Chapter 20

Profiling of macro and micronutrients in seafood

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Nutrients are organic and inorganic complexes contained in the food and it is essential for all living organisms for their normal body development. Macronutrients are the key biopolymers such as proteins, carbohydrates and lipids, which are needed in hefty quantities to provide the bulk of the energy while micronutrients (vitamins and minerals) are required in minor levels in the body; however, are indispensable for proper growth and development of living organisms. Food that cannot provide the right blend of energy including high-quality protein, essential fats, and carbohydrates as well as vitamins and minerals definitely impair growth and development, increase the risk of death from common childhood illness, or result in life-long health consequences. Fish and fishery products form a substantial part of human diet, both of poor and wealthy. Seafood is an excellent resource for proteins, vitamins, trace elements and polyunsaturated fat (omega-3 fatty acids). Profiling of nutrients is very much essential due to *the ever-increasing awareness about healthy food which finds more acceptance towards the consumption of fish and fish products owing to its special nutritional qualities.*

The knowledge of fish composition is essential for its maximum utilization. The percentage composition of the four major constituents of fish viz. water, protein, lipid and ash (minerals) is referred to as proximate composition (it may be noted that the term does not indicate any degree of inaccuracy in the analysis). These four components account for about 96-98% of total tissue constituents in most cases. The range of values for these constituents in the edible portion of common fish species from Indian coastal waters are given below:

Constituent	Percentage
Water	65-90 %
Protein	10-22 %
Fat	1-20 %
Mineral	0.5-5 %

Water

Water is important for all living organisms. It acts as medium of transport for nutrients, metabolites etc. It is required for normal functioning of biological molecules like proteins. The percentage of water is good indicator of its relative contents of energy, proteins and lipids. The lower the percentage of water would be greater the lipids and protein contents and higher the energy density of the fish. However, these values vary considerably within and between species, size, sexual condition, feeding season and physical activity.

Protein

In addition to the routine functions of protein, fish proteins are considered as superior to plant and animal protein in biological value and protein efficiency ratio due to its easily digestible and absorbable form of proteins. Fish protein comprises of all the ten essential amino acids in desirable quantity for human consumption and it accounts 30% higher than from plant origin. The highly digestible fish protein can be incorporated into protein supplement for human consumption. Presence of essential amino acids in required proportion like lysine, histidine, methionine and cysteine with high bio availability and minerals makes fish highly nutritious. Non-protein amino acid taurine is found to be rich in free amino acid pool which is beneficial in regulating heart function. Fish proteins lessen the risk of microalbuminuria. It also improves blood lipid profile of obese children. Fish protein powder can be used to formulate infant foods, soups and protein containing beverages to enhance their protein content and nutritive value. Fish protein hydrolysate prepared from low value fishes contains important bioactive peptide fraction like gastrin, calcitonin gene related peptides (CGRP) and some growth promoting peptides which play a key role in our metabolic path ways. Fish protein hydrolysate suppresses both hypertension and atherogenesis. Collagen found in skeleton, fins, skin and air bladder (source of pure collagen) of fish is a good source of amino acids required for the synthesis of extra cellular matrix protein of connective tissue. Its supplementation is also beneficial in the normal functioning of fragile bone joints.

Lpids

Quantity wise it is 3rd major component in the fish muscle. Major component of fish lipids includes phospholipids and triglycerides. Wide variation is occurs in the quantity of triglycerides. It is considered as stored form of energy and stored mainly in the form of depot fat in the

liver as well as in the muscle tissue. Phospholipid in the fish lipid mainly found in the cell membrane and plays a vital role in the functioning of the cells. It accounts 0.5 to 1% of the total lipid component. Fish fat is considered as unique, beneficial, healthy fat due to its high degree of unsaturation in their fatty acids composition. Fish and other marine life are rich sources of ω-3 Fatty acids [EPA & DHA], especially the low value fishes such as sardine, mackerel, anchovies etc. EPA and DHA are essential for the development of brain and heart tissue. EPA and DHA play a major role in modulating lipid and prostaglandin metabolism, required for proper functioning of vascular system in growing children. They also influence kidney function by modulating retention of water and removal of excess sodium, which plays a major role in a child's behavior. DHA enhances memory power and is critical to normal eye and vision development in the early and later stages of life of a human being.

Minerals

Minerals are inorganic substances required by the living organisms to accomplish many of their biological functions. Fish and shellfish are valuable sources of Ca & P, and trace elements, Fe, Cu, Se, Zn. Fish is also good source of magnesium, sodium, potassium, chlorine and iodine. Calcium powder from fish bones and back bone of tuna can be used to combat calcium deficiency in diet, particularly of children. Salt water fish have high content of iodine essential for brain and thyroid function. Sodium content in fish is relatively low, making it suitable for low sodium diets. Tuna is a rich source of macro minerals like Mg, which contributes to hardness of bone and acts as cofactor for certain enzymes important in nerve and muscle function. Tuna is also an important source of essential antioxidant trace element Se that provides protection against heavy metal poisonings and a variety of carcinogens. Crustaceans and shellfish are richest source of Cu essential for normal blood formation, maintenance of blood vessels, tendons and bones and health of central nervous system.

Carbohydrates

It is not nutritionally important component in the fish muscle. The major carbohydrate present in fish is glycogen and it is stored mainly in liver. Unlike fish and crustaceans, molluscs have high glycogen content in the range of 1-7%. It plays a significant bearing in post martom rigor changes in the fish flesh.

Vitamins

Vitamins are low molecular weight substances performing important roles in regulating the bodily functions. Fatty fishes are rich in

fat soluble vitamins (A, D & E). Fish is also relatively good source of water soluble vitamins of B group. Fish meat, liver, eggs, milt and skin are good sources of B vitamins, thiamine, riboflavin, pyridoxine, folic acid, biotin and cyanocobalamin. Regular consumption of fish helps in preventing anemia especially pernicious anemia. Fatty or semi fatty fishes are excellent sources of fat soluble vitamins, vitamin A- essential for normal vision, vitamin D-plays an important role in calcium and phosphorous metabolism; vitamin K- an anti-hemorrhage factor; vitamin E-a potent antioxidant involved in counteraction of free radical mediated oxidative damage to the cell membranes. Large quantity of vitamin A, D and E (500-3000 IU) are present in liver and body oils of shark and tuna.

Laboratory protocols for the analyses of biochemical constituents

Preparation of sample

Wash the fish with clean water to remove sand/mud debris, if any and cut the fish into small pieces after removing the fins, scales, bones etc. and homogenize well in a blender. Grind the pieces as smoothly as possible for obtaining a uniform mixed sample. Keep the material in an air-tight container, at low temperature, to prevent the loss of moisture during subsequent handling. This material can be used for most of the biochemical analysis.

Determination of Moisture Content

The water content of many fresh foods, including fish ranges from 60 to 95%. The water content of dried food stuffs are kept very low in order to extend shelf life. The most common methods for water analysis are the following:

- Oven drying (98-100°C)
- Vacuum oven drying (60-70°C)
- Infrared analysis
- Room temperature drying/vacuum desiccator
- Karl Fischer Method etc.

In the above mentioned methods oven drying is most easy and commonly used for determining moisture content

Oven drying

Apparatus

- Petridishes
- Weighing balance
- Hot air oven

- Desiccator

Procedure

Weigh2-10g of homogenized material in a flat bottomed metallic plate/or a clean dried petridish pre-dried at 98°C for 60 min. Dry the sample by heating for a period ranging from 2 to 3 h. to overnight in an hot air oven at 100+1°C. Weigh the sample periodically until it reaches a constant weight (+ 2mg). The percent moisture content can be calculated from the difference between the initial sample weight (W_1) and the final sample weight after drying (W_D)

$$\% \text{ Moisture} = \frac{W_1 - W_D}{W_1} \times 100$$

During oven drying, at high temperature, volatile components (flavour components) can be lost by evaporation along with moisture. Care is needed to avoid rehydration of dried material from moist air. Samples should be covered (preferably in desiccators) and weighed as soon as possible after drying and cooling.

Determination of Crude Protein (Kjeldahl Method) – AOAC Method

The most universally accepted method for determining total nitrogen or crude Protein in fish is the so called Micro Kjeldahl method. First, the food to be analysed is treated with concentrated sulphuric acid at high temperature, in presence of a catalyst, digestion mixture (a mixture of $CuSO_4$ and K_2SO_4 in the ratio 1:8). Digest over a burner till solution turns colourless. $CuSO_4$ acts a catalyst and K_2SO_4 elevates the boiling point of H_2SO_4 from 324°C to 400°C. $HgCl_2$ or SeO_2 can also be used as a catalyst.

The protein content in the food is calculated by estimating the nitrogen content and multiplying with a factor calculated based on the nitrogen content of the food. This is referred to as crude protein content because the non-protein nitrogen (NPN) is not excluded from the total nitrogen. The true protein content can be calculated by subtracting the NPN from the crude protein nitrogen before multiplying with the factor.

Principle

The nitrogenous compounds in the sample are converted in ammonium sulfate on treating with concentrated sulfuric acid. Upon distillation with excess alkali, the ammonia is liberated which is absorbed

in 2% boric acid and is estimated by titration with standard N/50 sulfuric acid.

Apparatus

- Kjeldahl digestion flask
- Kjeldahl distillation apparatus
- Standard flasks
- Conical flasks
- Pipettes

Reagents

- *Con. Sulfuric acid* (AR).
- *Digestion mixture*: Mix copper sulphate ($CuSO_4.5H_2O$) and potassium sulphate (K_2SO_4) in the ratio 1:8 and powder finely.
- *40% Sodium hydroxide*: Dissolve 40 g sodium hydroxide crystals in 100 mL distilled water and cool.
- *2% Boric acid solution*: Dissolve 20 g boric acid in 500 mL hot distilled water, cool and make up to 1 liter.
- *N/50 Sulfuric acid*: Standardized
- *Tashiro's indicator*: Stock solution: (a) 0.2 % alcoholic methyl red; (b) 0.2% alcoholic methylene blue. Mix 100 mL (a) with 50 mL (b)
- Working solution: Mix 1 vol. stock, 1 vol. Alcohol and 2 vol. Water.

Procedure

Weigh 1 to 2g of homogenized wet sample (or 0.2 to 0.5g of the well minced/powdered dry sample) into a Kjeldhal flask (digestion flask) of 100mL capacity. Add a few glass beads and a pinch of digestion mixture ($CuSO_4$ and K_2SO_4) and 10mL of AR sulphuric acid. Digestover a burner till solution turns colourless. $CuSO_4$actas catalyst and K_2SO_4 elevates the boiling point of sulphuric acid from 324°C to 400°C.

To the digested and cooled solution in the digestion flask add distilled water in small quantities with shaking and cooling till the addition of water does not generate heat. Transfer quantitatively into a 100mL standard flask and make up the volume. Transfer with a pipette 5mL of the made up solution to the reaction chamber of the micro-Kjeldal distillation apparatus. Rinse down with distilled water. Add two drops of

phenolphthalein indicator and 40% sodium hydroxide till the indicator changes to pink. Distill for four minutes and absorb the ammonia liberated in 2% boric acid (10mL) containing a drop of Tashiro's indicator and determine the amount of ammonia by titrating with $\frac{N}{50}$ sulphuric acid.

$$\% \text{ of crude protein} = \frac{\frac{14}{50} \times \text{Vol} \frac{N}{50} \times H_2SO_4 \times 100 \times 100 \times 6.25}{5 \times \text{weight of sample} \times 1000} \quad \text{OR}$$

$$\frac{\text{N} \times 14 \times 6.25}{\text{wt. of sample}}$$

Where: N is % nitrogen

- The main source of error is the presence of non-protein nitrogen compounds associated with some foods. High concentration of peptides, amino acids, nucleic acids or urea will give false positive results.
- Kjeldahl analysis can be used to determine nitrogen levels in a wide variety of agricultural materials ranging from fertilizer to feeds.

Determination of Crude fat (Soxhlet method)

Fat from dried sample is extracted repeatedly using petroleum spirit as solvent at high temperature. The fat soluble in the hot petroleum ether, except phospholipid, is extracted from the sample and quantified.

Principle

Fat soluble in organic solvents can be extracted from moisture free samples by using solvents like petroleum ether, ethyl ether etc. The solvent is evaporated and fat is estimated gravimetrically.

Apparatus

- Soxhlet extraction apparatus
- Thimble
- Flat bottom flask
- Water Condenser
- Desiccator

Reagent

- Diethyl ether or Petroleum spirit (60-80°C)

Procedure

Weigh, accurately 5 –10 g (W1) of dried sample in to a thimble and keep a cotton plug on top of it. Place the thimble in a soxhlet apparatus and add 1½ volumes of ether (approximately 200mL) in to a pre-weighed flat bottom flask (w_2) and distill for 16 h. (Cool the apparatus and filter the solvent in to a pre-weighed conical flask (W_2). Rinse the flask of the apparatus with small quantities of ether and then add washings to the above flask). Remove ether by evaporation and dry the flask with fat at 80 –100°C, cool in a desiccator and weigh (W3).

Calculations

$$\text{Fat content (g/100\%)} = \frac{(W3-W2) \times 100}{W1} = X$$

$$\text{Fat content (g/100\%)} = \frac{\text{Weight of fat} \times 100}{\text{Weight of sample}} = \text{'X'}$$

Where W1- weight of dry matter taken for extraction; W2 – weight of flat bottom flask and W3 – weight of flask with fat

Conversion of dry weight to wet weight basis

$$\text{Wetweight} = \frac{\text{'X'} \times (100-\text{Moisture})}{100}$$

Estimation of Ash content

Principle

Ash is the residue obtained after incineration of the dry material at high temperature and appears as grey- white colured powder.

Apparatus

- Crucible
- Muffle furnace
- Desiccator

Procedure

Heat a platinum or silica crucible to 600°C in a muffle furnace for one hour, cool in a desiccator and weigh (W1). Weigh accurately 2 g of the dried sample (w_2) in to a crucible and heat at low flame by keeping on a clay triangle to char the organic matter. Keep the charred material inside the previously set (600°C) muffle furnace and heat for 6-8 h to get white or greyish white ash. Cool the crucible in a desiccator and weigh (W3). Heat the crucible again for further 30 min to confirm completion of ashing, cool and weigh.

Calculation

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

Where;

W1 – weight of crucible and
W2- weight of dry matter with crucible taken for ashing
W3 – weight of crucible with ash

The minerals like sodium, potassium and calcium are estimated using Flame Photometer after dissolving the ash in dilute hydrochloric acid (6N). The reading is compared against standard solutions of the respective minerals.

Determination of carbohydrates

Principle

Carbohydrate is determined by the furfural colorimetric method after treatment with concentrate sulphuric acid. The intensity of the pink colour is measured at 520 nm.

Reagents

- Concentrated sulphuric acid AR
- 10% Trichloroacetic acid (TCA)
- Weigh 10g. of pure TCA dissolved in water and diluted to 100 mL.

- Silver sulphate
- Standard glucose solution
- Prepare a series of glucose standard for preparing the calibration curve

Procedure

Carbohydrate was determined by the furfural colorimetric method after treatment with con. H_2SO_4. About 30-50 mg of material was weighed out into a 20 mL centrifuge tube and heated in a boiling water bath for 30 minutes with 4 mL of 10% TCA and about 30 mg of Ag_2SO_4. After centrifuging, the clear supernatant and the subsequent washings of the residue with the TCA solution were transferred to a 25 mL graduated flask and made upto the volume. 2 mL aliquots were taken in duplicate and carefully layered over 6 mL of concentrated H_2SO_4 taken in a boiling tube. The tubes were quickly agitated to mix the contents thoroughly and then heated for 6.5 minutes in a vigorously boiling water bath. After rapid cooling to room temperature (~28°C) the optical density was measured at 520 nm. Blanks were run with each batch of analysis. Glucose was used to obtain the standard curve (Heath and Barnes, 1970).

Determination of Amino Acid Composition

Principle

Protein is hydrolysed to their constituent amino acids by 6N hydrochloric acid. The amino acids are separated in a HPLC equipped with an ion exchange column. Two buffers (pH 3.2 and pH 10.0) are used to elute the amino acids from the column and the individual amino acids are estimated by their fluorescence intensity, imparted on to the individual amino acids by reaction with o-phthalaldehyde in the presence of sodium hypochlorite solution, using a fluorescence detector.

Apparatus and facilities

- HPLC equipped with ion exchange column and fluorescence detector
- Rotary vacuum flash evaporator
- Fusing instruments
- Nitrogen gas

Reagents

a) 6N Hydrochloric acid

b) HPLC buffers -

Buffer A: Dissolve 13.31 g tri sodium citrate in 70 mL ethanol; add 12.8 mL citric acid (monohydrate), 3.74g. NaCl and 4 mL Brij; adjust pH to 3.2 and finally make up to 1L with distilled water.

Buffer B: Dissolve117.6 g Tri sodium citrate and 24.8g boric acid in 500 mL distilled water; add 45 mL 4N NaOH, adjust pH to 10 and make up to 2 L with distilled water.

c) O-pthalaldehyde(OPA) buffer – Dissolve 40.7g sodium carbonate, 13.57g boric acid and 18.8g potassium sulphate in distilled water and make up to 1 L with water.

d) O-phthalaldehyde (OPA) reagent : Dissolve 80 mg OPA, 1.4 mL ethanol, 0.2 mL 2-mercaptoethanol and brij 0.15 mL in distilled water and make up to 200 mL in OPA Buffer

e) Sodium hypochlorite solution**:** 0.2 mLsodium hypochlorite diluted to 200 mL in OPA buffer.

Preparation of sample

Weigh about 100 mg of finely homogenized fish mince in to a borosil test tube. Add 10 mL of 6N HCl in to the test tube. Seal the tubes after filling nitrogen and digest the contents of the tube by keeping at 110°C for 24 hours in an oven. Cool the test tubes and filter the contents using Whatman No.1 filter paper. Rinse the tubes with distilled water and filter. Evaporate filtrate in a vacuum flash evaporator. Add deionised water in to the tubes and continue evaporation until the contents are acid free. Generally, three washings with 50 mL water are required. Dissolve the amino acids in buffer A and inject in to HPLC.

HPLC Analysis

The separation and quantification of amino acids is carried out using an HPLC with an ion exchange column. Filter samples using 0.45μm syringe filter and inject appropriate quantities in to the HPLC system as per the specifications of the injector. The eluted amino acids are derivatized post column with O-phthaldehyde (for fluorescence detector).

Instrument details

- Equipment
- Model Hitachi L-2130 Elite La Chrom
- HPLC equipped with
- Auto sampler L-2200

- FL detector L-2485
- Wave length
- Ex: 340 nm
- Em: 450 nm
- Column Oven L-2350
- Column Shodex
- CX Pak
- P-421S and
- Two channel peristaltic pump

Calculation

$$\frac{\text{Conc. of Std AA } (\mu \text{ mol}) \times \text{Area of sample AA}}{\text{Area of Std AA}} = \text{Conc. of sample AA as } \mu \text{ mol}$$

$$\frac{\text{Conc. of sample AA} \times \text{Mol. Wt of AA} \times 1000 \times 100 \times 16 \times \text{Vol. made up}}{10 \times 1000 \times 1000 \times \text{wt. of sample} \times \% \text{ of } N_2} = \text{AAg}/16\text{gN}_2$$

Spectrophotometric Determination of Tryptophan

Tryptophan being labile to the conditions of hydrolysis described for other amino acids, it is estimated separately by spectrophotometer after alkali hydrolysis of the protein.

Principle

The 5-hydroxy furfural resulting from sucrose under acidic conditions of reaction forms pale green coloured condensation product with thioglycolic acid, which reacts with tryptophan in the hydrolysed protein giving a pink coloured complex. The colour intensity of the solution is measured at 500 nm.

Reagents

- **Sodium hydroxide**: 5% solution
- **Hydrochloric acid - 6 N solution**: Dilute A.R. concentrate Hydrochloric acid in the ratio 1:1 using ditilled water
- **Sucrose**: 2.5 % solution

- **Thioglycolic acid**: (80% solution) - 0.6 mL diluted to 100 mL (V/V) distilled water;
- **Sulphuric acid**: 50 % solution, v/v
- **Tryptophan standard**: Stock- 1 mg per mL solution in 0.1 N HCl;
- **Working standard**: 1 mL stock in 100 mL of 0.1N HCl to get 10 µg/mL solution

Procedure

Sample preparation

Weigh about 300 mg of finely homogenized fish mince in to a test tube. Add 10 mL of 5% NaOH in to the test tube. Seal the tube after filling with nitrogen gas and digest the contents of the tube by keeping at 110°C for 24 hours in an air oven. Neutralise the contents after hydrolysis to pH 7.0 using 6N HCl. Total volume is made to 100 mL and filter through Whatman No.1 filter paper

Estimation

Add 0.1 mL 2.5 % sucrose and 0.1 mL 0.6% thioglycolic acid successively in to a test tube containing 4 mL of 50% H_2SO_4. Keep the tubes in a water-bath at 45-50°C for 5 min. and cool. Aliquots (0.1 – 0.8mL) of sample was added to the test tube and mixed. Make the volume of the test tube to 5 mL with 0.1N HCl and leave aside for 5 min. Measure the colour intensity at 500 nm. For standards, add tryptophan standard solution in to a series of test tubes instead of sample and perform experiment as above. Calculate the concentration using a standard graph or by regression.

Calculation

$$\frac{\mu g \text{ of trypt obtained by regression x vol. made x 100 x 16}}{\text{Vol. for colour development x sample wt. x 1000 x 1000x } N_2\%} = g/16g\ N_2$$

Determination of Fatty Acid Profile by Gas Chromatograph

Fat Extraction

The wet fish/shellfish muscle is homogenized with 2:1 mixture of chloroform and methanol. The chloroform-methanol mixture extracts the

total lipid from the tissue in to a single phase of solvent. Disturbing the equilibrium between chloroform and methanol separates the chloroform soluble fat.

Apparatus

- High speed stirrer or mortar and pestle
- Buckner flask & Buckner funnel
- Filter paper
- Vacuum flash evaporator
- Water bath

Reagents

- Chloroform (Excelar grade)
- Methanol (AR grade)
- Chloroform- methanol mixture: (2:1)
- Anhydrous sodium sulphate

Procedure

Extract about 25 – 50 g (depending on the fat content) meat with 15 volumes of chloroform - methanol mixture for two minutes in the case of high speed stirrer or five minutes in the case of mortar and pestle (few grams of acid washed sand can be added at the time of grinding in mortar). The extraction and filtration is carried out in three steps. Filter the extract using a Buckner funnel with Whatman No.1 filter paper, applying little vacuum. Take the combined extract in to a separating funnel, add 20% of the volume water, mix well and allow to separate overnight. Collect lower layer and filter through sodium sulphate. Concentrate lipid to a known volume, say 10 ml, by evaporating the solvent in a vacuum flash evaporator and keep under nitrogen pending analysis. Take 1 mL of aliquot in a pre-weighed test tube and evaporated it off. The test tube is cooled in a desiccator and weighed.

Calculation

$$\text{Fat content (g/100g meat)}: \frac{W_2 \times V_1 \times 100}{V_2 \times W_1}$$

Where: V_1 : total volume of extract

V_2 : Volume of extract taken for drying

W_2: weight of dried lipid

W_1: weight of sample for fat extraction

Reagents

- 150% *Potassium hydroxide* – 3g Potassium hydroxide in 2 ml D/W
- *Petroleum ether*
- Std***: FAME*** mix (Fatty Acid Methyl Ester mix)
- *Methanol*
- *Boron trifluride-Methanol* **(BF_3–Methanol)**
- *Sodium chloride (saturated* solution)

Saponification and separation of non-saponifiable matter

Take appropriate volume of lipid (containing minimum 250 mg fat) evaporate solvent, add 30 mL methanol and 1.5 mL 150% KOH. Reflux for 30 min. in a boiling water bath under N_2. Cool slightly, transfer the solution into a separating funnel, add 20 mL D/W. Extract 3 times with 20 mL of Petroleum ether. Keep aqueous layer for fatty acid estimation. Pool extract (Non saponifiable matter) wash N.S. matter with D/W to make it alkali free (check washings with phenolphthalein). Note volume of NSM. Dry over Na_2SO_4. Keep aside for further analysis (cholesterol).

Extraction of fatty acids

Acidify the aqueous layer with Con. HCl. Check with pH paper. Extract 3 times with petroleum ether. Combine extract, wash with D/W 3 times. Filter through anhydrous Na_2SO_4. Flash evaporate to remove the solvent.

Preparation of fatty acid methyl ester

Add 6 mL BF_3-Methanol into the above flask containing fatty acids. Reflux in a boiling water bath for 6 min. Cool, add 6 mL.saturatedNaCl, transfer to a separating funnel. Extract 3 times with Petroleum ether, filter through Na_2SO_4, evaporate and make upto 1 mL in PE for GC analysis. The complete analysis should be done under N_2.

Fatty Acid Methyl Ester – Direct Method

To 100 mg of fat/oil, add 5 mL of 0.5 N Methanolic NaOH (1 g NaOH in 50 mL Methanol). Reflux for 5 min in a boiling water bath under N_2. Add 6mL BF_3-Methanol, boil for 5 more minutes. Cool and add 6 mL Sat. sodium chloride, extract thrice with Petroleum ether, pool extract and

wash with water. Filter through Na_2SO_4, evaporate and make upto 1 mL in Petroleum ether for Gas chromatographic analysis.

Gas Chromatographic Analysis

The Gas chromatograph programme for analysis of fatty acid is as follows:

Programme of GC; *Injector 265°C;*

Flame Ionisation Detector (FID) at 275°C; Capillary column (Elite -225) (30m, 0.25mm i.d, 0.25 µm);

Carrier gas, *Nitrogen* at 0.6 mL / min ; Temperature programme – 110°C for 4 min; temperature is programmed to raise at 2.7°C/ min to 240°C and maintained at that temperature for 5 min; *Split flow* 20 mL.

Sample 1 µl injection; Samples are identified by retention time by comparing with respective standards using Chromcard software; Area of each component is obtained from the computer-generated data and concentration calculated using the software by external standard method.

Calculation

$$\text{mg/g of sample (FAME)} = \frac{\text{Area of sample x Con. Std x total vol. of extract x final vol. made up}}{\text{Area of std. x vol. of lipid extract taken for FAME x wt. of sample}}$$

DETERMINATION OF FAT SOLUBLE VITAMINS

Principle

High performance liquid chromatography (HPLC) is now used regularly for the analysis of fat soluble vitamins in a wide range of foods. It offers many advantages over traditional methods of analysis in particular with regard to speed, sensitivity and selectivity. An extraction step prior to chromatographic determination is required for cleanup and concentration of vitamins.

Reagents

Chloroform :methanol 2:1; BHA or BHT; KOH -150%- 3 g KOH in 2 ml water; Methanol; Petroleum Ether; Water with 1% TFA; Acetonitrile with 1% TFA

Alpha tocopherol (vit E), cholcalciferol (vit D), retinol(vit A), vitamin K - Stock solution of 1ppm is prepared in hexane and stored in brown bottles at 20C. Working standard solutions were made by appropriate dilutions.

Procedure

Sample preparation

Grind fish tissue (20g) with anhydrous sodium sulphate and extract oil using 2:1 chloroform : methanol after adding BHA or BHT as antioxidants (Folch's method). To about 2g oil in a RB flask, add 25 ml alcohol, & 1.5 ml of 150% KOH. Reflux in a water bath for 30 min. Transfer the contents in to a 250 ml separating funnel after cooling; wash the flask with 50 ml petroleum ether and add to the separating funnel; shake the content of the separating funnel thoroughly and allow to separate. Extract the aqueous layer twice more and the pool solvent layer. Wash the solvent layer with two 20 ml portions of water to make it alkali free. Concentrate non-saponifiable matter in the ether fraction using a flash evaporator at 30 – 40°C to a definite volume. NSM is filtered through 0.45μ syringe filter and stored under refrigeration.

Chromatographic analysis

The HPLC consisting of a quaternary gradient pump, programmable variable wave length UV detector is used for the analysis.

Column: C18 RP 5μ 250 x 4 mm Atlantis (Waters Corporation) or related

The mobile phase - water with 1% TFA (A) and acetonitrile with 1% TFA (B) at 1 ml per min. The programme made in the HPLC is as follows.

Time (min)	Mobile phase B	Mobile phase A
0	50	50
5	80	20
6	100	0
10	100	0
20	100	0

The fat soluble vitamins elute from the column in the order vitA, vit D, vit E and vit K.

The wavelength used for eluting different vitamins is as follows. 265nm for vitamin D3, 325 for vitamin A, 291nm for vitamin E and 250 for vitamin K.

Calculation

The vitamin content in the unknown sample is determined from the linear graph drawn for the standard.

MINERAL PROFILING

The ash estimated under 'Experiment 4' of Biochemical analysis is dissolved in 100 ml 6N HCl quantitatively. The solution is appropriately diluted and aspirated in to the AAS for quantification.

The following steps are to be followed.

1. The AAS is switched on.
2. Three sets of concentrations are aspirated and the calibration line is drawn.
3. The cell tubes are washed with water
4. The sample is placed and aspirated and the reading is noted. The calculation is made based on the reading and dilution.

Calculation: The concentration of metal is detected in mg/l or PPM.

Metal mg/100g = (Conc. of metal in PPM x volume made) / weight of sample