about 1 to 6 g dry sample (3 significant figures) or pipette 10 ml of liquid sample into a 125 ml Erlenmeyer flask. For %recovery study, spike the recovery sample with 2 ml of 10 μg/ml standard solution (6.16.3).

Add about 60 ml 0.1 M hydrochloric acid (6.4) or ≥ 10 times dry weight sample in grams to all flasks then cap with aluminum foil and mix. Place the flasks in a boiling water bath for 30 minutes, with further mixing at 10 minutes intervals or autoclave mixture 30 minutes at 121°C.

WARNING: HOT ACID SOLUTIONS IN ERLENMEYER FLASKS ARE UNDER PRESSURE, WEAR RUBBER GROVES WHEN HANDLING.

Remove flasks from water bath, and then cool to below 50°C. Add 5 ml of 10% Takadiastase solution (6.6), cap flask, mix and incubate in a 37°C water bath overnight or 45-50°C water bath for 3 hours with intermittent mixing. Cool to room temperature and quantitatively transfer into 100 ml volumetric flask. Dilute to the volume with deionized water. Then filter through filter paper (7.5) and collect the filtrate in a 125 ml Erlenmeyer flask. Pass 10 ml filtrate through a 0.45 μm filter unit (7.7) and collect an aliquot into 5 ml amber glass vial which is ready for HPLC analysis.

8.2 Preparation of extracted standards for standard curves.

Pipette 5 ml of standard 6.16.4 into a Erlenmeyer flask. Treat the working standard in the same way as samples including diluting to 100 ml final volume. Prepare 3 concentrations of calibrating standards from the extracted standard as follows:

Calibrating Std.	Concentration (ng/ml)	Volume of extracted standard (ml)	Final volume (ml)
A	100	10	50
B	200	10	25
C	500	10	10

Pass 10 ml of each concentration through a 0.45 μm filter unit (7.7) and collect an aliquot into 5 ml amber glass vial which is ready for HPLC analysis.

A calibrating standard is repeated every ten samples or three times (beginning, middle and end) throughout the determination.

8.3 Determination

Set up the HPLC system using the following conditions:

Riboflavin

Column:	ZORBAX-ODS 5 μm column, 4.6 mm x 25 cm Stainless steel with a C_{18} guard column.
Mobile Phase:	50% methanol in water (6.13). Filter and degas before use.
Detector:	Fluorescence, excitation 440nm, emission 530 nm Gain 100, attenuation 16
Injection Volume:	10 μl
Flow Rate:	1.3 ml/min
Retention Time:	3-4 minutes.

Thiamin

Column:	As above
Mobile Phase:	0.005 M hexanesulphonic acid (sodium salt) in 85:15 methanol:water, pH 6.0 (6.14). Filter and degas before use.
Oxidant:	0.001 M potassium ferricyanide in 0.375 M sodium hydroxide (6.15). Prepare fresh before use.
Detector:	Fluorescence, excitation 360 nm, emission 435 nm, Gain 100, attenuation 8.
Injection Volume:	10 μl
Flow Rates:	1.3 ml/min (mobile phase) 1.0 ml/min (oxidant)
Retention Time:	3-4 minutes.

Oxidant is introduced at the T-piece, which is placed between the column and the detector.

Equilibrate the system until a stable baseline is obtained. Also ensure consistent peak height and retention times are obtained. Purge the injector, and then run the calibrating standards (8.2) as a test injection to check system performance.

8.4 Care of Equipment

After the analysis, flush HPLC and column with 50% methanol/water to remove traces of ion pair reagents and buffers. Also thoroughly flush oxidant delivery pump with deionized water to remove alkali.

9. CALCULATION

9.1 Preparation of standard curves using calibrating standards.

Prepare a calibration graph from the calibrating standard concentrations versus peak height.

9.2 Results Calculation

$$\text{Vitamin (mg/100g)} = \frac{\text{Sample conc. (ng/ml) X 100(ml) X 100(g)}}{10^6 \text{ X sample wt. (g)}}$$

The sample concentration is taken from the calibrating graph.

9.3 Recovery Calculation

$$\%\text{Recovery} = \frac{\text{The Recovery Sample Conc. (ng/ml) - Control Sample Conc. (ng/ml)}}{\text{Added Standard Conc. (ng/ml)}} \text{ X } 100$$

The concentration of the recovery sample and control sample are taken from the calibrating graph.

9.4 Limits of Detection and Limits of Quantification

Vitamin B_1:	Limits of Detection (mg/100g)	=	0.010
	Limits of Quantification (mg/100g)	=	0.10
Vitamin B_2:	Limits of Detection (mg/100g)	=	0.005
	Limits of Quantification (mg/100g)	=	0.05

9.5 REPORTING OF RESULTS

Results are reported to the nearest 0.01 mg/100g, or 2 significant figures.

10. ACCEPTANCE CRITERIA

The acceptance criteria are as follows:

10.1 The calibration for each vitamin, using the calibrating standards, should have a correlation coefficient for the linear regression of at least 0.9

10.2 The sample results are acceptable if the control sample result is within the level of two standard deviations of the mean on QC chart.

10.3 Sample duplicate

where, d1 = duplicate 1, d2 = duplicate 2, and $d1 \geq d2$;

then, $\frac{(d1-d2)}{d1} \times 100 \leq 5\%$

10.4 The standard addition or spiked recovery sample shall be between 90-110%.

11. CRITICAL CONTROL POINTS

Section	Control Item	Specification
6.2.2	Riboflavin standard	Ensure solution is completely clear and all solid is dissolved
8.1 and 8.2	Ambient light	Perform operation in tungsten light or subdued light
8.1	Extraction	Commence timing from the time water bath boils
8.3	Instrument performance	Ensure proper instrument operation / equilibration before running samples
9	Calculation	Check all weights, dilution factors, integration and calculations

Determination of Water-soluble Vitamin by Microbiological Assay

1. PURPOSE/SCOPE

The method is described for the determination of water soluble vitamins in foods by microbiological assay. Results are expressed as microgram or milligram per 100 gram food sample.

2. SAFETY

See specific vitamins.

3. REFERENCES

3.1 AOAC (2000) Microbiological Methods,17th Ed., Chapter 45, Method 960.46 pp. 44-48 and Chapter 50, pp 20–26.

3.2 Difco Manual of Dehydrated Culture Media and Reagent for Microbiology (1984). Media for the Microbiological Assay of Vitamins and Amino Acids, 10th Ed., Difco Laboratories, Detroit, Michigan, USA, pp1055-1114.

3.3 Augustin J, et al,. Methods of vitamin assay, 4th edition. A. Wisley-Interscience Publication, USA. 1985.

3.4 Ball GFM. Water-soluble Vitamin Assays in Human Nutrition, 1st edition, Chapman & Hall, Melbourn, 1994.

4. DEFINITION

See specific vitamins.

5. PRINCIPLE

Microbiological assays are based on the observation that certain microorganisms absolutely require specific vitamins for growth. Using basal medium containing all nutrients except that to be assayed, aliquots of a standard solution of vitamin to be determined or aliquots of the sample extract containing the vitamin are added. Following inoculation with the assay organism, the organism multiplies in proportion to the vitamin content of the standard or the sample. The extent of growth is ascertained by measuring the turbidity produced. Over a defined concentration range, the measured response will be directly proportion to the amount of vitamin present. Within this range, the sample solution and the standard vitamin can be compared accurately. The response depends very much upon whether bound forms of the vitamin are released during the extraction stage of the assay.

6. MATERIALS

6.1 Culture media

6.1.1 Micro inoculum broth (Difco Cat. No. 0320-02)

Dissolve 3.37 g in 100 ml distilled or deionised water. Dispense 5 ml quantities into tubes of 16-20 mm diameter. Sterile at 121-123^{o}C for 15 minutes. Final pH is 6.7$\pm$0.2 at 25^{o}C

6.1.2 Micro inoculum agar (Difco Cat. No. 0319-01-5)
To 100 ml liquid culture medium (6.1.1), add 1.5 g agar. Dissolve agar by heating with stirring on a steam bath. Add about 5 ml portions hot solution to a set of test tubes, cover to prevent contamination, and sterile for 15 min in an autoclave at 121-123^{o}C. Cool the tubes in upright position as rapidly as practicable to keep colour formation at minimum. Store at 4-10^{o}C.

6.2 Micro assay medium

Specific micro-assay medium is used for specific vitamin as shown in table below.

6.2.1 Weigh the assay medium used for each set of vitamin analysis according to an instruction on the label.

Vitamin	Assay medium
Niacin	Niacin assay medium
Vitamin B12	B12 assay medium
Folic acid	Folic acid casei assay medium
Vitamin B6	Pyridoxine Y medium

6.2.2 Suspend the assay medium in an appropriate volume of deionised water.

6.2.3 Boil for 2-3 minutes and cool to room temperature before use.

6.3 Stock culture of test organism

6.3.1 For appropriate test organism designated below, prepare stab culture $\geq$ 1 tubes (~ 3 tubes) in Micro inoculum agar (6.1.2). Incubate 16-24 h at any selected temperature between 30–37^{o}C (held constant to within $\pm$ 0.5^{o}C). Store at 4-10^{o}C. Before using the new culture, make several successive transfers of culture in 2-4 weeks.

Prepare fresh stab culture $\geq$ 1 time weekly and do not use for preparing inoculum if > 1 week old.

Note: Actively of slow-growing culture may be increased by daily or twice-daily transfer of stab culture, and is considered satisfactory when definite turbidity in liquid inoculum can be observed 2-4 h after inoculation. Slow-growing culture seldom gives suitable response curve and may cause erratic results.

Vitamin	Test organism	ATCC No.	Incubation temperature ^{o}C
Niacin	*Lactobacillus plantarum*	8014	35-37
Vitamin B_{12}	*Lactobacillus leichmanii*	7830	35-37
Folic acid	*Lactobacillus casei* subspecies rhamnosus	7469	35-37
Vitamin B6	*Saccharomyces carlsbergensis*	9080	30

6.3.2 Inoculum

- Subculture appropriate test organism from a stock culture into a tube containing 5 ml of Micro inoculum broth (6.1.1).
- Incubate 16-20 h at any selected temperature between 30–37^{o}C (held constant to within $\pm$ 0.5^{o}C).
- Under aseptic conditions, centrifuge culture at ca 2000 rpm for 5 min.
- Discard the supernatant and wash cells 3 times with 10 ml steriled 0.85% normal saline solution (NSS).
- Centrifuge the culture at ca 2000 rpm for 5 minutes, discard the last supernatant.
- Resuspend the cells in an appropriate volume of steriled NSS (for example to Mc Farland No 0.5-1.0).
- Mix thoroughly and use 1 drop of this diluted inoculum to inoculate the vitamin assay tubes.

6.4 Reagents

6.4.1 Steriled normal saline solution: 10 ml aliquots of 0.85% aqueous NaCl solution is distributed into 16x100 test tubes and autoclaved at 120^{o}C for 15 minutes.

6.4.2 1N NaOH: Dissolve 40 g sodium hydroxide pellets (Merck Cat. No. 1.06 498.1000) in 1 L deionised water.

6.4.3 1N HCl: Dilute appropriate volume of 37% hydrochloric acid (~83 ml) (Merck Cat. No. 1.00 317.2500) to 1 L with deionised water.

6.4.4 NaCl-anhydrous

6.4.5 25% Ethanol: Dilute 250 ml absolute ethanol to 1000 ml with deionised water.

6.4.6 Standard vitamins

Purity of each vitamin standard must be checked by molar extinction coefficient. % purity must be taken into account when the stock standard solution is prepared.

Purity of vitamin standards: characteristics of chromophore

Vitamin	Concentration of Standard	Wavelength Max λ	Molar extinction coefficient (ε) (OD units cm^{-1})
Thiamin hydrochloride	1% in 0.1M phosphate buffer, pH 2.9	246	425
Riboflavin	1 M in 0.1M phosphate buffer, pH 7.0	260	27 700
Niacin (Nicotinamide)	1% in 0.1 N H_2SO_4	261	478
Vitamin B6 (pyridoxine.HCl)	1% in Acidified solution, pH about 2	290.5	430
Folic acid	1M in 0.1M phosphate buffer, pH 7.0	282	27 000
Vitamin B12 Cyanocobalamin,	1 M in water	278	15 500

7. INSTRUMENT AND GLASSWARE

7.1 Instrument

- 7.1.1 Autoclave
- 7.1.2 Water bath
- 7.1.3 pH meter
- 7.1.4 Incubator
- 7.1.5 Spectrophotometer (e.g., MILTON ROY: Spectronic 401)
- 7.1.6 Vortex Mixer

7.2 Glassware

- 7.2.1 125-ml and 250-ml Erlenmeyer flasks
- 7.2.2 50-ml, 100-ml, 200-ml, 250-ml and 500-ml volumetric flasks
- 7.2.3 50-ml, 100-ml beakers
- 7.2.4 Filter papers (Whatman No. 42)
- 7.2.5 16x100 mm test tubes with caps
- 7.2.6 13x100 mm test tubes with caps
- 7.2.7 16x100 mm screw cap test tubes with caps
- 7.2.8 Steriled pasteured pipettes
- 7.2.9 Autopipette and autopipette tips, appropriate sizes of graduated and volumetric pipiettes class A

Note All glassware should be rinsed immediately after use and then cleaned by cleaning solution (meticulously cleanse by sodium lauryl sulfate USP has been found satisfactory as detergent). They are then rinsed with tap water, followed by deionised water to remove any of the cleansing agent. The test organism is highly sensitive to minute amounts of growth factors and to many cleansing agent. Therefore, it may be preferred to follow cleansing by heating 1-2 hours at $250^{O}C$. This is of importance in cobalamin and folate assay.

8. PROCEDURE

8.1 Sample preparation

Edible portion of a laboratory food sample must first be prepared, noting the weight proportion of non-edible portion. The sample is then homogenised using appropriate apparatus, e.g., high speed blender, food processor, meat mincer, grinder, sieves, etc. Care must be taken not to chop some foods too finely in the food processor because fat will liquefy and separate. Blending must not be excessively vigorous or prolonged, as aeration and heat generation can promote oxidation and decomposition of vitamins. After preparation, the sample in the form of powder, pulp, suspension, or solution should be analysed immediately. If the analysis cannot be performed on the same day, the sample must be kept in a screw-cap bottle and in a freezer.

A frozen sample must be thawed overnight in a refrigerator and brought to room temperature before remix thoroughly and then weighing or pipetting. Weigh accurately, in duplicate, 2-5 g dry powder (3 significant figures), or 5-10 semi-solid sample or 10-20 ml of liquid sample into a 250-ml Erlenmeyer flask. If the sample was powder or dry, add 3-5 ml water to moisten the sample.

8.2 Sample extraction

There is no common extraction method for all of the water soluble vitamins. The B-group vitamins occur naturally as coenzymes in animal and plant tissues tightly bound to their protein apoenzymes. Certain vitamin (vitamin B6) bound to carbohydrates, transport protein or being integral part of complex molecules (FAD, NAD).

Many of vitamins are extracted most efficiency by acids or alkaline hydrolysis at elevated temperature, some by enzymatic digestion and some use mixed condition. See specific extraction methods for specific vitamin.

For recovery study, spike an unknown sample with appropriate amount of vitamin standard solution. Follow the steps of extraction according to the specific vitamin.

8.3 Assay tubes

8.3.1 Prepare tubes containing appropriate standard solutions in duplicate (or replicate) as follows:

Standard set

Assay tubes	**1**	**2**	**3**	**4**	**5**	**6**
Working standard (ml)	0	1	2	3	4	5
Deionised water (ml)	0	4	3	2	1	0
Assay medium (ml)	5	5	5	5	5	5
Blank	2 sets of uninoculated blank for zero setting					

8.3.2 For unknown, % recovery and in-house control sample; prepare series of assay tubes in duplicate as follows:

Sample set

Assay tubes	**1**	**2**	**3**	**4**
Test solutions (ml)	1	2	3	4
Deionised water (ml)	4	3	2	1
Assay medium (ml)	5	5	5	5

8.3.3 After mixing, the tubes are steriled by boiling at 100^{o}C for15 min or autoclaving at 120^{o}C for 5 min according to the vitamin to be analysed.

8.3.4 Cool to room temperature in running water before inoculation.

8.4 Inoculation

Mix the diluted inoculum (6.3.2) thoroughly. Using a steriled pasteur pipette, under aseptic condition, inoculate one drop of the diluted inoculum into the vitamin assay tubes, except the uninoculated blank. Mix thoroughly on a vortex mixture.

8.5 Incubation

Incubate the whole set of assay tubes at appropriate temperature, 30-37^{o}C for 16-18 hours. Check the turbidity regularly after 16 h incubation.

8.6 Growth measurement

Stop growth of the test organisms by boiling at 100 ^{o}C, 5 min and cool in running water. Thoroughly mix the content of each tube (1 drop of 1-2 % of suitable antifoam agent solution may be added). Stand for about 30 second before measuring the turbidity of growth by reading the optical density at any specific wavelength between 540-660 nm. Uninoculated blank is used for zero setting.

8.7 Preparation of standard growth curve

Prepare a standard growth curve by plotting the series of standard concentrations of vitamin on x basis versus corresponding optical density (OD) on y basis. Drawing the standard curve and reading the concentration of vitamin in the unknown tubes from the linear part of the standard curve.

9. CALCULATION OF RESULTS

9.1 Vitamin concentration in unknown sample: vitamin B_{12} as an example

$$\text{Vitamin } B_{12} \text{ concentration } (\mu g/100g) = \frac{\text{Sample conc. (pg/tube) x dilution factor X 100}}{\text{weight or volume of sample x}10^{6}}$$

9.2 % Recovery

$$\% \text{ Recovery} = \frac{\text{vitamin in recovery sample - vitamin in control sample}}{\text{added vitamin}} \times 100$$

The concentration of the vitamin (pg/tube) in each tube is taken from the standard growth curve.

9.3 Report

In general, results are reported to the nearest 0.1 mg/100 g or nearest 0.1 μg/100g for vitamin B12, or with a round figure for folate.

10. ACCEPTANCE OF RESULTS

10.1 Duplicate results should not be more than 15 % variation, and ideally be within 10%.

10.2 To monitor the precision, the value from the in-house quality control Sample must fall within ± 2SD of the mean on the QC chart.

10.3 Percent recovery should be between 90-110%.

11. SUPPLEMENTARY NOTE

General critical control points

Control item	Specification
Ambient light	Analysis is performed under subdued light
Inoculum	Must be active. A drop of diluted test organism must be consistent to avoid large variation of the growth.
Calculation	Recheck calculation at all steps.

DETERMINATION OF NIACIN BY MICROBIOLOGICAL ASSAY METHOD

General information of microbiological assay is presented in section "Determination of Water-soluble Vitamin by Microbiological Assay"

1. PURPOSE/SCOPE

A method is described for the determination of niacin (nicotinic acid) in foods by microbiological assay. Results are expressed as milligram per 100g of food. This method can apply for all foods. In general, the vitamin is present in foods of animal origin.

2. SAFETY

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3. REFERENCES

1. AOAC (2000) Microbiological Methods, 17th Ed., Ch 50.1.19. pp. 21
2. Difco Manual of Dehydrated Culture Media and Reagent for Microbiology (1984) Media for the Microbiological Assay of Vitamins and Amino Acids, 10th Ed., Difco Laboratories, Detroit, Michigan, USA, pp1055-1114.

4. DEFINITION

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5. PRINCIPLE

See section 2 in "Determination of Water-soluble Vitamin by Microbiological Assay"

6. REAGENTS

6.1 Culture media

6.1.1 Micro Assay Culture Agar (Difco Cat. No. 0319-01-5)

6.1.2 Micro Inoculum Broth (Difco Cat. No. 0320-02)

6.1.3 Niacin Assay Medium (Difco Cat. No. 2003-03-31)

6.2 Assay medium

See section 8.2 in "Determination of Water-soluble Vitamin by Microbiological Assay".

6.3 Stock culture and test organism

6.3.1 Stock culture of *Lactobacillus plantarum* ATCC 8014

- Stab culture in 3 tubes of 10 ml micro assay culture agar (6.1.1).
- Incubate at 35-37^{o}C for 16 – 24 hours.
- Store the tubes at 4^{o}C and subculture every two weeks in triplicate.

6.3.2 Inoculum

- Subculture *Lactobacillus plantarum* from a stock culture to a tube containing 5 ml of micro inoculum broth (6.1.2).
- Incubate at 35-37^{o}C for 16 - 24 hours under aseptic condition.

- Centrifuge the culture at 2000 rpm for 10 minutes.
- Discard the supernatant and wash cells three times with 10 ml steriled normal saline solution (NSS).
- Centrifuge the culture at 2000 rpm for 10 minutes.
- Discard the last supernatant and dilute cell to an appropriate inoculum with steriled NSS. (Mc Farland No 1.0)
- Mix thoroughly and use 1 drop to inoculate the vitamin assay tubes.

6.4 Reagents

For general reagents, see section 6.4 in "Determination of Water-soluble Vitamin by Microbiological Assay".

6.4.1 NaOH, 1 N: Dissolve 40 g sodium hydroxide pellets (Merck Cat. No. 1.06498.1000) in 1 L deionised water.

6.4.2 H_2SO_4, 1 N: Dilute 28 ml Sulfulric acid to 1 L with deionesed water.

7. APPARATUS

See section 7 in "Determination of Water-soluble Vitamin by Microbiological Assay"

8. PROCEDURE

8.1 Preparation of sample

See section 8.1 in "Determination of Water-soluble Vitamin by Microbiological Assay".

8.2 Extraction

8.2.1 Weigh accurately about 1-2 g dry sample (3 significant figures) or pipette 10 ml of liquid sample into a 250 ml Erlenmeyer flask.

8.2.2 For %recovery study, spike the recovery sample (based on the in-house control sample) with 3 ml of 8 μg/ml standard solution.

8.2.3 Add about 100 ml 1 N Sulfuric acid (6.4.2) to all flasks, cover with aluminum foil and mix.

8.2.4 Autoclave at 121-123^{o}C for 30 minutes.

8.2.5 Cool to room temperature.

8.2.6 Adjust pH to pH 4.5 with 1 N NaOH (6.4.1) and quantitatively transfer into a 200 ml volumetric flask.

8.2.7 Dilute to the volume with deionised water.

8.2.8 Filter through filter paper (7.8) and collect the filtrate in a 125 ml Erlenmeyer flask. Adjust pH of a portion of the clear filtrate to pH 6.8 and dilute to concentration of about 20 ng niacin/ml.

8.3 Preparation of standard and sample set

See section 8.3 in "Determination of Water-soluble Vitamin by Microbiological Assay".

8.3.1 Stock niacin I, 100μg/ml. (for standard curves)

Accurately weigh 0.05g nicotinamide (BDH Cat. No. 44068) into a 500 ml volumetric flask. Dissolve and dilute to the volume with 25% ethanol.

8.3.2 Intermediate standard I, 1 μg/ml (for standard curves)
Pipette 5 ml of 8.3.1 into a 500 ml volumetric flask. Dilute to the volume with deionised water.

8.3.3 Working standard 40 ng/ml. (for standard curves)
Pipette 4 ml of 8.3.2 into a 100 ml volumetric flask. Dilute to the volume with deionised water

8.3.4 Stock niacin II, 100 μg/ml (for %recovery test)
Accurately weigh 0.05 g nicotinamide (BDH Cat. No. 44068) into a 500 ml volumetric flask. Dissolve and dilute to the volume with 25% ethanol.

8.3.5 Intermediate standard II, 8 μg/ml (for %recovery test)
Pipette 4 ml of 8.3.4 into a 50 ml volumetric flask. Dilute to the volume with deionised water.

Note : Stock standards kept at 4°C and protected from light in amber glass bottles.
: Prepare fresh stock standards every three months.

8.4 Inoculation

See section 8.5 in "Determination of Water-soluble Vitamin by Microbiological Assay".

8.5 Growth measurement

Incubate the whole set of tubes at 35-37 °C for 16-18 hours. Check the turbidity regularly after 16 h incubation.

8.6 Preparation of standard growth curve

See section 8.7 in "Determination of Water-soluble Vitamin by Microbiological Assay".

9. CALCULATION, UNIT OF EXPRESSION AND TEST REPORT

9.1 Vitamin concentration in unknown sample

$$\text{Vitamin (mg/100g)} = \frac{\text{Sample conc. (ng/ml) X the appropriate dilution factor X 100}}{\text{sample wt. (g)}}$$

The niacin concentration in the sample is taken from the standard growth curve.

9.2 Recovery Calculation

$$\% \text{ Recovery} = \frac{\text{the recovery sample conc. (ng/ml) - control sample conc. (ng/ml)}}{\text{added standard conc. (ng/ml)}} \text{ X } 100$$

The concentration of niacin in the recovery sample and control sample are taken from the standard growth curve.

9.3 Report

Results are reported to the nearest 0.1 mg/100g, or 1 significant figure.

10. ACCEPTANCE OF RESULTS

See section 10 in “Determination of Water-soluble Vitamin by Microbiological Assay”.

11. METHOD VALIDATION

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12. APPENDIX

See section 12 in “Determination of Water-soluble Vitamin by Microbiological Assay”.

DETERMINATION OF NIACIN BY COLORIMETRIC METHOD

1. PURPOSE/SCOPEC

This method is described for the determination of niacin (nicotinic acid) in foods by colorimetric method. Results are expressed as milligram per 100g of food.

2. SAFETY

Cyanogen bromide is extremely poisonous. All operations involving this reagent should be carried out in an efficient fume cupboard. Do not breathe any vapour, and if solution comes in contact with skin, wash immediately with water.

3. REFERENCE

3.1 Deutsch MJ (1984). Vitamins and other nutrients. In *Official Methods of Analysis of the Association of Official Analytical Chemists.* 14th Edition (Williams S, ed.), AOAC, Virginia; pp. 841-842.

3.2 Association of Vitamin Chemists, Inc. (1966). Methods of Vitamin Assay. 3rd Edition, Interscience Publishers, New York;pp. 169-172.

4. DEFINITION

Niacin, and the amide of niacin, niacinamide, is widely distributed in foods.

5. PRINCIPLE

Niacin, and the amide of niacin, niacinamide, are usually bound to other chemical compounds and must therefore be freed by hydrolysis with strong acid or alkali or by enzymatic treatment prior to analysis by chemical methods. Hence niacin derivatives such as coenzymes and niacinamide are converted into free niacin, which is being estimated by this procedure. To eliminate the interference of biologically inactive materials that may be present, purification procedures have to be carried out and blank determinations are necessary during colour development. Niacin in the extract is reacted with cyanogen bromide to give a pyridinium compound. The latter undergoes rearrangement yielding derivatives that couple with aromatic amines to produce coloured compounds. Under proper conditions the density of the colour produced is proportional to the niacin present and may be measured in a colorimeter.

It is to be noted that niacin and niacinamide are both stable in the dry form and in aqueous solutions and are unaffected by light and pH.

6. REAGENTS

6.1 Dilute ammonium hydroxide solution
Dilute 5 ml ammonia to 250 ml with water

6.2 1N sodium hydroxide

6.3 10 N sodium hydroxide

6.4 Dilute hydrochloric acid
Dilute concentrated hydrochloric acid with water in the ratio of 1:5

6.5 9N sulphuric acid

6.6 Cyanogen bromide solution, 10%
Warm 80 ml water to 40oC in a large flask and add 10 g cyanogen bromide (CNBr). Shake until dissolved, cool, and dilute to 100 ml. Store in refrigerator.

6.7 Sulphanilic acid solution, 10%
Add ammonia drop by drop to mixture of 20 g sulphanilic acid ($C_6H_7NO_3S$) and 120 ml water until dissolved. Adjust pH to 4.5 with a solution of a mixture of hydrochloric and water (1+1), using pH meter. Dilute to 200 ml. Solution should be almost colourless.

6.8 Ammonium sulphate [$(NH_4)_2SO_4$)]

6.9 Niacin standard solutions
a. Stock solution: dissolve 50 mg pure niacin standard in 25% alcohol to make 500 ml. Store in refrigerator. Concentration of solution is 100 μg per ml.
b. Working solution: dilute 6 ml stock solution to 100 ml with water. Concentration of solution is 6 μg per ml.

7. APPARATUS

7.1 Analytical balance
7.2 Spectrophotometer
7.3 pH-meter
7.4 Glassware: test tubes, volumetric flasks, pipettes, beakers, cylinder, etc.

8. PROCEDURE

8.1 Preparation of extract

1) Weigh 5-20 g of the sample (containing about 0.5-1.0 mg niacin) into a beaker.
2) Add 20 ml 1N sulphuric acid and 20 ml water, mix, and heat 1 hour over a boiling water-bath.
3) Cool and adjust pH to 4.5 with 10 N sodium hydroxide and 9N H_2SO_4 .
4) Dilute to 100 ml (V_1) with water and filter.
5) Add 17 g ammonium sulphate into 50 ml (V_2) of the filtered extract in a stopper measuring cylinder.
6) Dilute to 100 ml (V_3) with water and shake vigorously.
7) Filter, mix well, and use 1.0 ml (V_4) for colour development. This solution should contain approximately 3 μg niacin per ml.
8) Pipette 50 ml of the working standard solution (6 μg per ml) into 17 g ammonium sulphate in a 100 ml stoppered measuring cylinder.
9) Dilute to volume with water and filter.
10) Use 1.0 ml of this standard solution, containing 3.0 μg niacin, for colour development.

8.2 Colour development

Prepare tubes and add reagents in the order as outlined in the following table. Detail instructions for the addition of these solutions and reagents are given below the table and should be strictly followed. Prepare separate sample blank for each sample being analysed.

	Standard Blank	Standard	Test Blank	Test
Standard solution (ml)	1.0	1.0	-	-
Test solution (ml)	-	-	1.0	1.0
Water (ml)	5.0	-	5.0	-
Dilute NH_4OH (ml)	0.5	0.5	0.5	0.5
10% sulphanilic acid (ml)	2.0	-	2.0	-
CNBr solution (ml)	-	5.0	-	5.0
10% sulphanilic acid (ml)	-	2.0	-	2.0
Dilute HCl (ml)	0.5	-	0.5	-
Water (ml)	-	0.5	-	0.5

Caution: add sulphanilic acid and CNBr solutions in fume cupboard from burette or pipettes filled by mechanical suction.

Note : After adding the test, standard solution and water to the respective tubes, the subsequent reagents must be added to a single tube and measure the color before proceeding to the next tube.

Starting with the standard blank:

1. Swirl tube to impart a rotary motion in liquid immediately.
2. Add dilute ammonium hydroxide, swirl again.
3. Add sulphanilic acid solution, and swirl.
4. Immediately add 0.5 ml dilute hydrochloric acid [and mix again.
5. Place in colorimeter with wavelength adjusted to 450 nm (or any specific wavelength between 430 and 450 nm)
6. Adjust instrument to 0 absorbance within about 30 seconds after addition of sulphanilic acid solution.

Treat standard solution in the same way as for the standard blank with respect to addition of dilute ammonium hydroxide.

1. Immediately swirl tube, add cyanogen bromide solution and swirl again.

2. At 30 seconds after addition of cyanogen bromide solution, swirl tube, add sulphanilic acid solution and swirl again.

3. Immediately add 0.5 ml water, mix again and stopper.

4. Read absorbance of standard solution at maximum, with instrument set at 0 absorbance for standard blank, as performed above.

   Colour reaches maximum in about l.5 minutes after addition of sulphanilic acid solution, remains at peak for about 2 minutes, and then fades slowly.

5. With test blank set at 0 absorbance, determine absorbance of test solution in a similarly manner.

Niacin content is proportional to absorbance if standard and test solutions are of approximately the same concentrations.

9. CALCULATION, UNIT OF EXPRESSION AND TEST REPORT

$$\text{mg niacin per 100 g sample} = \frac{\text{test O.D.}}{\text{std O.D.}} \times C \times \frac{V_3}{V_4} \times \frac{V_1}{V_2} \times \frac{100}{W} \times \frac{1}{1000}$$

where,

C = μg niacin standard in reaction tube (3.0 μg)
W = weight, in g, of food taken for analysis
V_1 = }
V_2 = } see explanation given in procedure
V_3 = }
V_4 = }

10. ACCEPTANCE OF RESULTS

11. METHOD VALIDATION

12. APPENDIX

DETERMINATION OF NIACIN IN FOOD
(HIGH PRESSURE LIQUID CHROMATOGRAPHY METHOD)

1. PRINCIPLE

In food, niacin and the amide of niacin, niacinamide are usually occur both in the free and bound forms. Therefore, they must be freed by hydrolysis with strong acid or alkaline prior to analysis with chemical or HPLC method.

The HPLC method is described in this procedure. The first step involves the use of alkaline digestion on food sample. Hence niacin derivatives such as coenzymes and niacinamide are converted into total niacin by alkaline digestion with aqueous calcium hydroxide. Following alkaline extraction of food, niacin is purified and concentrated using C18 and cation exchange cartridge (SCX). The purified extract is determined by HPLC at a detection wavelength of 254 nm using C8 column and PIC A reagent in 15 % methanol.

2. REAGENTS

1) Methanol, HPLC grade, BDH.
2) Ethanol, HPLC grade, BDH.
3) Ultra high quality (UHQ) water of 18 MΩ.
4) 10% and 1% oxalic acid solutions.
5) Weigh 10 g of oxalic acid (BDH, Analar) into a 100 ml volumetric flask and make up to the mark with UHQ water to produce a 10% oxalic acid solution.
6) Prepare 1% oxalic acid by pipetting 10 ml of 10 % oxalic acid solution into a 100 ml volumetric flask and make up to volume with UHQ water.
7) Calcium hydroxide, $Ca(OH)_2$, M=74.09 g/mol, Merck brand.
8) Niacin (nicotinic acid: Pyridine-3-carboxylic Acid), Anhydrous MW = 123.1 (Sigma brand)
   a. Preparation of niacin stock standard, 100 ug/ml :-
      Using an analytical balance, weigh accurately 10 mg (0.01 g) of dry niacin into a 100 ml volumetric flask and make up to the mark with 25% ethanol (25 ml pure ethanol and 75 ml UHQ water). Store in refrigerator at 4^{0} C and discard after 3 months.
   b. Preparation of niacin working standard, 10 ug/ml
      Pipette 1 ml of 100 ug/ml niacin stock solution into a 10 ml volumetric flask and make up the volume with UHQ water. Prepare a new working standard for every batch of samples.
9) 2 % ammonia solution in methanol.
   Pipette 2.4 ml of concentrated ammonia solution (25%) (Merck) into a 100 ml volumetric flask and make up to mark with 100% methanol.
10) PIC A Reagent (tetrabutylammonium phosphate), Workable UV range in mobile phase of 240+ nm, 5 vials per package, (Waters brand), product no. WAT 085101.
11) Preparation of HPLC mobile phase:
    Mix the content of 1 vial (15 ml) of PIC A reagent with 835 ml UHQ water (85 % aqueous phase), mix well and add 150 ml methanol (15 % organic phase). Mix thoroughly and filter through a 47 mm, 0.45 μm Whatman nylon

membrane filter using a Waters solvent filtration apparatus. After this, degas in an ultrasonic bath.

3. APPARATUS

1) A hot air oven (a substitute for an autoclave)
2) Refrigerated centrifuge
3) Vac master® (IST) 10 vacuum manifold or sample processing station with 10 place and a vacuum system
4) 50 ml graduated conical polypropylene centrifuge tubes with screw caps
5) IsoluteTM SPE column : SCX cation exchange cartridge, 500mg of 3 cc
6) Waters Sep-Pak® cartridge, Vac C 18, 500mg of 3 cc
7) 10 ml disposable syringe barrels
8) Test tubes, glass,100 x 15 mm

4. HPLC SYSTEM

1) (Jones Genesis) C 8 column which is a stainless steel HPLC column of 15cm x 4.6 mm I.D with particle size of 4 μm. (from Jones Chromatography Limited).
2) Guard column holder housing a disposable C18 pre-column insert.
3) A Gilson HPLC system comprising of:
   (i) A computer with a Gilson UniPoint™ System Controller Software.
   (ii) A Gilson 232 Bio auto sample injector with a 100 ul sample loop.
   (iii) A Gilson 305 piston pump to deliver the mobile phase.
   (iv) A Gilson 805 manometric module to dampen the pulsations of the pump and to supply the current pressure value to the 305 piston pump.
   (v) Gilson UV 116 detector
   (vi) A HP LaserJet 6 L printer to print the results and chromatograms.

5. PROCEDURE

A. Sample preparation:

1) Food samples should be finely ground and mixed well before taking sample aliquot.
2) Use an analytical balance to weigh accurately 1 g of sample and put into a 50 ml PP centrifuge tube.

B. Alkaline Extraction

1) Weigh 0.75 g $Ca(OH)_2$ and add into the centrifuge tubes which contain the 1.0 g sample. Include a 'duplicate' sample, a 'control' sample, a 'recovery' sample, a 'niacin standard' and a 'blank'.
2) To the 'recovery' sample, weigh 1.0 g of food sample and then add 1.0 ml niacin stock standard of 100 ug/ml. and then 0.75 g $Ca(OH)_2$.
3) To the 'niacin standard' tube is added with 1 ml stock standard (100 ug/ml) and then add 0.75 g of $Ca(OH)_2$.
4) The 'blank' sample contain only water and $Ca(OH)_2$.
5) Use a 25 ml measuring cylinder to add 10 ml UHQ water into all tubes.
6) Use a glass rod to mix each tube well, and then add 10 ml of UHQ water rinsing the glass rod as well.

7) Loosely screw caps on, and then put into a preheated hot air oven of 121^{o} C for 2 hrs (Make sure the oven reaches the desired temperature before starting to time for 2 hours).
8) At the end of 2 hours, switch off the oven and leave it until the temperature is about 700^{o}C. Then open and take out the tubes.
9) Open the caps and use glass rod to stir well while is still hot. Allow them to cool to room temperature and then make up to 50 ml with UHQ water.
10) The digest may be stored in refrigerator at 40^{o}C overnight (or for up to 3 days).
11) Centrifuge at 2500 rpm for 15 mins at 0$_{o}$C. Allow the tubes to warm up to room temperature before pipetting out 15 ml supernatant (use a pipetter set at 5000 ul, pipette 5000 ul x 3) into a clean 50 ml graduated PP centrifuge tube.
12) Adjust to pH 7 using 10 % oxalic acid initially, and then use 1 % oxalic acid when closer to the desired pH of 7. Note: Care and patience is required. If pH drops to less than pH 7, slowly add a drop of saturated $Ca(OH)_2$ solution until pH 7 is reached.
13) Adjust the final volume to 25 ml with UHQ water, cap it and then mix well.
14) Centrifuge at 2500 rpm for 15 min at 0^{o}C.
15) Connect a 500 mg C18 Sep-Pak cartridge and a 500 mg SCX cartridge in series (C18 cartridge on top) using a column adaptor. Attach a 10 ml disposable syringe barrel to the C18 Sep-Pak cartridge. Then attach the entire cartridge series to the vacuum manifold. (Make sure the pressure is below 20" Hg).
16) Condition the cartridges by passing 10 ml pure methanol follow by 10 ml UHQ water through the entire cartridges (drip-rate: 1-2 drops/sec). The cartridges should not be allowed to dry out; therefore, the vacuum is turned off during sample loading.
17) Pipette 10 ml supernatant (sample) and load onto the column. Turn the vacuum on and allow the sample supernatant to pass through the cartridge series.
18) Wash the entire cartridge series with 5 ml UHQ water. Then discard the C18 cartridge.
19) Wash the SCX cartridge with 5 ml methanol.
20) Elute niacin from the SCX cartridge with 5 ml of a freshly prepared 2 % ammonia solution in methanol. Collect the eluent into test tube.
21) Evaporate the eluent with a stream of nitrogen and redissolve the residue in 2 ml of UHQ water and mix thoroughly.

C. Instrument set up procedure

1) Warm up instrument for at least 1 hour before starting.
2) Set the Gilson UV 116 detector to the single wavelength mode of 254 nm at 0.01 AUFS
3) Check that the solvent bottles are filled with the filtered and degassed solvents as listed below:
   a) 100 % methanol,
   b) 10 % methanol,
   c) 50 % methanol.

d) The mobile phase (PIC A)

4) Let filtered water run through the 305 pump head at a slow flow rate all the time i.e. until the system is being shut down.
5) Attach the syringe to the fitting of the low pressure prime valve.
6) Draw liquid into the syringe from the 50% methanol solvent bottle with the low pressure prime valve in the 'open' position. (Open by turning anticlockwise the knob at the prime head and note the pressure should read 0 psi.)
7) Press the PRIME key on the front panel of the 305 piston pump; the pump will start running at its maximum speed (i.e. 10 ml/minute).
8) When no bubbles can be seen at the outlet tubing, press the STOP soft key to end the priming procedure.
9) Turn the valve to the 'close' position, remove the syringe from the low pressure prime valve and remember to turn the prime knob clockwise to close the prime head so as to allow 50 % methanol to flow through the C 8 column.
10) Using the Gilson Unipoint™ software, open the 'mobile phase window' and gradually increase the flow rate of the 50 % methanol to 0.5 ml/min in 2 minutes. Allow to flow for at least 15 mins.
11) Slow down the flow rate to 0 ml/min gradually for 2 mins.
12) Then switch to the mobile phase (PIC A) and repeat steps no. 6 to step no 11.
13) In the 'mobile phase window', increase the flow rate of (PIC A) to 0.9 ml/min gradually in 3 minutes duration.
14) Allow the column to equilibrate for 1 hour.
15) Using the Gilson UniPoint™system software, create a control method by keying in all the necessary parameters.
16) Filter all the reconstituted samples (refer to B.20 & 21) using 13mm, 0.45 μm, Whatman nylon membrane filter into a 2-ml sample vial.
17) Use the sample controller keypad and the UniPoint™system software to initiate the autosampler to inject 25 ul of the niacin standard (done in duplicate) into the HPLC column and then subsequently all the other samples.
18) After the HPLC run, stop the pump gradually. Then switch to 10 % methanol, and wash the column for about 10 minutes at 0.8 ml/min.
19) Stop the pump gradually, switch to 50 % methanol and wash the column at a slow flow rate of 0.5 ml/min. for 3 hours. Stop the pump gradually.
20) Switch to 100 % methanol and wash the column at 1 ml/min for 1 hour before shut down.
21) Wash the autosampler and injection sample loop with 10 % methanol.

D. Chromatography of niacin

1) Identification and quantitation of niacin is carried out by comparing the retention time with the niacin standard and then with the recovery.
2) The 'peak areas' from the chromatograms are used to determine the total niacin content by calculation against the standard.

6. CALCULATIONS

The concentration of niacin in the samples is obtained by the calculation below:

Niacin (mg/100g sample) =

$$\frac{\text{Peak area of sample}}{\text{Peak area of std.}} \times \frac{\text{Amount of std. in } \mu g}{1000} \times \frac{\text{(ml) vol. reconstituted}}{\text{Inject volume in ml}} \times \frac{50}{15} \times \frac{25}{10} \times \frac{100}{\text{Wt. of sample}}$$

7. REFERENCES

Caroline M.Ward & V.Craige Trenerry, Food Chemistry, Vol 60, pp 667-674, 1997. The determination of niacin in cereals, meat and selected foods by capillary electrophoresis and high performance liquid chromatography.

DETERMINATION OF VITAMIN C BY MICROFLUOROMETRIC METHOD

1. PURPOSE/SCOPE

The method describes a procedure for the quantitative determination of vitamin C in foodstuffs (both fresh and stored foodstuffs intended for immediate consumption and in particular to those of complex composition, for example, vegetables, fruits, fruit juices, complete meals, etc). The limit of quantitation (LOQ) is 1 mg/100 g.

2. SAFETY

Handle o-phenyenediamine with care as it is a carcinogen. This reagent should be weighed on the hooded balance. Anti-static gloves should be worn.

3. REFERENCES

a) Horwitz W. 2000. Official Method of Analysis of AOAC International. 17th Edition. Vol. II AOAC International. Maryland, USA.
b) Brubacher G, Muller-Mulot W and Southgate DAT. 1985. Methods for the determination of vitamins in food. Elsevier Applied Science Publishers. London.

4. DEFINITION

Vitamin C content is understood to be the ascorbic acid + dehydroascorbic acid content calculated as ascorbic acid determined by the procedure described here. The total vitamin C (ascorbic acid and dehydroascorbic acid) of natural origin and, if required, vitamin C added during the production of the foodstuff. It is given in mg of ascorbic acid per 100 g of original sample.

5. PRINCIPLE

Ascorbic acid is oxidized to dehydroascorbic acid in the presence of Norit activated carbon. The oxidized form is reacted with o-phenylenediamine (1, 2 phenylenediamine) to produce a fluorescent quinoxaline compound, whose fluorescent intensity is proportional to concentration. The fluorescence of the derivative of the vitamin is prevented by forming a H3BO3 dehydroascorbic acid complex prior to addition of diamine solution. Any remaining fluorescence is due to extraneous materials and serves as a “blank”. A standard calibration curve is used for the calculations. Fluorescence is measured at 350/430 nm.

6. REAGENTS

If not otherwise specified AR grade chemicals are to be employed. The water used must be distilled and taken from glass containers or be of corresponding purity.

1) Extracting solutions

a. Metaphosphoric acid-acetic acid solution

Dissolve, with shaking, 15 g HPO_3 pellets or freshly pulverized stick HPO_3 in 40 ml acetic acid and 200 ml H_20; dilute to approximately 500 ml and filter rapidly through fluted paper into glass-stoppered bottle.
(HPO_3 slowly changes to H_3PO_4, but if stored in refrigerator, solution remains satisfactory 7-10 days).

b. Metaphosphoric acid-acetic acid-sulphuric acid solution

Proceed as in (a), except use 0.3N H_2SO_4 in place of H_2O.

2) Ascorbic acid standard solution

a. Accurately weigh 50 mg ascorbic acid that has been stored in desiccator away from direct sunlight. Transfer to 50 ml volumetric flask. Dilute to volume with metaphosphoric acid-acetic acid solution [(1)(a)]. Prepare just before use. Concentration of solution is 1 mg/ml.

b. Dilute l0 ml ascorbic acid standard solution (2 a) to 100 ml with metaphosphoric acid-acetic acid solution (1)(a). Concentration of solution is 100 μg/ml.

3) *o*-Phenylenediamine solution
For each 100 ml solution required, weigh 20 mg o-phenylenediamine.2HCl. Dilute to volume with H_2O immediately before use.

(Chemical is a possible carcinogen. Use with extreme caution)

4) Thymol blue pH indicator, 0.04%
Dissolve 0.l g indicator by triturating in agate mortar with 10.75 ml 0.02N NaOH and dilute to 250 ml with H_2O. Transition range: l.2 (red) - 2.8 (yellow).

5) Sodium acetate solution
Dissolve 500 g $CH_3COONa.3H_20$ in H20 and dilute to l litre.

6) Boric acid-sodium acetate solution
Dissolve 3 g H_3BO_3 in 100 ml sodium acetate solution [reagent (5)]. Prepare fresh for each assay.

7) Acid-washed Norit
Add 1 litre HCl (1 volume HCl and 9 volumes H_2O) to 200 g Norit Neutral, heat to boiling point and filter with vacuum. Remove cake to large beaker. Add 1 litre H_2O, stir and filter. Repeat washing with H_20 and filtering. Dry overnight at 110-120°C.
Commercially available prepared acid-washed activated charcoal may also be used.

7. APPARATUS

1) Spectrofluorophotometer
2) Analytical balance
3) Vortex mixer
4) Erlenmeyer flask
5) pipettes

6) Glass filter funnel, 15-20 cm diameter.
7) Filter paper

8. PROCEDURE

8.1 Glassware setup - the following glassware should be placed in a line from front to back: A 100 ml volumetric flask with funnel for sample, a 250 ml Erlenmeyer flask (wide mouth works well) containing 2 gram acid-washed Norit and a 125 ml Erlenmeyer flask with funnel and filter (#42 Whatman) for each sample and standard.

8.2 Extraction of sample

A. Preliminary Test for Appreciable Amount of Basic Substances

1) Grind representative sample or express contents from capsule and add approximately 25ml metaphosphoric acidacetic acid solution [reagent (1)(a)].
2) Test pH by placing a drop of thymol blue pH indicator on pestle or by using spot plate.
(A pH of > l.2 indicates appreciable amounts of basic substances).
3) For liquid preparations, dilute representative sample approximately two-fold with reagent (1 a) before testing with indicator.

B. Preparation of Sample Assay Solution

a. For dry materials containing no appreciable amount of basic substances

1) Pulverize sample by gentle grinding, add metaphosphoric acid-acetic acid solution [reagent (1 a)], and triturate until sample is in suspension.
2) Dilute with reagent (1 a), to approximately 100 μg ascorbic acid per ml. Designate this volume as V ml.
3. Filter solutions containing large amounts of suspended solids through Whatman No.12 paper, or equivalent. Designate as sample assay solution.

b. For dry materials containing appreciable amounts of basic substances

1) Pulverize sample by gently grinding, add metaphosphoric acid-acetic acid-sulphuric acid solution [reagent (1 b)] to adjust pH to approximately l.2, and triturate until sample is in suspension.
2) Dilute with metaphosphoric acid-acetic acid solution [reagent (1 a)] to approximately 100 μg ascorbic acid per ml. Designate this volume as V ml.
3) Filter solutions containing large amounts of suspended solids through Whatman No.12 paper, or equivalent. Designate as sample assay solution.

c. For liquid materials

1) Take amount of sample containing approximately 100 mg ascorbic acid.
2) If appreciable amounts of basic substances are present, adjust pH to approximately l.2 with metaphosphoric acid-acetic acid-sulphuric acid solution [reagent 1 b].

3) Dilute with metaphosphoric acid-acetic acid solution [reagent 1 a] to approximately 100 μg ascorbic acid per ml. Designate this volume as V ml.
4) Filter solutions containing large amounts of suspended solids through Whatman No.12 paper, or equivalent. Designate as sample assay solution.

d. For gelatin-encapsulated pharmaceutical products

1) Place sample in small beaker and heat gently with enough proper extracting solution, reagent (1 a) or (2 a) to cover.
2) If capsules do not disintegrate readily, crush with glass rod.
3) Cool rapidly to room temperature.
4) If appreciable amounts of basic substances are present, adjust pH to approximately 1.2 with reagent (1 b).
5) Dilute with reagent (1 a) to approximately 100 μg ascorbic acid per ml. Designate this volume as V ml. 6. Filter solutions containing large amounts of suspended solids through Whatman No. 12 paper, or equivalent. Designate as sample assay solution.

Note:

1) For samples that are difficult to filter, proceed as in applicable section (a), (b), (c) or (d), except dilute sample assay solution with metaphosphoric acid-acetic acid solution [reagent 1 a] to approximately 50 μg ascorbic acid/ml. Compare with standard solution prepared by diluting 5 ml ascorbic acid standard solution (reagent 2 b), of concentration 1 mg/ml] to 100 ml with reagent (1 a). Concentration of this standard solution is 50 μg ascorbic acid per ml.
2) Filtrate can be kept for up to 10 days at 4^{o} C.

8.3 Oxidation of ascorbic acid in the extract

1) Dilute standards (1, 3, 5, 10 ml of solution E) corresponding to 1, 3, 5, 10 mg of ascorbic acid, respectively, are made up to the mark in a 100 ml vol flask with solution A. The final concentration of each standard is 10, 30, 50 and 100 μg per ml, respectively.
2) Transfer sample solution or standards to each 250 ml Erlenmeyer flask containing 2 g acid-washed Norit and swirl vigorously for two minutes. Immediately filter through whatman #42 paper into a 125 ml Erlenmeyer flask, discarding the first few cloudy drops of filtrate. Note: If the filtrate is gray or cloudy, it must be refiltered through a 0.45 micron syringe tip filter before continuing with the procedure.
3) To the blank volumetrics (containing 5 ml of H_3BO_3 / NaOAc solution), add 5.0 ml sample or standard filtrate and allow to stand for 15 minutes with occasional swirling. It is important that all blanks sit at least 15 minutes.

8.4 Fluorimetry (Formation of Quinozaline)

1) Add 5.0 ml sample or standard filtrate to the other 100 ml volumetrics flask containing the 5.0 ml NaOAc solution. Dilute to volume with deionized water and mix well.

2) After 15 minutes has elapsed, dilute blanks to volume with deionized water and mix well. Transfer 2.0 ml from each 100 ml volumetric flask into two test tubes. Two sample tubes and two blank tubes for each sample or standard - four tubes in all).
   Note: The rest of the procedure should be performed in subdued lighting.
3) Add 5.0 ml o-phenylenediamine solution to tubes and stir with vortex mixer.
4) Let tubes stand 35 minutes in the dark at room temperature. The 35 minute time period can be started after the first set of four tubes has been stirred with the vortex mixer, as the time elapse during tube reading is about the same as for *o*-phenylenediamine and stirring.
5) Allow 15 minutes for the spectrofluorometer and computer system to warm up.
6) The emission at 430 nm with a 350 nm excitation is then measured (main and blank values). Record the fluorescence of the blank, sample, standards.

9. CALCULATION

1) Standard test values for the ascorbic acid standard concentrations of ascorbic acid per ml can be plotted or calculated by linear regression.
2) The values of sample in micrograms of ascorbic acid per ml can be read off the standard calibration curve or calculated.
3) The ascorbic acid content in 100 g of the sample material investigated can be calculated.

mg ascorbic acid/g or mL = [(average X – average D)/(average C – average B)] x (20 x (20 x S x V/E)

Where, V = 100 = initial assay solution volume

E = number of g or mL of sample

S = concentration of standard in mg/mL added to reading tube

10. ACCEPTANCE OF RESULTS

1) In-house control sample using orange juice powders (Tang).
2) The linearity of the calibration curve is in the range 5-150 μg ascorbic acid per ml of standard test solution.
3) A recovery of 100 ± 5% is found in the supplementary test.

11. METHOD VALIDATION

An amount of 5 mg of ascorbic acid per 100 g of sample or 10 μg per ml of standard test solution can be quantitatively evaluated. The upper detection limit for this method is 150 mg per 100 g of sample.

12. APPENDIX

12.1 Charcoal activated (Sigma, C-4386) can be used instead of Norit.

12.2 Instrument Performance Checks

12.2.1 Sensitivity

Check frequency: When use

Perform an instrument sensitivity check with a 100 ug/mL standard solution and plot the signal on the control chart. The signal should be within the acceptable control limit.

12.2.2 Detection Limit

Frequency: Yearly

Determine a reagent blank and a 0.25 ug/mL standard solution 10 consecutive times. The standard solution should give a minimum response of three times the standard deviation of the blank. Compare the detection limit against previous results.

12.3 Recommended QA/QC protocols include:

12.3.1 An instrument sensitivity check using the 100 ug/mL standard solution and plotting the signal on the control chart. The signal should be within the acceptable control limit.

12.3.2 QC standard check of 100 ug/mL (sec. 8.2) per batch of 10 samples. A 15% acceptance criteria applies.

12.3.3 1 reagent blank per batch of 10 samples.

12.3.4 1 spike per batch of samples if batch is larger than 10 samples or per every 10 samples tested if batch is smaller than 10 samples.

12.3.5 1 duplicate sample per batch of samples if batch is larger than 10 samples or per every 10 samples if batch is smaller than 10 samples.

VITAMIN C ANALYSIS IN JUICES BY TITRATION METHOD

1. PURPOSE/SCOPE:

The method is applicable to determination of reduced ascorbic acid in food products, juices and vitamin preparation. The method is not applicable to juices with dark red, blue and violet color.

2. SAFETY:

2.1 Carry out sample preparation and analysis under subdued light
2.2 Wear gloves to reduce contact of corrosive substances with skin.
2.3 Use fume hood to reduce inhalation of acids.
2.4 If you do not know the hazards of the chemicals involved, consult MSDS.

3. REFERENCES:

Official Methods of Analysis of AOAC International. 1997, 16th ed., 3rd rev.
Association of Vitamin Chemists Inc. 1951 Methods of Vitamin Assay. 2nd ed. on. Interscience Publishers, Inc., New York.

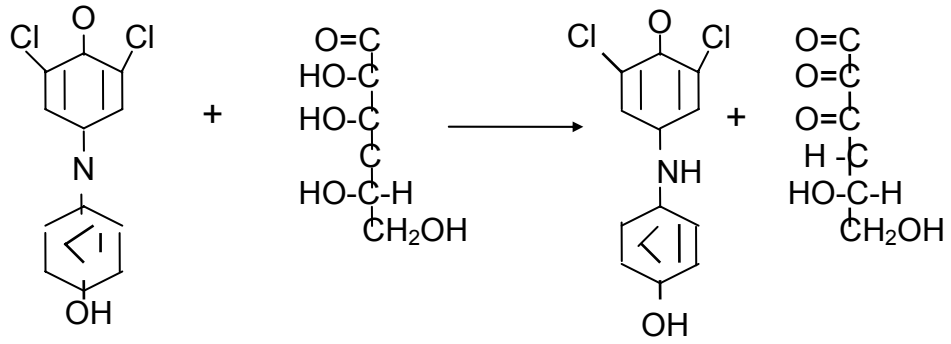
4. DEFINITION:

Vitamin C is the ascorbic acid determined by the reduction with 2, 6-dichloroindophenol solution to a colorless dye.

5. PRINCIPLE:

Ascorbic acid reduces oxidation-reduction indicator dye, 2, 6-dichloroindophenol to colorless solution. After the ascorbic acid is oxidized to dehydroascorbic acid, excess dye remains pink in acid solution. At end point, excess unreduced dye is rose pink in acid solution. Vitamin is extracted and titration performed in presence of HPO_3-CH_3COOH or HPO_3-CH_3COOH-H_2SO_4 solution to maintain proper acidity for reaction and to avoid autoxidation of ascorbic acid at high pH.

REACTION



2,6 Dichloroindophenol Ascorbic acid colorless dye Dehydroascorbic acid

6. REAGENTS AND MATERIALS:

Water used in this method is deionized water

6.1 Ascorbic acid Standard Solution, 1 mg/mL.
Weigh accurately 50 mg Ascorbic Acid Reference Standard to a 50 mL volumetric flask. Dissolve and dilute to volume immediately before use $HPO_3.CH_3COOH$.

6.2 2,6-Dichloroindophenol Standard Solution.

6.2.1 Dissolve 50 mg 2, 6 dichloroindophenol and 42 mg $NaHCO_3$ in approximately 50 mL water and dilute to 200mL with water.

6.2.2 Filter through Whatman filter paper #541into amber glass bottle and store in refrigerator.

6.2.3 Standardize before use. Pipet in triplicate 2mL aliquots of ascorbic acid standard solution to a 50 mL erlenmeyer flask containing 5mL $HPO_3.CH_3COOH$ solution. Titrate rapidly with indophenol solution from 50 mL digital burette until the solution turn light rose pink in color. Distinct rose pink color persist for $\geq$5 seconds. Record the volumes (mL) used and get the mean.

6.3 Metaphosphoric acid-acetic acid solution.

6.3.1 Dissolve with shaking, 15 g HPO_3 pellets or freshly pulverized stick HPO_3 in 40 mL CH_3COOH and 200 mL water.

6.3.2 Dilute to about 500 mL with water.. (HPO_3 slowly changes to H_3PO4, but if stored in refrigerator, solution remains satisfactory 7-10 days).

6.4 Sulfuric acid, 0.3N
Pipet 8.33 mL conc H_2SO_4 into 200 mL water and and dilute to 1L

6.5 Metaphosphoric acid-acetic acid-sulfuric acid solution.
Proceed as in (6.3.), except use 0.3N H_2SO4 in place of water.

6.6 Standard Buffer solution, Fisher, pH 4, 7, & 10

6.7 Filter paper, Whatman No. 541

7. APPARATUS:

7.1 Burette digital, 50 mL, 0.01 mL graduation
7.2 Volumetric flask, 25, 50, & 100 mL
7.3 Volumetric pipet, 1, 2, 3, 4, 5, & 10 mL
7.4 Measuring pipet, 5, 10 mL, 0.1 mL graduation
7.5 Erlenmeyer flask, 50 mL
7.6 Desiccator with desiccant
7.7 pH meter, capable of measuring pH ranging 1.0 to 7.0
7.8 Glass funnel, suitable for use with a fluted filter paper
7.9 Graduated cylinder, 100, 250 mL
7.10 Beaker, 250 & 500 mL
7.11 Tissue Homogenizer
7.12 Analytical Balance
7.13 Blender

8. PROCEDURE:

8.1 Preliminary test for appreciable amounts of basic substances

8.1.1 Weigh representative amount of sample (approx. 2g) into a beaker and add approx. 25mL $HPO_3.CH_3COOH$ solution.

8.1.2 Test pH by using pH meter pH > 1.2 indicates appreciable amounts of basic substances. For liquid preparations, dilute representative sample approx. two fold with $HPO_3.CH_3COOH$ solution, before testing with indicator.

8.2 Sample Preparation

8.2.1 For dry materials containing no appreciable amounts of basic substances.

8.2.1.1 Pulverize sample by gentle grinding in a blender

8.2.1.2 Weigh 5-10 g samples (depending on ascorbic acid content) in 100 mL beaker and homogenize with approximately 50 mL $HPO_3.CH_3COOH$ solution using tissue homogenizer until sample is in suspension.

8.2.1.3 Transfer homogenized sample into a 100mL volumetric flask and dilute to volume with HPO_3. CH_3COOH solution. If very low conc. of ascorbic acid is expected, dilute to a smaller volume. Use 10mL extracting solution per g dry sample. The final solution should contain 10-100 mg ascorbic acid/100 mL.

8.2.2 For dry materials containing appreciable amounts of basic substances.

8.2.2.1 Pulverize sample by gentle grinding in a blender.

8.2.2.2 Weigh 5-10g sample (depending on ascorbic acid content) in 100 mL beaker, add $HPO_3.CH_3COOH.H_2SO_4$ solution, to adjust pH to ca 1.2, and homogenize using tissue homogenizer until sample is in suspension.

8.2.2.3 Transfer homogenized sample into a 100mL volumetric flask and dilute to volume with HPO_3. CH_3COOH solution. If very low conc. of ascorbic acid is expected, dilute to a smaller volume. Use 10mL extracting solution per g dry sample. The final solution should contain 10-100 mg ascorbic acid/100 mL.

8.2.3 For liquid materials.

8.2.3.1 Weigh 5-10g sample (depending on ascorbic acid content) in a 100 mL volumetric flask. If appreciable amounts of basic substances are present, adjust pH to ca 1.2 with $HPO_3.CH_3COOH.H_2SO_4$ solution using pH meter.

8.2.3.2 Dilute to volume with HPO_3. CH_3COOH. The final solution should contain 10 to 100mg ascorbic acid /100 mL.

8.2.4 For fruit and vegetables juices.

8.2.4.1 Prepare fresh juices by pressing the fruit pulp using a cheese cloth or commercial device and filter through absorbent cotton or Whatman No. 541 filter paper. Collect juice in a clean container.

8.2.4.2 Mix juice thoroughly using stirring rod or by shaking to ensure a uniform sample.

8.2.4.3 Weigh 40-50g sample (depending on ascorbic acid content) in a 100 mL volumetric flask and dilute to volume with $HPO_3.CH_3COOH$ solution. Mix and filter using Whatman filter paper # 541.

8.3 Determination:

8.3.1 Blank test

Measure in triplicate 7mL $HPO_3.CH_3COOH$ solution into a 50 mL erlenmeyer flask and titrate with indophenol solution until rose pink color persist for $\geq$5 seconds. Record the volumes (mL) used and get the mean.

8.3.2 Titration of the sample

Pipet aliquots ranging from 1 to 5 mL of sample containing ca 2 mg ascorbic acid into each of two 50 ml erlenmeyer flasks. Prepare necessary dilutions if sample is too concentrated. Add enough $HPO_3.CH_3COOH$ solution to make a total volume of 7 mL. Titrate with indophenol solution using a digital burette. Record the volume in mL.

9. CALCULATION:

9.1 Calculate for mg ascorbic acid per mL indophenol solution (Factor)

$$\text{mg/mL Ascorbic acid standard} = \frac{\text{Weight Ascorbic acid standard}}{\text{Total dilution volume}}$$

$$\text{Factor} = \frac{\text{2 mL x mg/mL Ascorbic acid standard}}{\text{(Mean vol (mL) indophenol std – Mean vol (mL) blank)}}$$

9.2 Calculate for mg ascorbic acid per 100g sample

$$\text{mg/100 Vit C} = \frac{\text{(Vol indophenol soln-blk) Factor x Total vol x Dil x 100}}{\text{Weight x Aliquot}}$$

9.3 Test Report:

Report results of analysis in mg/100g edible portion as whole number.

10. ACCEPTANCE OF RESULTS:

Test result is accepted if the following are satisfied

10.1 Results of duplicate analysis should not vary by more than 10%.

10.2 Recovery range should be between 70-120%.

11. METHOD VALIDATION:

11.1 Precision as repeatability (r), within day

Parameter	Orange powder, Tang
Number of analysis (n)	10
Mean (mg/100g)	301
Standard deviation (s.d.)	6.26
Relative Standard Deviation %(RSD)	2.08
Repeatability at 95% confidence limit(r)	20.00

11.2 Precision as reproducibility (R)

Parameter	Orange powder, Tang
Number of analysis(n)	5
Mean (mg/100g)	292
Standard deviation (s.d.)	11.55
Relative Standard Deviation %(RSD)	3.96
Repeatability at 95% confidence limit(r)	45.38

11.3 Accuracy (% Recovery range)

Parameter	Orange powder, Tang
Number of days (n)	5
Conc. of standard added (mg/100g)	100-400
Recovery range (%)	86.4-107.4

11.4 Limit of detection

Parameter	Orange powder, Tang
Number of days (n)	10
Mean (mg)	0.00843
Standard deviation	0.0016
Limit of detection	0.0132

11.5 Limit of quantitation/reporting

Parameter	Orange powder, Tang
Number of days (n)	10
Mean (mg)	0.00843
Standard deviation	0.0016
Limit of quantitation	0.02443

12. Appendix

The value of the indophenol reagent for the determination of ascorbic acid is limited by the presence of reducing substances in the foods such as ferrous iron, stannous tin, cuprous copper, sulphur dioxide, sulphite, or thiosulphate. Another limitation of the method arises when the sample to be analysed gives a highly intense coloured solution, such as the reddish-purplish colours of certain fruits.

Ascorbic acid is a highly unstable vitamin; it is sensitive to alkalis and to oxidation. The sampling and extraction of the sample material must therefore be carried out with minimum delay. As much of the combination as possible should be conducted in the presence of stabilising acids mentioned above. These acids are able to retard the oxidation of ascorbic acid by inactivating the catalytic effects of ascorbic acid oxidase, copper and iron. They also aid in maintaining proper acidity for the reaction with the indophenol dye. It is also desirable to perform the titration rapidly.

DETERMINATION OF VITAMIN C BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

1. PURPOSE/SCOPE

The method detects vitamin C (ascorbic acid) of natural origin using HPLC method. Ascorbic acid added during the processing of the foodstuff may also be detected. The method is applicable to both fresh and stored foodstuffs intended for direct consumption such as vegetables, fruits, fruit juices, milk and milk preparations. Vitamin C content of over 0.5 mg per 100 g can be determined quantitatively.

2. SAFETY

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3. REFERENCES

1) Lakshanasomya N. 1998. Determination on Vitamin C in Some Kinds of Food by HPLC. Bull. Dept. Med Sci 40(3): 347-357.
2) Knelfel W and Sommer R. 1985. HPLC methode zur bestimmung von vitasmin C in milch, molke and molkegetranken. Z Lebensm Unters Forsch. 181: 107-110. (in German)

4. DEFINITION

Vitamin C content is understood to be the content of ascorbic acid plus dehydroascorbic acid. This method determined ascorbic acid of natural origin and, if required, vitamin C added during the production of the foodstuff. It is given in mg of ascorbic acid per 100 g of original sample.

5. PRINCIPLE

The material under investigation is homogenised in 3% metaphosphoric acid. The sample extract, obtained after filtering the homogenate, is chromatographed without preliminary clean-up on an RP C18 column by means of HPLC. Evaluation is carried out by comparing the peak area against an ascorbic acid standard.

6. REAGENTS

Unless otherwise indicated AR grade chemicals are to be used. The water used must be double distilled and taken from glass containers or corresponding purity.

1) Metaphosphoric acid, rods, ($60\pm2\%$ HPO_3, $36\pm1\%$ $NaPO_3$)
2) Potassium dihydrogen phosphate
3) Ortho-phosphoric acid
4) L (+)-Ascorbic acid (vitamin C) AR grade and biochemical purposes, e.g. Merck: reference standard is kept tightly in a refrigerated desiccator. The bottle of standard is allowed to come to room temperature in another desiccator before a portion is removed. Opened bottles of ascorbic acid reference standards are stable for three months.
5) Metaphosphoric acid solution, 3%; dissolve 30 g of metaphosphoric acid in water, make up to 1000 ml, can be stored for seven to ten days when frozen.

6) 3 M potassium dihydrogen phosphate in 0.35% (v/v) ortho- phosphoric acid : dissolve 0.408 g KH_2PO_4 in 1 L of 0.35% (v/v) ortho-phosphoric acid.
7) Ascorbic acid standard solution; dissolve 100 mg of ascorbic acid in 3% metaphosphoric acid, make up to 100 ml volumetric flask (=stock solution), ascorbic acid content 1 mg/ml; make up new daily: 0.25, 0.5, 1, 2, and 3 ml of stock solution diluted to 100 ml volumetric flask with 3% metaphosphoric acid. Mix well. (=ascorbic acid standard solution with : 2.5, 5.0, 10, 20 and 30 μg per ml)

7. APPARATUS

Instruments & glassware

1) Usual basic laboratory equipment
2) Vortex mixer
3) Analytical balance
4) Volumetric flask 100 ml
5) Glass filter funnel, 15-20 cm diameter.
6) Filter paper
7) Ultrasonic bath
8) HPLC apparatus consisting of:
   Pump: e.g. Waters model 510
   Injector: 20 μl
   Column: 5 μm Lichrocard Lichrospher 100 RP18, 125 x 4 mm, Merck
   UV detector: 248 nm
   Integrator
   Chromatographic conditions:
   Stationary phase: 5 μm Lichrocard Lichrospher 100 RP 18, Merck
   Mobile phase: 0.3 mM potassium dihydrogen phosphate in 0.35% (v/v) ortho- phosphoric acid
   Flow rate: 0.5 ml/min.
   Detection: by means of the retention time for ascorbic acid and/or co-eluted added reference ascorbic acid, at λ = 248 nm.
   Integrator: data module
   Peak evaluation: by peak area.

8. PROCEDURE

8.1 Sample Preparation

8.1.1. The weighed portion of food (about 2.5 g) is homogenised in 100 ml volumetric flask with a suitable volume of 3% metaphosphoric acid.

8.1.2. Shake vigorously 2 min and sonicate in ultrasonic bath for 5 min.

8.1.3. The sample extract is made up to the mark with 3% metaphosphoric acid.

8.1.4. Filter through filter paper Whatman #4 and further pass through membrane filter 0.45 μm. The resulting sample test solution should contain 0.5 – 2.5 mg ascorbic acid.

8.2 Analysis

Transfer the sample test solution into a vial and analyse using HPLC

9. CALCULATION, UNIT OF EXPRESSION AND TEST REPORT

9.1 Standard test values for the ascorbic acid standard concentrations of ascorbic acid per ml can be plotted or calculated by linear regression.

9.2 The values of sample in micrograms of ascorbic acid per ml can be read off the standard calibration curve or calculated.

9.3 The ascorbic acid content in 100 g of the sample material investigated can be calculated.

$$\text{Vitamin C (mg/100 g)} = \frac{A2 \times C1 \times V}{A1 \times 10 \times W}$$

A1 = peak area of standard solution
C1 = concentration of standard solution (μg/ml)
A2 = peak area of sample
V = final volume of sample (ml)
W = weight of sample (g)

The result of the determination is in mg/100 g sample.

10. ACCEPTANCE OF RESULTS

In-house control sample using orange juice powders (Tang) can be used.

11. METHOD VALIDATION:

11.1 **Range of linearity**: In the measurement range 2.5 – 30 μg ascorbic acid per ml, the peak areas are linearly proportional to the concentrations.

11.2 **Recovery**: The recovery of ascorbic acid for infant formula fortified juice, supplementary food for infant and children and in fortified candy were 96.67, 99.93, 103.23 and 102.45%, respectively.

11.3 **Repeatability**: The relative standard deviation for this method is 3.46%.

11.4 **Comparison with other methods for determination of vitamin C**: The method was compared with AOAC titrimetric method on the same samples of foodstuffs. The agreement was very good, correlation coefficient, r = 0.987; pooled standard deviation, Sp = 1.49, 1.89, respectively.

12. SUPPLEMENTARY NOTE:

When in solution, vitamin C is sensitive to oxygen, oxidising agents and catalytically active traces of metal. The material to be analysed should be prevented from coming into contact with metals (steel, copper, zinc equipment).

DETERMINATION OF PYRIDOXINE (VITAMIN B6) BY MICROBIOLOGICAL ASSAY METHOD

General information of microbiological assay is presented in the main protocol "Determination of Water-soluble Vitamin by Microbiological Assay"

1. PURPOSE OR SCOPE

This method is described for the determination of vitamin B_6 (pyridoxine) in foods by microbiological assay. Results are expressed as milligram per 100 g food sample.

2. SAFETY

3. REFERENCES

3.1 AOAC (2000) Microbiological Methods, 17th Ed., Ch 45 pp.47-48.
3.2 See other references in the mail protocol

4. DEFINITION

Vitamin B6 is generic descriptor for all 3-hydroxy –2-methlpyridine derivatives. Six vitamin B6 vitamers exist in all foods of both animal and plants, namely pyridoxine or pyridoxol (PN), pyridoxal (PL) and pyridoxamine which possess, respectively, alcohol, aldehyde and amine group in the 4-position; their 5' phosphate ester are designed as PNP, PLP and PMP. High concentration is present in yeast extract, wheat bran and liver. Whole grain cereals, nuts, pulses, lean meat, fish, kidney, potatoes and other vegetables are important sources. The major form of vitamin B6 in animal and fish tissue is PLP which is reversible bound to protein, PN and PNP are virtually absent. Plant tissue contains mostly PN. A ubiquitous bound form of PN is a PN-glucoside.

5. PRINCIPLE

Heating food sample with diluted mineral acid under autoclaving conditions liberates the B6 vitamers from their protein complex and also hydrolyses phosphorelated forms to the free vitamers. The process must be protected from light. This heat-treatment is necessary for the determination of total vitamin B6 in foods because the assay organism, Saccharomyces *carlsbergensis*, utilises only the non phosphorelated form of the vitamin. For general principle, see section 5 in the main protocol, **"**Determination of Water-soluble Vitamin by Microbiological Assay"

6. MATERIALS

6.1 Culture media

For general preparation of culture media, see section 6.1 in main protocol.

6.1.1 Potato Dextrose Agar; PDA (Difco Cat. No. 0013-17-6)
6.1.2 Micro inoculum broth (Difco Cat. No. 0320-02)

6.2 Assay medium

Pyridoxine Y Medium (Difco Cat. No. 0951-15-2) is used. For media preparation, see section 6.2 in main protocol.

6.3 Stock culture of test organism

6.3.1 Stock culture of *Saccharomyces carlsbergensis*

Streak the culture in 3 tubes of 5 ml PDA agar slant (6.1.1).
Incubate at 30°C for 24 hours.
Store the tubes at 4°C and subculture every two weeks in triplicate.

6.3.2 Inoculum

- Subculture *Saccharomyces carlsbergensis* from a stock culture to a tube containing 5ml of micro inoculum broth (6.1.2).
- Incubate at 30°C for 16-20 hours under aseptic condition.
- Centrifuge the culture at 2000 rpm for 10 minutes.
- Discard the supernatant and wash cells three times with 10ml of steriled NSS normal.
- Centrifuge at 2000 rpm for 10 minutes.
- Discard the last supernatant and dilute cell to an appropriate inoculum with steriled 0.85% NSS solution. (Mc Farland No 0.5)
- Mix thoroughly and use 1 drop to inoculate the vitamin assay tubes.

6.4 Reagents

For general reagents, see section 6.4 in main protocol "Determination of Water-soluble Vitamin by Microbiological Assay".

6.4.1 0.055N HCl

Dilute 4.6 ml hydrochloric acid fuming 37% (Merck Cat. No. 1.00317.2500) to 1 L with deionised water.

6.4.2 3M CH_3COONa

Dissolve 408.2 g sodium acetate trihydrate ($CH_3COONa.3H_2O$)Merck Cat. No. 1.06267.1000) in 1 L of deionised water.

6.4.3 Standards

6.4.3.1 Stock pyridoxine I, 100 μg/ml. (for standard curves preparation)

Accurately weigh 0.01215 g pyridoxine hydrochloride (Sigma Cat. No. P-9755) into a 100 ml volumetric flask. Dissolve and dilute to the volume with 25% ethanol.

6.4.3.2 Intermediate standard I, 1 μg/ml.

Pipette 2 ml of 6.4.3.1 into a 200 ml volumetric flask. Dilute to the volume with deionised water.

6.4.3.3 Working standard I, 4 ng/ml.

Pipette 2 ml of 6.4.3.3 into a 500 ml volumetric flask. Dilute to the volume with deionised water.

6.4.3.4 Prepare another set of stock standard pyridoxine (100 g/ml), intermediate standard II (1 μg/ml) and working standard II (4 ng/ml) according to the set of standard solution I for calibration curve. This set of standard is used for checking % recovery.

Note: Stock standard solution is kept in an amber glass bottles at 4°C. Prepare fresh every three months.

7. INSTRUMENT AND GLASSWARE

See section 7 in "Determination of Water-soluble Vitamin by Microbiological Assay"

8. PROCEDURE

8.1 Sample preparation

See section 8.1 in the main protocol, "Determination of Water-soluble Vitamin by Microbiological Assay".

8.2 Sample Extraction

8.2.1 Weigh accurately about 2-5 g dry powder (3 significant figures) or pipette 10 ml of liquid sample into a 250-ml Erlenmeyer flask.

8.2.2 For % recovery study, spike a sample (based on the in-house control sample) with 10 ml of 4 ng/ml standard solution (3.3.3).

8.2.3 Add about 100 ml 0.055N HCl (3.2.1) to all flasks. Cover with aluminum foil and mix thoroughly.

8.2.4 Autoclave at 121^{o}C for 3 hours.

8.2.5 Cool to room temperature in running water.

8.2.6 Adjusted pH to pH 4.6 with 3M CH_3COONa (3.2.6) and quantitatively transfer into a 200-ml volumetric flask.

8.2.7 Dilute to the volume with deionised water.

8.2.8 Then filter through filter paper Whatman No.42 or equivalent and collect the filtrate in a 125-ml Erlenmeyer flask. Dilute a portion of the clear filtrate to concentration of about 6-10ng pyridoxine/ml.

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8.3 Preparation of standard and sample set.

See section 8.3 in the main protocol "Determination of Water-soluble Vitamin by Microbiological Assay".

8.3.1 Standard set

Pipette working standard (4 ng/ml, 6.4.3.3), to give a standard set of 0, 4, 8, 12, 16, and 20 ng/tube

8.3.2 Sample set

Pipette diluted samples solution (8.2.8), deionised water and assay medium into the tubes in duplicate according to section 8.3.2 in the main protocol "Determination of Water-soluble Vitamin by Microbiological Assay".

After mixing, the tubes are steriled by autoclave at 121-123 ^{o}C for 10 min. Cool to room temperature in running water.

8.4 Inoculation

See section 8.4 in "Determination of Water-soluble Vitamin by Microbiological Assay".

8.5 Incubation

Incubate the whole set of tubes at 30^{o}C in shaking water bath or sloping the rack at 60^{o} for 16-18 hours.

Check the turbidity regularly after 16 h incubation.

8.6 Growth measurement

See section 8.6 in the main protocol "Determination of Water-soluble Vitamin by Microbiological Assay".

8.7 Preparation of standard growth curve

See section 8.7 in "Determination of Water-soluble Vitamin by Microbiological Assay".

9. CALCULATION

9.1 Vitamin B_6 concentration in unknown sample

$$\text{Vitamin } B_6 \text{ (mg/100g)} = \frac{\text{Sample conc. (ng/ml) X the appropriate dilution factor X 100}}{\text{sample wt. (g) x } 10^6}$$

Sample concentration as ng per ml, of diluted extract is taken from the standard growth curve.

9.2 % Recovery

$$\% \text{ Recovery} = \frac{\text{the recovery sample conc. (ng/ml) - control sample conc. (ng/ml)}}{\text{added standard conc. (ng/ml)}} \text{ X } 100$$

The concentration of the vitamin in the recovery sample and the control sample are taken from the standard growth curve.

9.3 Report

Results are reported to the nearest 0.1 mg/100g, or 2 significant figures.

10. ACCEPTANCE OF RESULTS

See section 10 in the main protocol, "Determination of Water-soluble Vitamin by Microbiological Assay".

11. SUPPLEMENTARY NOTE

See section 11 in "Determination of Water-soluble Vitamin by Microbiological Assay".

DETERMINATION OF FOLIC ACID BY MICROBIOLOGICAL ASSAY

General information of microbiological assay is presented in the main protocol "Determination of Water-soluble Vitamin by Microbiological Assay"

1. PURPOSE/SCOPE

This method is described for the determination of Folic acid in various kinds of foods by microbiological assay. Results are expressed as microgramme per 100 g food sample.

2. SAFETY

3. REFERENCE

3.1 AOAC (2000) Microbiological Methods,17th Ed., Ch 50 pp.24-26

3.2 See other references in the main protocol

4. DEFINITION

Folate is a term which includes all forms of folic acid. Natural folates exist as reduced forms of tetrahydrofolate and are predominantly attached to a polymeric chain of glutamate. The most common forms in foods are 5-methylhydrofolate, tetrahydrofolate and 5-formyl tetrahydrofolate. Free folic acid or Pteroyglutamic acid is the monoglutamate of pteroic acid and is produced synthetically. It is used for fortification of foodstuffs and supplements.

5. PRINCIPLE

To liberate of the natural folate (generally bound to protein), two-steps procedure is conducted involving thermal treatment to denature protein, following by enzymatic deconjugation with conjugase which converts the polyglutamate forms of folic acid to the mono or di glutamate. Then total folic acid is measured by microbiological assay. For general principle, see section 5 in "Determination of Water-soluble Vitamin by Microbiological Assay"

6. MATERIALS

6.1 Culture media

For general preparation of culture media, see section 6.1 in "Determination of Water-soluble Vitamin by Microbiological Assay".

6.1.1 Micro assay culture agar (Difco Cat. No. 0319-01-5)

6.1.2 Micro inoculum broth (Difco Cat. No. 0320-02)

6.2 Assay medium

Folic acid casei assay medium (Difco Cat. No. 0822-15) is used. For media preparation, see section 6.2 in "Determination of Water-soluble Vitamin by Microbiological Assay".

6.3 Stock culture of test organism

6.3.1 Stock culture of *Lactobacillus casei*

Stab culture in 3 tubes of 5 ml Micro assay culture agar (6.1.1).
Incubate at 35-37^{o}C for 24 h.
Store the tubes at 4^{o}C and subculture every two weeks in triplicate.

6.3.2 Inoculum

- Subculture *Lactobacillus casei from* a stock culture to a tube containing 5 ml of micro inoculum broth (6.1.2).
- Incubate at 35-37^{o}C for 16-20 h under aseptic condition.
- Centrifuge the culture at 2000 rpm for 10 min.
- Discard the supernatant and wash cells three times with 10ml of steriled 0.85% NSS solution.
- Centrifuge at 2000 rpm for 10 minutes.
- Discard the last supernatant and dilute cell to an appropriate inoculum with steriled 0.85% NSS solution. (Mc Farland No 0.5)
- Mix thoroughly and use 1 drop to inoculate the vitamin assay tubes.

6.4 Reagents

For general reagents, see section 6.4 in "Determination of Water-soluble Vitamin by Microbiological Assay".

6.4.1 Phosphate buffer, pH 6.1

Stock buffer, 0.2 M

Solution A: weigh 31.2 g $NaH_2PO_4 \cdot 2\ H_2O$* (molecular weight 156.01). Dissolve and dilute to 1000 ml with deionised water.

Solution B: weigh 28.39 g Na_2HPO_4* (molecular weight 141.96). Dissolve and dilute to 1000 ml with deionised water.

(**weight of salts depends on the forms available*)

Working buffer, containing 0.5% ascorbic acid

Working buffer must be freshly prepared by mixing 212.5 ml of solution A and 35.5 ml of solution B.

Adjust pH to 6.1 with solution A or solution B and dilute the solution to nearly 1000 ml.

Add ascorbic acid to the buffer at the concentration of 0.5% (w/v).

Readjust the pH and dilute to 1000 ml with deionised water.

6.4.2 Chicken pancreas γ-glutamyl hydrolase, 1% w/v

Prepare enzyme solution containing 10 mg enzyme per ml in working buffer (6.4.1) at pH 7.2 (optimum pH for conjugase chicken pancreas). Stirred and centrifuge at about 1500 rpm for 5 min before use. Add 2 ml (20 mg of the enzyme) of the diluted enzyme per 1 g of sample

6.4.3 Standards

6.4.3.1 *Stock folic acid I,* 100 μg/ml (for %recovery test)

Accurately weigh 0.025 g folic acid (Merck Cat. No. 103984) into a 250 ml volumetric flask. Dissolve and dilute to the volume with 25% ethanol

6.4.3.2 Intermediate *standard I–1,* 2 μg/ml (for %recovery test)

Pipette 5 ml of stock standard solution (6.4.3.1) into a 250-ml volumetric flask. Dilute to the volume with deionised water.

6.4.3.3 *Intermediate standard I–2,* 200 ng/ml (for %recovery test)
Pipette 5 ml of 6.4.3.2 solution into a 50-ml volumetric flask. Dilute to the volume with deionised water.

6.4.3.4 *Stock folic acid II,* 100 μg/ml. (for standard curves)
Prepare another set of stock solution following the same method as 6.4.3.1.

6.4.3.5 *Intermediate standard II–1,* 1 μg/ml. (for standard curves)
Pipette 2 ml of 6.4.3.4 into a 200-ml volumetric flask. Dilute to the volume with deionised water.

6.4.3.6 *Intermediate standard II–2,* 10 ng/ml. (for standard curves)
Pipette 2 ml of 6.4.3.5 solution into a 200-ml volumetric flask. Dilute to the volume with deionised water.

6.4.3.7 *Working standard*, 0.5 ng/ml. (for standard curves)
Pipette 10.0 ml of 6.4.3.6 into a 200-ml volumetric flask. Dilute to the volume with deionised water

Note: Stock standards is kept at 4°C and protected from light in amber glass bottles. Prepare fresh stock standards every three months.

7. INSTRUMENTS AND GLASSWARE

See section 7 in "Determination of Water-soluble Vitamin by Microbiological Assay"

8. PROCEDURE

8.1 Sample preparation

See section 8.1 in "Determination of Water-soluble Vitamin by Microbiological Assay".

8.2 Sample Extraction

8.2.1 Weigh accurately about 2-5 g dry powder (3 significant figures) or pipette 10 ml of liquid sample into a 250-ml Erlenmeyer flask.

8.2.2 For recovery study, weigh accurately about ≥ 2g dry sample (3 significant figures) or pipette 10 ml of liquid sample into a 250 ml Erlenmeyer flask. Spike the sample with 5 ml of 200 ng/ml standard solution (amount added depending on the endogenous folate in the in-house control sample).

8.2.3 Add 30-40 ml buffer (6.4.1) to each g of sample, and then close the flask with aluminum foil and mix.

8.2.4 Autoclave at 121-123 °C for 10 minutes. Cool to room temperature in running water.

8.2.5 Adjust pH of the heat-treated sample to 7.2. Add 20 mg chicken pancreas to each g of sample (e.g. 4 ml of diluted chicken pancreas solution (6.4.2) to 2 g sample) and 1-2 drop of toluene, mix and incubate in a water bath at 37°C for 16 hour.

8.2.6 Stop enzyme (and remove toluene) by boiling in a water bath for 15 minutes (or autoclave at 121-123°C for 5 minutes).

8.2.7 Cool in running water and quantitatively transfer into a 200-ml volumetric flask. Dilute to the volume with deionised water.

8.2.8 Filter through filter paper, discard the first portion and collect the filtrate in a 125-ml Erlenmeyer flask.

8.2.9 Adjust a portion of the clear filtrate to pH 6.2 and dilute to concentration of about 0.25-0.3 ng folic acid /ml with deionised water.

8.3 Preparation of standard and sample set

See section 8.3 in the main protocol "Determination of Water-soluble Vitamin by Microbiological Assay".

8.3.1 Standard set

Pipette working standard (0.5 ng/ml, 6.4.3.7) 1 to 5 ml into a series of tubes to give a standard set of 0, 0.5, 1.0, 1.5, 2.0, 2.5 ng/tube.

Dilute the working standard to 5 ml with phosphate buffer pH 6.1. Then follow steps of standard set preparation in section 8.3.1. in the main protocol.

8.3.2 Sample set

Pipette diluted samples solution (8.2.9), deionised water and assay medium into the tubes in duplicate. Dilute the sample to 5 ml with phosphate buffer pH 6.1. Then follow steps of unknown set preparation according to section 8.3.2 in the main protocol.

After mixing, the tubes are steriled by autoclave at 121-123 ^{o}C for 10 min. Cool to room temperature in running water.

8.3.3 Enzyme blank

Small amount of folate in 4 ml diluted chicken pancreas is analysed following the same method as unknown samples. The folate content obtained is then used to correct the amount of folate found in the unknown sample.

8.4 Inoculation

See section 8.4 in "Determination of Water-soluble Vitamin by Microbiological Assay".

8.5 Incubation

Incubate the whole set of tubes at 35-37^{o}C for 16-18 hours. Check the turbidity regularly after 16 h incubation.

8.6 Growth measurement

See section 8.6 in the main protocol "Determination of Water-soluble Vitamin by Microbiological Assay".

8.7 Preparation of standard growth curve

See section 8.7 in the main protocol "Determination of Water-soluble Vitamin by Microbiological Assay".

9. CALCULATION

9.1 Total folic acid concentration in unknown sample

$$\text{Folic acid } (\mu g/100g) = \frac{\text{Sample conc. (ng/ml)* x dilution factor x 100}}{\text{sample wt. (g) x } 10^3}$$

*Sample concentration, as ng per ml, of diluted extract is taken from the standard growth curve. Correction for folic acid in the diluted conjugase must be included in the process of calculation.

9.2 % Recovery

$$\%\text{Recovery} = \frac{\text{the recovery sample conc. (ng/ml) - control sample conc. (ng/ml)}}{\text{added standard conc. (ng/ml)}} \text{ X } 100$$

The concentration of the vitamin in the recovery sample and the control sample are taken from the standard growth curve. Correction for folic acid in the diluted conjugase must be included in the process of calculation

9.3 Report

Results are reported to the nearest 0.1 μg/100g, or 2 significant figures.

10. ACCEPTANCE OF RESULTS

See section 10 in "Determination of Water-soluble Vitamin by Microbiological Assay".

11. SUPPLEMENTARY NOTE

For general critical control points see section 11. In the main protocol "Determination of Water soluble Vitamin by Microbiological Assay". Some specific critical control points for folate are as follows.

Section	Control Item	Specification
6.4.1	Phosphate salt	Check formula carefully before use, adjust the weight of salts needed
	pH of buffer	Phosphate buffer at pH 6.1 is used for sample extraction by heat treatment while pH 7.2 is the optimum pH at the deconjugation step which carried out by enzyme conjugase from chicken pancreas
6.4.2	Chicken pancreas	A single enzyme is used in this protocol. In the future, tri-enzyme treatment (amaylase, protease and conjugase) may be required especially for samples with high protein and carbohydrate. Correction of folic acid in the commercial enzyme is necessary. Purification of the enzyme can be conducted. See reference 3.3 Augustin J et al, 1985, in the main protocol.

DETERMINATION OF CYANOCOBALAMIN (VITAMIN B12) BY MICROBIOLOGICAL ASSAY METHOD

General information of microbiological assay is presented in section "Determination of Water-soluble Vitamin by Microbiological Assay"

1. PURPOSE/SCOPE

This method is described for the determination of vitamin B_{12} (cyanocobalamin) in foods by microbiological assay. Results are expressed as microgram per 100 g food sample.

This method can apply for all foods. In general, the vitamin is present in foods of animal origin.

2. SAFETY

Potassium cyanide is a toxic substance; perform in the fume hood to avoid inhaling and directly contact.

3. REFERENCE

AOAC (2000) Microbiological Methods, 17th Ed., Ch 45 pp.47-48

4. DEFINITION

5. PRINCIPLE

See section 2 in **"**Determination of Water-soluble Vitamin by Microbiological Assay"

6. REAGENTS

6.1 Culture media

6.1.1 B_{12} culture agar USP (Difco Cat. No. 0541-15-9)

6.1.2 B_{12} inoculum broth USP (Difco Cat. No 0542-15-8)

6.1.3 Vitamin B_{12} (Lactobacillus) assay broth base (Merck Cat. No. 1.11988), or Difco B_{12} assay medium (Difco Ref No. 245710)

6.2 Reagents

For general reagents, see section 6.3 in "Determination of Water-soluble Vitamin by Microbiological Assay".

6.2.1 Extraction buffer (0.04M acetate buffer with KCN), freshly prepared

Dissolve 5.44 g $CH_3COONa{\cdot}3H_2O$ (MW 136.08) in deionised water and adjust pH to 4.5 with glacial acetic acid (about 3.5 ml). Dilute to 1000 ml with deionised water and add 1 % KCN solution to provide 5 mgKCN per 1000 ml buffer.

6.2.2 Tween 80 (Merck Cat. No. 822187) *(if assay medium from Merck is used)*

Prepare 1:25 Tween 80 solution by weighing 1 g of Tween 80 into a 25-ml volumetric flask and dilute to the volume with deionised water.

6.3 Standards

6.3.1 Stock standard vitamin B_{12} (100 μg/ml)

Accurately weigh 0.025g vitamin B_{12} (Sigma Cat. No. V-2876) into a 250 ml volumetric flask. Dissolve and dilute to the volume with 25% ethanol (6.2.5).

6.3.2 Intermediate standard I (1 μg/ml)

Pipette 5 ml of the stock standard (6.3.1) into a 500-ml volumetric flask and dilute to the volume with deionised water.

6.3.3 Intermediate standard II (10 ng/ml)

6.3.4 Pipette 5 ml of the intermediate standard I (6.3.2) into a 500-ml volumetric flask and dilute to the volume with deionised water.

6.3.5 Intermediate standard III, 1ng/ml. (for standard curves)

6.3.6 Pipette 10 ml of intermediate standard II (8.3.3) into a 100-ml volumetric flask. Dilute to the volume with deionised water.

6.3.7 Working standard, 40 pg/ml. (for standard curves)

6.3.8 Pipette 4 ml of intermediate standard III (6.3.4) into a 100-ml volumetric flask. Dilute to the volume with deionised water.

6.3.9 Prepare another set of stock standard vitamin B_{12} (100 μg/ml), intermediate standard I (1 μg/ml) and intermediate standard II, (10 ng/ml) according to the set of standard solution for calibration curve. This set of standard is used for checking % recovery.

Note: Stock standard solution should be kept in an amber bottle

6.4 Reference Culture

6.4.1 Stock culture of *Lactobacillus leichmannii* ATCC 7830:

- Stab culture of *Lactobacillus leichmannii* in 3 tubes of 10 ml B_{12} assay culture agar (6.1.1).
- Incubate at 35-37°C for 24 h.
- Store the tubes at 4°C and subculture, in triplicate, every week.

6.4.2 Inoculum

- Subculture *Lactobacillus leichmannii* from a stock culture to a tube containing 5 ml of B_{12} inoculum broth (6.1.2).
- Incubate at 35-37°C for16-18 hours.
- Centrifuge the culture at 2000 rpm for 10 minutes.
- Discard the supernatant and wash cells 3 times with 10 ml steriled 0.85% normal saline solution (NSS).
- Centrifuge the culture at 2000 rpm for 10 minutes, discard the last supernatant and dilute cell to an appropriate inoculum with steriled NSS.
- Mix thoroughly and use 1 drop to inoculate the vitamin assay tubes.

6.5 Assay medium

6.5.1 Calculate the medium used for each set of analysis (2 ml of medium solution for each analysed tube).

6.5.2 To prepare a single strength medium of 100 ml, suspend 8.3 g of the Vitamin B_{12} (Lactobacillus) assay broth base (6.1.3) in deionised water Add 5 ml of diluted Tween-80 (3.2.6) and dilute to 100 ml with deionised water.

6.5.3 Boil for 2-3 minutes and cool to room temperature before use.

6.5.4 If the Difco assay medium is used, suspend 8.5 g in 100 ml deionised water and boil 2-3 min. Use without adding any Tween 80.

7. APPARATUS

See section 7 in "Determination of Water-soluble Vitamin by Microbiological Assay"

8. PROCEDURE

8.1 Sample preparation

See section 8.1 in "Determination of Water-soluble Vitamin by Microbiological Assay".

8.2 Sample Extraction

8.2.1 Weigh accurately about 2-5 g dry powder (3 significant figures) or pipette 10 ml of liquid sample into a 250-ml Erlenmeyer flask.

8.2.2 For recovery study, spike an unknown sample with 5 ml of 10 ng/ml standard solution (6.3.3 set II).

8.2.3 Add about 40 ml buffer (6.2.1) for each gram of sample, mix and close the flask with aluminum foil.

8.2.4 Boil the sample in a boiling water bath, in a fume hood, for 30 minutes or autoclave at 120^{o}C for 10 minutes.

8.2.5 Cool to room temperature with running water.

8.2.6 Quantitatively transfer the content in each flask into a 200-ml volumetric flask. Dilute to the volume with deionised water.

8.2.7 Then filter through filter paper, discard the first portion and collect the filtrate in a 125-ml Erlenmeyer flask.

8.2.8 Adjusted pH of a portion of the clear filtrate to pH 6.1

8.2.9 Dilute to a concentration of about 20-30 pg vitamin B_{12}/ml.

8.3 Preparation of standard and sample set.

See section 8.3 in "Determination of Water-soluble Vitamin by Microbiological Assay".

8.3.1 Standard set

Pipette working standard (40 pg/ml, 6.3.5), to give a standard set of 0, 10, 20, 40, 60, and 80 pg/ml

8.3.2 Sample set

Pipette diluted samples solution (6.2.9), deionised water and assay medium into the tubes in duplicate according to section 8.3 "Determination of Water-soluble Vitamin by Microbiological Assay".

After mixing, the tubes are steriled by boiling at 100°C for15 min (or autoclave at 120°C for 5 min). Cool to room temperature in running water.

8.4 Inoculation

See section 8.5 in "Determination of Water-soluble Vitamin by Microbiological Assay".

8.5 Incubation

Incubate the whole set of tubes at 35-37°C for 16-18 hours. See section 8.5 in "Determination of Water-soluble Vitamin by Microbiological Assay". Check the turbidity regularly after 16 h incubation.

8.6 Growth measurement

See section 8.6 in "Determination of Water-soluble Vitamin by Microbiological Assay".

8.7 Preparation of standard growth curve

See section 8.7 in "Determination of Water-soluble Vitamin by Microbiological Assay".

9. CALCULATION, UNIT OF EXPRESSION AND TEST REPORT

9.1 Vitamin concentration in unknown sample

$$\text{Vitamin } (\mu g/100g) = \frac{\text{Sample conc. (pg/ml) X the appropriate dilution factor X 100}}{\text{sample wt. (g)}}$$

The sample concentration as pg per ml of diluted extract is taken from the standard growth curve.

9.2 % Recovery

$$\%\text{Recovery} = \frac{\text{the recovery sample conc. (pg/ml) - control sample conc. (pg/ml) X 100}}{\text{added standard conc. (pg/ml)}}$$

The concentration of the vitamin in the recovery sample and the control sample are taken from the standard growth curve.

9.3 Report

Results are reported to the nearest 0.1 μg/100g, or with 1 significant figure.

10. ACCEPTANCE OF RESULTS

See section 10 in "Determination of Water-soluble Vitamin by Microbiological Assay".

11. METHOD VALIDATION

-

12. APPENDIX

- See section 12 in “Determination of Water-soluble Vitamin by Microbiological Assay”.
- Do not forget to add diluted Tween 80 when the assay medium from Merck is used.

DETERMINATION OF FATTY ACIDS BY GAS CHROMATOGRAPHY METHOD

1. PURPOSE/SCOPE

The method is used for the quantitative determination of fatty acids in foods. This method covers a procedure for conversion of animal and vegetable fatty acids into methyl esters of the fatty acids suitable for analysis by gas-liquid chromatography (GLC).

2. SAFETY

2.1 All organic solvents and boron trifluoride should be used under fume cupboard.

2.2 Laboratory safety glasses and gloves should be used during extraction.

2.3 There are some precautions on some chemicals such as

2.3.1 Boron trifluoride (BF_3), 14% solution in methanol: corrosive! Avoid contact with eyes and skin and avoid breathing its vapour. Wear gloves and eye protection and use only in a fume hood.

2.3.2 Methanol AR: harmful if swallowed.

2.3.3 Sodium hydroxide AR pellets: caustic! Avoid contact with skin and eyes.

2.3.4 Iso-octane (trimethyl pentane) AR, or n-Heptane AR, inflamma-ble, avoid contact with skin or breathing vapour.

3. REFERENCES

3.1 AOAC-IUPAC Method. Preparation of methyl esters boron trifluoride method in: Firestone D, ed. AOAC Official Method of Analysis. 16 th ed. Virginia: AOAC International, 1995; 2: Chapter 41, 17-22.

3.2 AOCS.Official Methods 1990 Fourth edition Ce 2-66 (?)

3.3 AOCS .Official Method. Fatty acid composition by GC in: Firestone D,ed. Official Methods and Recommended practices. 4 th ed. USA: AOCS Press, 1994; 1: Ce 1-62 (?)

3.4 Jham, G.N., Teles, F.F.F. and Campos, L.G. 1982. Use of aqueous HCl/ MeOH as esterification reagent for analysis of fatty acids derived from soybean lipids. JAOCS. 59(3): 132-3.

3.5 Horwitz W (2000) (editor). Official Method of Analysis of AOAC Interna-tional. 17th Edition. AOAC International, Maryland, USA. 969.33, 963.22, 991.39, 41.1.30 and 41.1.28A.

3.6 Sullivan DM, Carpenter DE. 1993. Methods of Analysis for Nutrition Labeling. AOAC International, Arlington, Virginia USA. Pp. 258-262.

3.7 Official and Tentative Methods of the Americal Oil Chemist's Society (AOCS) Vol.1; Ce 1-62 (1975) Fatty Acid Composition be Gas Chromatography; Ce 2-66 (1973) Preparation of Methyl esters of Long Chain Fatty Acids.

4. DEFINITION

Fatty acids are one of the components of lipid or fat that are present in structures called triglycerides. Fatty acids are either saturated or unsaturated. In a saturated fatty acid the alkyl chain does not contain any double bonds. Unsaturated fatty acids contain one or more double bonds.

5. PRINCIPLE

For fatty acids profile, the lipid fraction is obtained by cold extraction using a mixture of chloroform and methanol. For saturated fat determination in plant source origin and mixed diet, the lipid fraction is obtained by acid digestion in the presence of antioxidant, followed by solvent extraction. In this case pyrogallic acid is added to minimize oxidative degradation of fatty acids during analysis. Lipid portion is then saponified, derivatised to fatty acid methyl esters with boron trifluoride/methanol, and determined by gas chromatography using Flame Ionisation Detector (FID). This method determines the percentage area of the fatty acids and the absolute concentration of specific fatty acids can be estimated using fatty acids internal standards.

6. REAGENTS

6.1 Boron trifluoride (BF_3), 14% or 20% solution in methanol
6.2 Methanol, AR
6.3 Sodium hydroxide, AR pellets
6.4 0.5N Methanolic sodium hydroxide: dissolve 2 g NaOH with 100 mL AR methanol.
6.5 Sodium chloride, AR: prepare saturated solution by dissolving approxima-tely 36 g NaCl in 100 mL in water.
6.6 Hexane
6.7 Heptane
6.8 Petroleum or iso-octane, AR
6.9 Chloroform, AR
6.10 Na_2SO_4, anhydrous, AR grade
6.11 Standard of fatty acid methyl esters (FAMEs)
6.12 Fatty acid internal standards, (C11:0, C17:0, C19:0, C23:0)
6.13 Antioxidant: pyrogallic acid, AR grade

7. APPARATUS

7.1 Intruments

7.1.1 Gas chromatograph with FID detector, split mode injector, oven temperature programming sufficient to implement a hold-ramp-hold sequence. Operating conditions: temperature: injector 225^{o}C, detector 285^{o}C, initial temp 100^{o}C (hold 4 min), ramp 3^{o}C/min, final temp 240^{o}C, hold 15 min; carrier gas, He; flow rate 0.75 mL/min; split ratio 200:1

7.1.2 Capillary column: 60 m x 0.25 mm id, 0.20 μm film, fused silica capillary, e.g. DB 5 (5% Phenyl methylpolysiloxane), DB 17 (50% Phenyl methylpolysiloxane), DB 23 (50% Cyanopropyl methylpolysiloxane) or equivalent

7.1.3 Rotary evaporator
7.1.4 Analytical balance, accurate to 0.0001 g
7.1.5 Water bath, capable of maintaining temperature of 100^{o}C
7.1.6 Refrigerated centrifuge

7.2 Glassware
7.2.1 Flatted or round bottom flasks 125 mL
7.2.2 Glass funnels
7.2.3 Separating funnel 100 or 250 mL
7.2.4 Screw cap test tubes
7.2.5 Pasteur pipette

8. PROCEDURE

8.1 Fat extraction

8.1.1 Cold extraction for fatty acids profile

8.1.1.1 Accurately weigh sample into a 125 mL flatted or round bottom flask.
8.1.1.2 Pour 50 mL chloroform/methanol (2+1) into the flask, add a magnetic bar, and stir with magnetic stirrer for at least 30 min.
8.1.1.3 Filter the solution through filter paper No. 1 into a separating funnel.
8.1.1.4 Rinse the flask with 2 x 25 mL chloroform/methanol (2+1) and add into the same funnel.
8.1.1.5 Pour 20 mL distilled water into the separating funnel and swirl gently.
8.1.1.6 Stand overnight or until two layers are completely separated.
8.1.1.7 Collect the lower layer into a flatted or round bot-tom flask, and then evaporate off the solvent by rotary evaporator until the solution is nearly dry.
8.1.1.8 Dissolve crude oil with 1-2 mL of each iso-propanol (to remove water) and hexane (to remove iso-propanol), and continue to evaporate off the sol-vents until nearly dry.
8.1.1.9 Add 4 mL hexane and evaporate off the solvent until nearly dry.
8.1.1.10 Blow the residue with oxygen free nitrogen (OFN) until dry.
8.1.1.11 The residual obtained is extracted lipid and ready to be analysed for fatty acids.

8.1.2 Hydrolytic extraction with antioxidant for saturated fat from plant source origin and mixed diet.

8.1.2.1 Similar to crude fat analysis, but in the presence of 100 mg antioxidant (pyrogallic acid) (AOAC Inter, 2000, 41.1.28A) to protect unsaturated fatty acid oxidation and maintain saturated fatty acid concentration.

8.1.2.2 Lipids in the sample is extracted from the food sample by acid digestion (AOAC,1990, 922.06) fol-lowed by organic solvent extraction.

8.2 Saponification and methylation

8.2.1 Weigh 0.05-0.1 g of extracted fat into screw cap tube (or test tube containing 5-10 mg of internal standard, see appendix 13.1).
8.2.2 Add 1-4 mL 0.5 N NaOH in methanol (see appendix 13.2) to the tube and shake.
8.2.3 Place the tube in a water bath at 85-100°C for 5-10 min.
8.2.4 Cool and then add 1-5 mL BF_3 (carried out in a fume cupboard)
8.2.5 Shake and heat at 85-100°C for 10-15 mins.
8.2.6 Cool and add 3 mL of iso-octane or other selected organic solvent.
8.2.7 Add 3 mL saturated NaCl solution, shake vigorously then centrifuge or stand until the iso-octane (upper) layer separates from the aqueous (lower) phase.
8.2.8 Transfer the upper layer through small amount of anhydrous Na_2SO_4 (placed on top of some cotton wool in a filter liner) into a test tube with a pasteur pipette.
8.2.9 Re-extract the aqueous phase twice more with 2 mL iso-octane and transfer the iso-octane layer through the Na_2SO_4 into the same test tube with the same Pasteur pipette.
8.2.10 Add a small amount of anhydrous Na_2SO_4.
8.2.11 Evaporate off solvent in the oven or by rotary evaporator under a stream of OFN.
8.2.12 Dissolve and dilute the FAMEs to an appropriate volume with iso-octane in a volumetric flask.
8.2.13 Sample is ready to be injected to GC column

9. CALCULATION

9.1 Identify the fatty acid methyl esters in the sample by comparing each retention time with the retention time of fatty acid methyl ester standards.
9.2 Quantification of FAME's
9.2.1 Using percentage area as reported by integrator (normalisation method)

$$\% \text{ Area of each fatty acids} = \frac{A_x}{A_T - A_{IS}} \times 100$$

where A_x = area of each fatty acid methyl ester
A_T = total area of all fatty acid methyl ester
A_{IS} = area of fatty acid internal standard

9.2.2 Quantifying as absolute concentration of specific fatty acids

$$\text{Conc of fatty acids (mg/g)} = \frac{A_x \times W_{IS} \times CF_x \times 1000}{A_{IS} \times W_S \times F_x}$$

where:

A_x = area of each fatty acid methyl ester
A_{IS} = area of fatty acid internal standard
W_{IS} = weight of internal standard (mg)
W_S = weight of sample (g)
CF_x = correction factor of detector for particular fatty acid obtained from AOCS reference mixture
F_x = factor necessary to express result as mg fatty acid/g oil (rather than as methyl ester).

$$F_x = \frac{\text{MW (molecular weight) of ester}}{\text{MW of fatty acid.}}$$

9.2.3 P/M/S ratio is calculated from the following equation:

$$\text{P/M/S} = \frac{\sum \% \text{ PUFA}}{\sum \% \text{ SFA}} : \frac{\sum \% \text{ MUFA}}{\sum \% \text{ SFA}} : 1$$

Report the P/M/S ratio to one decimal place.

10. ACCEPTANCE OF RESULTS

Accept test results if one or more of the following condition is satisfied.

10.1 Duplicate results should not differ by more than 10% of the mean.

10.2 Mean concentration of saturated fatty acid in control sample should be within $\pm$ 3SD in the control chart based on established acceptance criteria.

11. APPENDIX

11.1 Type and concentration of internal standard used.

Sample	Internal Standard Use		Fat Extraction	Reference
	Fatty acid	Conc. (mg/mL)		
Encapsulated Fish Oils	C 23:0	10	---	AOAC Inter. 2000, 41.1.28A

11.2 The amount of reagent needed.

Oil extracted (mg)	Flask (mL)	0.5N NaOH (mL)	14%BF_3-methanol (mL)
100 - 250	50	4	5
250 - 500	50	6	7
500 - 750	100	8	9
750 - 1000	100	10	12

Note: from ref. 3.7

DETERMINATION OF CHOLESTEROL BY GAS CHROMATOGRAPHY METHOD

1. PURPOSE/ SCOPE

The method is used for the quantitative determination of cholesterol in foods and food products by gas chromatography.

2. SAFETY

2.1 Trimethylchlorosilane may be carcinogenic. Trimethylchlorosilane and dimethylformamide may be mutagenic. Dimethylformamide may be teratogenic. Exposure to these compounds should be reduced to the lowest possible level. Avoid inhalation of vapors and contact with skin. Wear safety glasses.

2.2 Dispose of waste solvent in appropriate manner compatible with applicable environmental rules and regulations.

3. REFERENCES

3.1 Horwitz W (2000) (editor). Official Method of Analysis of AOAC International. 17th Edition. AOAC International, Maryland, USA.

3.2 Greenfield H and Southgate DAT (1992). Food Composition Data: Production, Management and Use. Elsevier Applied Science, UK.

3.3 Kirk RS and Sawyer R (1991). Pearson's Composition and Chemical Analysis of Foods, 9th Edition. Longman Scientific & Technical, Essex, England.

3.4 Kovacs, M.I.P., Anderson, W.E. and Ackman, R.G. (1979) *J. of food Science*, vol. 44, 1299-1301, 1305.

4. DEFINITION

Cholesterol in foods and food products is determined quantitatively as total cholesterol.

5. PRINCIPLE

The method for the determination of cholesterol involves alcoholic KOH saponification to extract lipid from samples. Then the non-saponifiable fraction containing cholesterol and other sterols is extracted with hexane. Sterols are derivatized to form trimethylsilyl (TMS) ethers, which are determined quantitatively by gas chromatograph, using 5α-cholestane as internal standard.

6. REAGENTS

6.1 *n*-hexane, AR grade

6.2 50 % aqueous KOH, AR Grade

6.3 Dimethylformamide (DMF) for GLC

6.4 Dimethyldichlorosilane (DMCS)

6.5 *n*-heptane, AR grade

6.6 Standard cholesterol solution

6.6.1 Stock solution: 2.0 mg/mL in dimethylformamide (DMF)

6.6.2 Working solution: dilute stock solution with DMF to obtain 5 solutions at concentrations 0.0025-0.2 mg/mL (i.e., 0.0025, 0.005, 0.01, 0.05, 0.1, and 0.2 mg/mL)

6.7 Standard 5α-cholestane solution (0.1 mg/mL). Dissolve 10 mg 5α-cholestane in 100 mL n-heptane and store in a closed container in the freezer. Allow reaching room temperature before use.

6.8 Hexamethyldisilane (HMDS)

6.9 Trimethylchlorosilane (TMCS)

6.10 95 % ethanol

7. APPARATUS

7.1 Analytical balance

7.2 Water bath or heating block

7.3 Pasteur pipettes

7.4 100 mL flat-round bottom flasks (evaporating flask)

7.5 Air oven

7.6 Vacuum rotary evaporator

7.7 Micropipette

7.8 Test tube with screw caps, 16 x 150 mm., 20 x 150 mm.

7.9 Pipette

7.10 Vortex mixer

7.11 Gas chromatograph:

7.11.1 Capillary column: 30 m, 0.32 mm, id 0.25 μm, HP-5 or equivalent

Operating condition: Injector Temp. 250^{o}C
Detector Temp. 300^{o}C
Column Temp. 190^{o}C; hold 2 min
Increase 20^{o}C /min to 230^{o}C; hold 3 min
Increase 40^{o}C /min to 255^{o}C; hold 25 min

Flow rate: Carrier gas (N_2 or He) 2.0 mL/min
H_2 40 mL/min
Air 400 mL/min
Split ratio 50:1
Make-up gas flow 40 mL/min

7.11.2 Packed column: Glass column 2.4 m x 4 mm diameter, 0.5% Apiezon L Chrom Q80/100

Operation condition: Oven temp. 240^{o} C
Injector 280^{o} C
Detector FID 290^{o} C
Flow rate N_2 40 mL/min
H_2 30 mL/min
Air 300 mL/min

8. PROCEDURE

8.1 Silylation

8.1.1 Rinse clean tubes with AR grade methanol and dry 30 min in an oven at 100^{o}C.

8.1.2 Transfer tubes to desiccator to cool.

8.1.3 Fill tubes with a 10% solution of DMCS in toluene, screw on tops, and let stand 10 min.
8.1.4 Drain tubes and rinse thoroughly with AR Grade methanol.
8.1.5 Dry in 100^{o}C oven before using for derivatization of samples.

Note: After use, tubes can be cleaned with methanol, water, and methanol, in that order. Dry tubes in 100^{o}C oven before using for derivatization. Tubes can be re-used without silylation, as long as strong alkali wash is avoided. Label tubes clearly to keep separate for re-use. Resilanize tubes at least every 6 months.

8.2 Saponification and extraction

8.2.1 Accurately weigh sufficient food sample (about 1-5 g) into a screw capped test tube.
8.2.2 Add 1 mL 50% aqueous KOH and 4 mL 95% ethanol to the test tubes.
8.2.3 Attach screw cap, place in heating block or water bath set at 85-120^{o}C and reflux solution 1 hr. It is important that all the homogenate be suspended and not adhered to the wall above the solution.
8.2.4 Turn off heating block or water bath and after cooling add 2.5mL water.
8.2.5 Add 5 mL n-hexane, shake vigorously 15 sec using vortex, let the layers separate.
8.2.6 Remove the upper organic layer with a Pasteur pipette and place in an evaporating flask.
8.2.7 Repeat for a total of four extractions (4 x 5 mL n-hexane).
8.2.8 Evaporate the total extract to dryness on a vacuum rotary evaporator at 40^{o}C.

8.3 Derivatization

8.3.1 Prior to analysis by GLC, dissolve residue in 3 mL DMF and transfer 1 mL of DMF sample solution into a silanized test tube.
8.3.2 In the fume cupboard, add 0.2 mL HMDS and 0.1 mL TMCS to each tube, stopper, and shake vigorously 1 min using vortex mixer. Let the solution stand undisturbed for 15 min.
8.3.3 Add 1.0 mL 5 α-cholestane internal standard (0.1 mg/mL) and 10 mL distilled water (in fume cupboard). Vigorously shake it for 1 min.

Note: Solution should fume when water is added.

8.4 GLC Measurement

8.4.1 Inject 1 μl of upper heptane layer into gas chromatograph.
8.4.2 Inject 1 μl each of standard solution into gas chromatograph
8.4.3 Keep silane residues in separate flasks for proper disposal.

9. CALCULATION

The amount of cholesterol in mg/100 g sample is calculated as follows

$$\text{Cholesterol (mg/100g)} = \frac{\text{Cholesterol from standard curve (mg/mL)} \times \text{dilution} \times 100}{\text{Weight of sample (g)}}$$

Cholesterol from standard curve (mg/mL) obtains from correlation between the ratio of cholesterol standard and internal standard compare with each concentration.

Report test results (in g per 100 g sample) to one decimal place.

10. ACCEPTANCE OF RESULTS

10.1 The level of cholesterol in control sample must be within $\bar{x} \pm 2$ SD on the control chart.

10.2 The different between duplicate results should be less than 10 %.

DETERMINATION OF AMINO ACID BY AMINO ACID ANALYZER

1. SCOPE

Applicable to determination of amino acid composition in Food and Food products by Amino acid Analyzer.

2. SAFETY

2.1 Wear eye / hand protection when preparing 6N HCl

2.2 Prepare performic acid in Fume hood

2.3 Wear hand protection when use Ninhydrin

3. REFERENCES

Instruction manual for the L – 8500 High Speed Amino Acid Analyzer. Hitachi, Ltd. 1986.

4. DEFINITION

Amino Acid Composition refer to:

- Aspartic acid
- Serine
- Glycine
- Valine
- Isoluecine
- Tyrosine
- Lysine
- Arginine
- Cystine
- Threonine
- Glutamic acid
- Alanine
- Methionine
- Luecine
- Phenylalanine
- Histidine
- Proline

(Not including tryptophan)

5. PRINCIPLE

The food sample is preliminary acid treated to hydrolyse proteins and peptides to free amino acids. The free amino acids obtained are separated by ion-exchange chromatography using different pHs buffers. The amino acids emerging from the column are quantitatively determined by mixing with ninhydrin to develop a proportional purple blue colour. The product of ninhydrin reaction,

Diketohydrindyliden Diketohydrindamin (DYDA), is known to exhibit maximum light absorbance at wavelength of 570 nm. For studies of α–imino compounds such as proline and hydroxyproline, where the resultant product of the ninhydrin reaction is not DYDA, the maximum absorbance will occur at some wavelength other than 570 nm, and a special 440 nm filter is provided for this.

This method can not be applied directly to sulphur-containing amino acids and to tryptophan due to their instability during acid hydrolysis. Separate procedures for these amino acids are necessary. It is, therefore, the amino acids analysis is divided in to 3 determinations:

1) 6 N HCl Hydrolysis: Acid – stable amino acids.
2) Performic Acid Oxidation followed by 6 N HCl Hydrolysis: Sulphur – containing amino acids.
3) Alkaline hydrolysis: Tryptophan

6. REAGENTS

6.1 Hydrochloric acid

6.2 Formic acid

6.3 Hydrogen peroxide

6.4 Sodium citrate 2 H2O

6.5 Sodium hydroxide

6.6 Sodium chloride

6.7 Citric acid

6.8 Ethyl alcohol

6.9 Benzyl alcohol

6.10 Thiodiglycol

6.11 Brij – 35

6.12 Caprylic acid

6.13 Lithium acetate dihydrate

6.14 Glacial acetic acid

6.15 Propylene glycol monomethyl ether

6.16 Ninhydrin

6.17 Sodium borohydrile

6.18 Standard

6.18.1 Amino acid standard solution Type H Lot. No. TWE 4765 from Wako Pure Chemical Industries. Ltd.

6.18.2 L – Cystine (Mercks)

7 APPARATUS:

7.1 Instruments

7.1.1 Amino acid analyzer: Hitachi Model 8500 L

7.1.2 Rotary evaporator

7.1.3 Water bath

7.1.4 Vacuum Pump

7.1.5 Analytical balance, 200 g. cap 0.1 mg sensitivity

7.1.6 Autoclave

7.2 Glasswares

7.2.1 Hydrolyzate tube: Bombelroll tube

7.2.2 Erlenmeyer flask: 50, 25 ml

7.2.3 Volumetric flask: 50, 25, 10 ml, 1 L

7.2.4 Vial 1.5 ml

7.2.5 Funnel 65 mm

7.2.6 Measuring pipette : 0.5, 1, 2, 5, 10 ml

7.2.7 Measuring cylinder : 500, 100 ml

7.2.8 Dropping pipette

7.2.9 Membrane filters (cellulose Nitrate)

8. PROCEDURE:

8.1 Preparation of reagents

8.1.1 6 N HCl: Dilute 531 ml of concentrate Hydrochloric acid (sp. gr =1.18) to 1 L with deionization water.

8.1.2 Buffer solution

Name	PH-1	PH-2	PH-3	PH-4	PH-5
Vessel (elute)	B1	B2	B3	B4	B5
Sodium concentration	0.16	0.2	0.2	1.2	0.2
1. Distilled water (approx.)	700 ml	700 ml	700 ml	700 ml	700 ml
2. Sodium citrate (2 H_2O)	6.19 g	7.74 g	13.31 g	26.67 g	-
3. Sodium hydroxide	-	-	-	-	8.00 g
4. Sodium chloride	5.66 g	7.07 g	3.74 g	54.35 g	-
5. Citric acid (H_2O)	19.80 g	22.00 g	12.80 g	6.18 g	-
6. Ethyl alcohol	130.0 ml	20.0 ml	4.0 ml	-	100.0 ml
7. Benzyl alcohol	-	-	-	5.0 ml	-
8. Thiodiglycol	5.0 ml	5.0 ml	5.0 ml	-	-
9. Brij – 35	4.0 ml	4.0 ml	4.0 ml	4.0 ml	4.0 ml
10. pH (nominal)	3.3	3.2	4.0	4.9	-
11. Total (adjust)	1.0 L	1.0 L	1.0 L	1.0 L	1.0 L
12. Caprylic acid	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml

8.1.3 0.2 N Sodium citrate pH 2.2

8.1.3.1 Weigh out 98.5 g of tri-sodium citrate dihydrate.

8.1.3.2 Dissoved the sodium citrate in ca 4.0 L of deionized water, Check that all the crystals have been completely dissolved.

8.1.3.3 While stirring, add 82.5 ml hydrochloric acid, phenol 5 g and 100 ml of Thiodiglycol.

8.1.3.4 Add deionized water to make 5 L of solution.

8.1.3.5 Add 40 ml of 25% Brij – 35 solution and mix.

8.1.4 Ninhydrin solution and ninhydrin buffer solution

Vessel	Step	Reagent	Measurement
R2 for ninhydrin buffer solution	1	Distilled water	671 ml
	2	Lithium acetate dihydrate	407 g
	3	Glacial acetic acid	245 ml
	4	Propylene glycol monomethyl ether	801 ml
	5	Total	2000 ml
	6	Nitrogen bubling	10 min minimum
R1 (ninhydrin)	1	Propylene glycol monomethyl ether	1957 ml
	2	Ninhydrin	77 g
	3	Nitrogen bubling, dissolution	5 min minimum
	4	Sodium borohydrile	161 mg
	5	Nitrogen bubling	20 min minimum

8.1.5 Performic acid: Mix 1 volume of 30% hydrogen peroxide and 9 volume of 98% formic acid allow to stand for 1 h at room temperature, Keep at 0^{o} to 5^{o}C until use.

8.2 Preparation of Standard

8.2.1 Preparation of Standard Amino Acid Mixture Sample

Sample 2 ml out of a furnished ampoule Amino Acid Calibration Mixture where 18 amino acid components are mixed, dilute it with 0.02 N HCl and make the total 10 ml.

It means that 10 μl contains 5 nmol of each amino acid. Use it as a standard sample for a standard assay.

After diluting, the sample remains stable for about 1 month in a refrigerator.

8.2.2 Standard cystine (500 ng/10ml)

Dissolve 0.125 g of cystine in 2 ml of 0.1N NaOH and dilute to 10 ml with distilled water. The solution should be used within 1-2 h. Use 0.1 ml of the standard and subject to the below procedure as of the unknown sample (8.3.2).

8.3 Sample preparation

8.3.1 Acid hydrolysis method

8.3.1.1 Weigh a quantity of sample accurately, to obtain approximately 100 mg of protein, into a 250 ml. Bombelroll tube, Add hydrochloric acid to 50 ml. Of volume, shake gently and close the tube tightly with a stopper.

8.3.1.2 Heat the tube 110 - 115^{o} C in an autoclave for 7 hours. Let the tube cool to room temperature.

8.3.1.3 Evaporate to dryness by rotary vacuum evaporator.

8.3.1.4 Dissolve the residue in 50 ml of sodium citrate pH 2.2

8.3.1.5 Fillter through Whatman no. 42 paper.

8.3.1.6 Take aliquot of solution, filter the solution through 0.45 μm membrane filter and load to amino acid analyzer.

8.3.2 Performic acid oxidation followed by acid hydrolysis method

8.3.2.1 Weigh a quantity of sample accurately to obtain approximately 10-50 mg of protein into a 50 ml erlenmeyer flask.

8.3.2.2 Add 4 ml of cooled performic acid mix gently cover the flask with beaker and leave in the freezer overnight.

8.3.2.3 Transfer the mixture to 25 ml of Bombelroll tube. Wash the residue in the erlenmeyer flask with 2-3 ml of 6 N HCl, 3-4 times and transfer to the Bombelroll tube. Close the tube tightly with stopper.

8.3.2.4 Heat at 115^{o} C in autoclave for 7 hours and cool to room temperature.

8.3.2.5 Evaporate to dryness by rotary vacuum evaporator

8.3.2.6 Dissolve the residue in 25 ml of sodium citrate buffer pH 2.2

8.3.2.7 Take an aliquot of the diluted hydrolysate and load to the Amino acid analyzer.

9. Calculations and Unit of Expression

9.1 Compute mg/100 g sample using the formula

$$\text{mg / 100 g sample} = \frac{x\ v}{10\ aw}$$

when x = amount of amino acid (ng/volume sample injected)

v = total volume of sample

a = volume sample injected

w = Sample weight (g)

9.2 Unit of expression

The amino acid components in sample are reported in term of milligrom per 100 gm sample

10. Acceptance of results

10.1 Duplicate results for major component should not differ by more than 5 % of the mean.

10.2 Standard solution is injected before series of sample injections and at frequent regular's intervals such as every 10 runs. The result of standard solution should not differ by more than 5 % of the first injection

11. Method validation

12. Appendix

Alternative method for analysis of amino acid is separately provided.

ANALYSIS OF AMINO ACID IN FOODS USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

1. SCOPE

Applicable to determination of amino acid composition in Food and Food products by high performance liquid chromatography.

2. SAFETY

2.1 Wear eye / hand protection when preparing 6N HCl

2.2 Prepare performic acid in Fume hood

3. REFERENCES

4. DEFINITION

5. PRINCIPLE

Hydrolysis of proteins with 6 N HCl at 110^{o}C for 24 hours under nitrogen atmosphere destroys cysteine/cystine, tryptophane and give low recovery of methionine. In order to quantitate all amino acids present in the protein three hydrolysis procedures must be used for each sample of foods. These are:

1) 6N HCl Hydrolysis: for all amino acids except cysteine/cystine, methionine and tryptophan
2) Performic Acid Oxidation followed by 6N HCl Hydrolysis: for cysteine/cystine (oxidised to cysteic acid) and methionine (oxidised to methionine sulphone)
3) Alkaline Hydrolysis: for tryptophan.

With few exceptions, amino acids do not absorb UV radiation. When an amino acid is allowed to react with Phenylisothiocyanate (PITC) at room temperature the derivative formed is UV absorbing. The amino acid derivatives (which are stable at room temperature) can be injected on to a reverse phase column to separate them and the separated derivatives are detected by UV absorbance at 254 nm.

6. REAGENTS

6.1. Pico-Tag Column 15 cm

6.2. Amino Acid Standard H: Protein hydrolyzate standard 2.5 μmole/ml for each amino acid (except as noted) in 0.1 N HCl. Ammonia, L-Alanine, L-Arginine,

L Asperatic Acid, L-Cystine (1.25 μmole/ml), L-Glutamic Acid, Glycine, L-Histidine, L-Isoleucine, L-Leucine, L-Lysine.HCL, L-Methionine, L-Phenylalanine, L-Proline, L-Serine, L-Threonine, L-Tyrosine, L-Valine (PIERCE H Standard AA).

6.3. Phenylisothiocyanate (PITC)

6.4. Triethylamine (TEA)

6.5. L- Alpha-Amino-Butyric Acid (AABA)

6.6. L-Methionine sulfone

6.7. L- Cysteic Acid

6.8. L-Tryptophan

6.9. 10 mM HCl

6.10. Methanol, HPLC Grade

6.11. 0,2 N Sodium Acetate

6.12. Ethylenediamine Tetraacetic Acid (EDTA)

6.13. Disodium Hydrogen Phosphate (Na2HPO4)

6.14. 10% Phosphoric Acid (H3PO4)

6.15. Acetonitrile, HPLC grade

6.16. Sodium Acetate Trihydrate

6.17. Glacial Acetic Acid, 100%

6.18. Nitrogen gas

6.19. 6 N HCl

6.20. Dry ice

6.21. Water, HPLC grade such as Milli-Q water or equivalent

Additional items needed for Performic Acid Oxidation.

1) 30% Hydrogen Peroxide
2) 99% Formic acid
3) 47% Hydrogen Bromide
4) 1-octanol

Additional items needed for Alkaline Hydrolysis.

5) Barium Hydroxide

7. APPARATUS

7.1. Equipments

7.1.1. Ice-bath

7.1.2. Rotary Vacuum Evaporator, complete with vacuum pump

7.1.3. Oven

7.1.4. Vortex Mixer

7.1.5. Pyrex tube with teflon-lined screw tops

7.1.6. Volumetric pipettes 1 ml, 5 ml and 10 ml

7.1.7. Analytical balance, capable of reading to 5 places of decimals

7.1.8. pH meter, capable of reading to 2 places of decimals

7.1.9. Magnetic stirrer

7.1.10. Vacuum pump

7.1.11. HPLC system

7.2. Glasswares

7.2.1. Volumetric flasks 10 ml, 50 ml, 100 ml, 250 ml and 1 L

7.2.2. Measuring glass cylinders

7.2.3. Vial

7.2.4. Syringe 25 μL, 100 μL

8. PROCEDURE

8.1. Preparation of Stock Standard, Working Standard, Derivatization Reagent, Mobile Phases and other Solutions.

8.1.1. Stock Internal Standard Alpha-Amino-Butyric Acid (AABA) 6.25 μmole/ml. Weigh 0.1613 g AABA and dilute to volume with 10 mM HCl in a 250 ml volumetric flask.

8.1.2. Stock Cysteic Acid (CYA), 25 μmole/ml: Weigh 0.1058 g CYA and dilute to volume with 10 mM HCl in a 25 ml volumetric flask.

8.1.3. Stock Methionine Sulfone (METO2), 25 μmole/ml: Weigh 0.1133 g METO2 and dilute to volume with 10 mM HCl in a 25 ml volumetric flask.

8.1.4. Stock Tryptopan (TRP), 25 μmole/ml.

Weigh 0.5106 g TRP and dilute to volume with 10 mM HCl in 100 ml volumetric flask. Add minimum amount of 6N HCl to dissolve Tryptophan.

8.1.5. Dilute Stock Standard

Combine 4 ml of stock AABA, 1 ml stock CYA, 1 ml stock METO2, and 1 ml stock TRP and dilute to volume with 10 mM HCl in a 10 ml volumetric flask

8.1.6 Working Standard

Combine equal volume (each 0.5 ml) of dilute stock standard and PIERCE H Standard.

8.1.7 Working Internal Standard for Samples, AABA, 2.5 μmole/ml

Dilute 100 ml of stock AABA to volume with 10 mM HCl in a 250 ml volumetric flask

8.1.8 Redry solution

Add 200 μl HPLC grade methanol, 200 μl 0.2 N Sodium Acetate, and 100 μl TEA to a vial and mixing

8.1.9 Derivatization reagent

Add 350 μl HPLC grade methanol, 50 μl HPLC grade water, 50 μl TEA, and 50 μl PITC to a vial and mixing. Reagent must be fresh. Reagent should be clear on mixing. If not, methanol may have high moisture content-replace with fresh.

8.1.10 Stock Disodium Calcium Ethylenediamine Tetraacetic Acid (EDTA) solution

- Weight 100 mg EDTA
- Add 100 ml HPLC grade water
- Sonicate to dissolve
- Store refrigerated up to 1 month

8.1.11 Sample diluent

- Weight 710 mg disodium hydrogen phosphate (Na_2HPO_4)
- Add 1 liter of HPLC grade water
- Titrate to pH 7.40 with 10% phosphoric acid (H_3PO_4)
- Mix the resulting solution with acetonitrile so that acetonitrile equals 5% by volume.

8.1.12 Mobile Phase A

- Weight 19.0 g sodium acetate trihydrate
- Add 1000 ml HPLC grade water
- Filter through an aqueous solvent filter (using solvent clarification kit)
- Add 0.5 ml triethylamine (TEA) while stirring
- Adjust pH to 5.70 with glacial acetic acid using magnetic stirrer and pH meter capable of reading to 2 places of decimal

- Add 200 μl of stock EDTA solution and mix
- Pour into brown bottle, pump in Nitrogen gas, store at 0-5^{o}C
- Before use, filter again through an aqueous solvent filter (using solvent clarification kit); then degas under vacuum in ultrasonic bath for 20 seconds.

8.1.13 Mobile Phase B

- Filter approximately 625 ml HPLC grade acetonitrile through an organic solvent filter (using solvent clarification kit).
- Filter approximately 425 ml HPLC grade water through an aqueous solvent filter (using solvent clarification kit)
- Combine 600 ml filtered acetonitrile and 400 ml filtered water (measure separately)
- Add 200 μl of stock EDTA solution and mix
- Store in brown bottle, pump in nitrogen gas
- Before use, filter again through an aqueous solvent filter (using solvent clarification kit); then degas under vacuum in ultrasonic bath for 20 seconds.

8.2 Working Standard Derivatization

8.2.1 Filter approximately 1 ml of working standard through Millipore filter (HATF 0.45 μm)

8.2.2 Mark Pyrex tube for standard identification: **"STD"**

8.2.3 Carrefully pipet 10 μl of filtered working standard into bottom of **"STD"** tube

8.2.4 Place tube into drying vessel and dry in vacuum station. Vacuum gauge should read approximately 50 millitor, and drying should be completed in 10-15 minutes.

8..2.5 Remove tube from drying vessel. Add 30 μl of redry solution to tube and mix well using vortex mixer. Avoid contact of inside of tube with fingers

8.2.6 Dry again in vacuum station as in step 4.

8.2.7 Add 30 μl of derivatization reagent to tube and mix well using vortex mixer as in step 5. Cap and allow to react for 20 minutes.

8.2.8 Dry in vacuum station for approximately 15 minutes.

8.2.9 Remove tube from vessel and add 30 μl of HPLC grade methanol to tube and mix again using vortex mixer to re-suspend solution.

8.2.10 Dry in vacuum station, until vacuum gauge read approximately 70 millitor. Total time will be approximately 1 hour. The pyrex tube should appear dry, indicated with the appearance of white crystal.

8.2.11 Add 100 μl sample diluent to tube, and then mix well with vortex mixer.

8.2.12 Centrifuge, if necessary, to put residue in bottom of tube.

8.2.13 Working standard solution ready to be injected to HPLC column.

8.3 Acid Hydrolysis

8.3.1 Weight sample corresponding to approximately 40 mg protein to nearest 0.01 mg into Pyrex tube

8.3.2 Add 15 ml 6 N HCl, mix carrefully

8.3.3 Purge with nitrogen for 30 seconds.

8.3.4 Cap immediately.

8.3.5 Soak the tube in boiling water for 10 minute

8.3.6 Place in 110^{o}C oven for 24 hours.

8.3.7 Remove from oven and allow to cool

8.3.8 Add 10 ml working internal standard AABA 2.5 μmole/ml

8.3.9 Vortex for 10 seconds

8.3.10 Quantitatively transfer above to 50 ml volumetric flask, rinsing the sample Pyrex tube with HPLC grade water, and filling flask to mark with washings

8.3.11 If derivatization does not immediately follow, store hydrolysed sample capped in freezer

8.4. Sample Derivatization

8.4.1 Filter approximately 1 ml of hydrolysed sample (see step 10 on acid hydrolysis) through Millipore filter (HATF 0.45 μm)

8.4.2 Mark Pyrex tube for sample identification: **“SPL”**

8.4.3 Carefully pipet 10 μl of filtered hydrolyse sample into bottom of **“SPL”** tube

8.4.4 Place tube into drying vessel and dry in vacuum station. Vacuum gauge should read approximately 50 millitor, and drying should be completed in 10-15 minutes.

8.4.5 Remove tube from drying vessel.

8.4.6 Add 30 μl of redry solution to tube and mix well using vortex mixer. Avoid contact of inside of tube with fingers

8.4.7 Dry again in vacuum station as in step 4.

8.4.8 Add 30 μl of derivatization reagent to tube

8.4.9 Mix well using vortex mixer as in step 6. Cap and allow to react for 20 minutes.

8.4.10 Dry in vacuum station for approximately 15 minutes. Remove tube from vessel.

8.4.11 Add 30 μl of HPLC grade methanol to tube and mix again using vortex mixer to re-suspend solution.

8.4.12 Dry in vacuum station, until vacuum gauge read approximately 70 millitor. Total time will be approximately 1 hour. The Pyrex tube should appear dry, indicated with the appearance of white crystal.

8.4.13 Add 100 μl sample diluent to tube, and then mix well using vortex mixer.

8.4.14 Centrifuge, if necessary, to put residue in bottom of tube.

8.4.15 Hydrolysed and derivatised sample solution ready to be injected to HPLC column.

8.5 Performic Acid Oxidation, followed by Acid Hydrolysis (for determination of Cystine as Cysteic Acid, and Methionine as Methionine Sulfone; for quantitation of Sulphur–containing Amino Acids).

8.5.1 Preparation of performic acid solution:

- Add 1 volume of 30% Hydrogen Peroxide H_2O_2 to 9 volumes of 99% formic acid HCOOH
- Let mixture stand for 1 hour, swirling frequently
- Chill performic acid on ice bath for 30 minutes
- Use immediately

8.5.2 Weight sample corresponding to approximately 10 mg protein to nearest 0.01 mg into Pyrex tube.

8.5.3 Chill tube containing sample in an ice-bath for 30 minutes

8.5.4 Add 8mlof cold Performic Acid to sample

8.5.5 Swirl gently and cap the tube

8.5.6 Store at 4^{o}C overnight (16 hours) by placing sample (still in plenty of ice) in refrigerator.

8.5.7 After 16 hours, with sample still at 4^{o}C, add 3 drops of octanol and then 1.2 ml of cilled 47% Hydrogen Bromide (HBr) slowly while swirling. Do this step in a hood.

8.5.8 Allow to stand at 4^{o}C for 30 minutes

8.5.9 Evaporate to dryness on rotary vacuum evaporator at 44^{o}C. This will take 1-2 hours. Sample should be relatively dry.

8.5.10 Vacuum to 200 millitor for approximately 15 minutes

8.5.11 Add 15 ml 6 N HCl and purge with nitrogen for 30 seconds. Cap immediately

8.5.12 Place in 110oC oven for 24 hours

8.5.13 Remove from oven and allow to cool

8.5.14 Add 10 ml working internal standard AABA 2.5 μmole/ml

8.5.15 Mix well using vortex mixer for 10 seconds

8.5.16 Quantitatively transfer above to 50 ml volumetric flask.

8.5.17 Rinsing the sample Pyrex tube with HPLC grade water, and filling flask to mark with washings

8.5.18 If derivatization does not immediately follow, store sample capped in freezer.

8.5.19 For derivatization process of this sample, follow the same procedure as in point IV. Sample Derivatization.

8.6 Alkaline Hydrolysis for Tryptophan

8.6.1 Weigh sample (pass thru 0.5 mm sieve) containing 20 mg protein into Pyrex tube

8.6.2 Add 1 g Barium hydroxide $Ba(OH)_2.8H_2O$ and 1 ml $SnCl_2.2H_2O$/Glycerol solution.

8.6.3 Flush with N2 gas and cap immediately

8.6.4 Heat at 120oC for 15 hours

8.7 HPLC Analysis

8.7.1 Set the column heater at 40^{o}C

8.7.2 Set the elution program as follow:

Time	Flow (ml/min)	% A	% B	Curve
Initial	1.0	89	11	*
5.0	1.0	68	32	6
13.0	1.0	52	48	6
13.5	1.0	0	100	6
15.5	1.0	0	100	6
16.0	1.5	0	100	6
16.5	1.5	0	100	6
17.0	1.5	89	11	6

8.7.3 Samples can be injected every 26 minutes.

8.7.4 To start the analysis

8.7.5 Reinject the standard after 10 injections of sample

8.7.6 To store the instrument and column, flush with HPLC grade water followed by HPLC grade methanol.

9. CALCULATION

The principle behind quantitation is the area of each component peak in the sample is compared to the area for a known amount of the corresponding component peak in the calibration standard.

The area comparison is generally done indirectly, through the use of a response factor (RF), calculated from the area produced when a known quantity of component is injected.

APPENDIX 1.

List of contributors, addresses and areas of expertise
ASEANFOODS Workshop: ASEAN Manual of Food Analysis
9 - 11 October 2002

PARTICIPANTS	CONTACT ADDRESS	PHONE / FAX / E-MAIL ADDRESS	ANALYTICAL AREA OF EXPERTISE
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Dr. Julia Kantasubrata	Research Center for Chemistry, Indonesian Institute of Sciences, Jl. Cisitu-Sangkuriang Bandung 40135, **Indonesia**	Tel. No. 022-2503051 Fax No. 022-2503240 e-mail: juliakan@indo.net.id	Proximate composition, amino acids, sugars
Dr. Komari	Nutrition and Food Research and Development Center, Jl. Dr. Sumeru 63 Bogor 16112, **Indonesia**	Tel. No. 0251-348764 Fax No. 0251-326348 e-mail: kom_2000@minister.com	Macro- micro-nutrients
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Ms. Elinda M. Castillo	Food and Nutrition Research Institute, Department of Science and Technology, Bicutan, Taguig, Metro Manila, **Philippines**	Tel.no. (0632)- 837-29-34 Fax no. {0632} -837-31-6 e-mail: trpfnri@yahoo.com, emc@fnri.dost.gov.ph	Minerals: Ca, Fe, I, P Proximate composition, total reducing sugars, vitamin A, amino acids

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Ms. Amornrat Pigoolthong	Food Analysis Laboratory, Thailand Institute of Scientific and Technological Research196 Phahonyothin Road, Chatuchak, Bangkok 10900, **Thailand**	Tel. No. 66-2-323-1672 to 80 Fax No. 66-2-323-9165 E-mail: parameepe@yahoo.com	Fat soluble vitamins
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Dr. Kunchit Judprasong	Institute of Nutrition, Mahidol University, Salaya, Phuttamonthon, Nakhon Prathom 73170, **Thailand**	Tel. No. 66-02-800-2380 Fax No. 66-02-441-9344 E-mail: nukjp@mahidol.ac.th	Fatty acids, cholesterol, dietary fibre, proximate composition

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Ms. Nuntaya Chongchaithet	Food Research for Nutrition Section, Ministry of Public Health, Tiwanon road, Muang, Nonthaburi 11000, **Thailand**	Tel No. +66-2-968-7610-11 Fax: No. +66-2-968-7616 E-mail: pattaya@health3-moph.go.th	Amino acids, iodine, water soluble vitamins
Dr. Paramee Pengprecha	Food Analysis Laboratory, Thailand Institute of Scientific and Technological Research, 196 Phahonyothin Road, Chatuchak, Bangkok 10900, **Thailand**	Tel. No. 66-2-323-1672 to 80 Fax No. 66-2-323-9165 E-mail: parameepe@yahoo.com	Minerals
Ms. Pimporn Watcharangkul	Food Research for Nutrition Section, Ministry of Public Health, Tiwanon road, Muang, Nonthaburi 11000, **Thailand** .	Tel. No. +66-2-968-7610-11 Fax No. +66-2-968-7616 E-mail: pattaya@health3-moph.go.th	Fatty acids, cholesterol
Dr. Pongtorn Sungpuag	Institute of Nutrition, Mahidol University, Salaya Phuttamonthon, Nakhon Prathom 73170, **Thailand**	Tel. No. 66-02-800-2380 Fax No. 66-02-441-9344 E-mail: grpsp@mahidol.ac.th	Fat soluble vitamins, and all nutrients

List of contributors, addresses, and areas of expertise (continued)

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Dr. Prapasri Puwastien	Institute of Nutrition, Mahidol University, Salaya, Phuttamonthon, Nakhon Prathom 73170, **Thailand**	Tel. No. 66-02-800-2380 Fax No. 66-02-441-9344 E-mail: nuppw@mahidol.ac.th	Water soluble vitamins : folate, B6, B12, niacin, and other nutrients
Dr. Ratchanee Kongkachuichai	Institute of Nutrition, Mahidol University, Salaya, Phuttamonthon, Nakhon Prathom 73170, **Thailand**	Tel. No. 66-02-800-2380 Fax No. 66-02-441-9344 E-mail:nurkk@mahidol.ac.th	Proximate composition, minerals
Dr. Somkiat Kosulwat	Institute of Nutrition, Mahidol University, Salaya, Phuttamonthon, Nakhon Prathom 73170, **Thailand**	Tel. No. 66-02-800-2380 Fax No. 66-02-441-9344 E-mail: nusks@mahidol.ac.th	Sugars, fatty acids, cholesterol
Dr. Songsak Srianujata	Institute of Nutrition, Mahidol University, Salaya, Phuttamonthon, Nakhon Prathom 73170, **Thailand**	E-mail: rassn@mahidol.ac.th	Chairman of the ASEANFOODS Workshop 2002
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Mr. Le Hong Dung	National Institute of Nutrition, 48B Tang Bat Ho St. Hanoi, **Vietnam**	Tel. No 844-9717090 Fax No. 844-9717885 E-mail: nin_vstp@hn.vnn.vn	Vitamin A, cholesterol