Tools used in characterization of high value products from fishery waste

Niladri Sekhar Chatterjee, R. Anandan & Suseela Mathew
Biochemistry and Nutrition Division, ICAR-Central Institute of Fisheries Technology, Cochin-29, Kerala, India

India is the third largest producer of fish and the second largest producer of fresh water fish in the world. The fish production during 2009-10 was found to be 78.51 lakh tonnes (Ministry of Agriculture, Government of India, 2011). Fish processing operations produce huge amount of solid waste in the form of fish carcasses, shrimp scales, viscera, skin and head. Disposal and storage of this waste, so as to prevent contamination to the environment, is a problem of gigantic proportions. India generates greater than 2 million metric tonnes of by-products due to fish processing activities. This waste biomass can be valuable source of bioactive lipids, proteins, peptides, amino acids. Various chromatographic and mass spectrometric tools are used for assessment of quality and safety of such high value products from fishery waste.

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. For pure oil sample directly 250-500 mg oil is taken for saponification.

Apparatus, materials and reagents

High speed stirrer or mortar and pestle, Buckner flask & Buckner funnel, Filter paper, Vacuum flash evaporator, Water bath, Chloroform, Methanol, Chloroform- methanol mixture: (2:1), Anhydrous sodium sulphate, 150% Potassium hydroxide – 3g Potassium hydroxide in 2 mL D/W, Petroleum ether, Std: FAME mix (Fatty Acid Methyl Ester mix), Boron trifluride-Methanol(BF3–Methanol), Sodium chloride (saturated solution).

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

$$\text{Fat content (g/100g meat):} \quad \frac{W2 \times V1 \times 100}{V2 \times W1}$$

Where: V1 : total volume of extract
V2 : Volume of extract taken for drying

W2: weight of dried lipid

W1: weight of sample for fat extraction

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.BF3-Methanol into the above flask containing fatty acids. Reflux in a boiling water bath for 6 min. Cool, add 6 mL saturated NaCl, and transfer to a separating funnel. Extract 3 times with Petroleum ether, filter through Na_2SO_4, evaporate and make up to 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 usingChromcard software; Area of each component is obtained from the computer-generated data and concentration calculated using the software by external standard method.

Calculation

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

Determination of total amino acid composition by HPLC

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, materials and reagents

HPLC equipped with ion exchange column and fluorescence detector, Rotary vacuum flash evaporator, Fusing instruments, Nitrogen gas, 6N Hydrochloric acid, 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; 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; 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; Sodium hypochlorite solution: 0.2 mL sodium 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).

Calculation

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

$$\frac{\text{Conc. of sample AA x Mol. Wt of AA x 1000 x 100 x 16 x Vol. made up}}{\text{10 x 1000 x 1000 x wt. of sample x \% of N2}} = \text{AAg/16gN2}$$

Sample preparation and GC-MS/MS analytical method for detection of contaminants in fish

Poly-halogenated endocrine disrupting compounds and some of the pesticide endocrine disruptors are amenable to GC-MS/MS analysis. We have developed a GC-MS/MS multiresidue analytical method for analysis of multiclass pesticides in fatty fish18. To achieve desired trace level sensitivity, a programmable temperature vaporizer -large volume injection (PTV-LVI) technique was used. Validation of the modified analytical method was done as per DG SANTE guideline SANTE/11813/201719.

The analyses of samples were performed using a GC equipped with a CTC Combipal auto sampler (CTC Analytics, Switzerland) attached to a triple quadrupole mass spectrometer (GC: 7890A, MS: 7000B, Agilent Technologies, Palo Alto, USA). The system was controlled using Mass Hunter software (ver B.05.00.412). The analytical separation was performed using a VF-5MS (30 m x 0.25 mm, 0.25 µm) capillary column (Agilent Technologies) with mid-point back flush set up for the 15 m column towards the injector port end, for which additional helium flow was supplied through a purged ultimate union. A gooseneck liner (78.5 mm x 6.5 mm, 4 mm) from Restek Corporation (PA, USA) was used. The carrier gas (Helium) flow was set at a constant rate of 1.2 mL/min for the first column, and 1.24 mL/min for the second column. The oven temperature program was set at initial temperature of 70 °C (1 minute hold), ramped to 150 °C at 25 °C/minute (0 minute hold), then at 3 °C/minute up to 200 °C (0 minute hold) and

finally to 285 °C at 8 °C/minute (9 minute hold) resulting in a total run time of 40.49 minute. The transfer line temperature was maintained at 285 °C. During a 3 minute post-run period, the oven temperature was maintained at 285 °C with the carrier gas flow rate in column 1 set at -3.4 mL/minute.

The multi-mode inlet (MMI) was operated in solvent vent mode and 5 µL of sample was injected. The programmable temperature vaporizer (PTV) program was set at the initial temperature of 70 °C (0.07 minute hold), raised to 87 °C at 50 °C/ minute (0.1 minute hold) followed by rapid heating at 700 °C/ minute up to 280 °C (3 minute hold). The purge flow to solvent vent was maintained at 50 mL/ minute, at a pressure of 11.266 psi until 0.17 minutes after injection. Next, the split vent was closed for 2.7 minutes to transfer the analytes to the column. Then, the split vent was opened to remove the high boiling matrix compounds from the inlet. The mass spectrometer was operated in MS/MS mode with acquisition starting at 4.4 minutes. Electron impact ionization (EI+) was achieved at 70 eV and the ion source temperature was set at 280 °C.

Approximately 2 kg fish meat was separated from bones and skin and crushed thoroughly in a homogenizer. A subsample of 5 g homogenized meat was weighed into a 50 mL centrifuge tube, mixed with 5 mL of distilled water and vortexed for 1 minute. Next, 15 mL of acetonitrile (+ 1% acetic acid) and 2 mL of hexane were added, and the tube was vortexed again for 1 minute. Subsequently, 6 g of MgSO4 and 1.5 g of NaAC were added to each tube, followed by vortexing for 2 minutes and centrifugation at 5000 rpm for 5 minutes. A portion of the middle organic layer (1.5 mL acetonitrile) was pipetted out of each tube and kept in a 15 mL centrifuge tube at -20 °C for 20 minutes. Adsorbents (100 mg CaCl2 + 150 mg MgSO4) were added to the tube for dSPE cleanup. The supernatant (1 mL) was further cleaned with 50 mg PSA + 50 mg florisil + 150 mg C18 + 150 mg MgSO4, vortexed for 1 minute and, centrifuged at 10000 rpm for 5 minutes. The supernatants from each tube were filtered through a PTFE membrane and analyzed by GC-MS/MS.

Suggested Readings

- AOAC (2000). Official Methods of Analysis. 16th Edition. Association of Official Analytical Chemists, Washington.
- AOAC (1990).Official Methods of Analysis.15thedn.Association of Official Analytical Chemists.Vol. 1 Arlington.Virginia USA.247 pp.
- Organochlorines pesticides, APHA, 1998, Standard methods for the examination of water and waste water, 20th Edn., American Public Health Association, 6-91)