File No. 11014/07/2021-QA

Food Safety and Standards Authority of India

(A statutory Authority established under the Food Safety and Standards Act, 2006)

(Quality Assurance Division)

FDA Bhawan, Kotla Road, New Delhi – 110002

Dated, the 17th July, 2023

Order

Subject: FSSAI Manual of Methods of Analysis of Foods – Fish and Fish Products - reg.

The FSSAI Manual of Methods of Analysis of Foods-Fish and Fish Products which has been approved by the Food Authority in its 42nd meeting held on 30.05.2023 is enclosed herewith.

2. This manual shall be used by the laboratories with immediate effect. It supersedes the test Methods for Fish and Fish Products specified under the Manual of Methods of Analysis of Foods-Meat and Meat Products & Fish and Fish Products issued vide Office Order No. 1-90/FSSAI/SP (MS&A)/2009 dated 09.01.2017.

3. Since the process of updation of test methods is dynamic, any changes happening from time to time will be notified separately. Queries/concerns, if any, may be forwarded to email: sp-sampling@fssai.gov.in, dinesh.k@fssai.gov.in.

Encl: as above

Dr. SATYEN KUMAR PANDA

Digitally signed by Dr. SATYEN KUMAR PANDA
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(Dr. Satyen Kumar Panda)
Advisor (QA)

To:

1. All FSSAI Notified Laboratories
2. All State Food Testing Laboratories
3. CEO, National Accreditation Board for Testing and Calibration Laboratories (NABL)

फा. सं. 11014/07/2021 – **क्यूए**

भारतीय खाद्य सुरक्षा और मानक प्राधिकरण

(खाद्य सुरक्षा और मानक अधिनियम**, 2006** के अंतर्गत स्थापित एक वैधानिक प्राधिकरण**)**

(**गुणवत्ता आश्वासन विभाग**)

एफडीए भवन, कोटला रोड, नई दिल्ली-110002

दिनांक: 17 जुलाई, 2023

आदेश

विषय: खाद्य पदार्थों के विश्लेषण के तरीकों की एफएसएसएआई मैनुअल – मछली और मछली उत्पाद- के संबंध में।

खाद्य पदार्थों के विश्लेषण के तरीकों की एफएसएसएआई मैनुअल - मछली और मछली उत्पाद, जिसे खाद्य प्राधिकरण ने 30.05.2023 को आयोजित अपनी 42वीं बैठक में अनुमोदित किया है, इसके साथ संलग्न है।

2. इस मैनुअल का प्रयोग प्रयोगशालाओं द्वारा तत्काल प्रभाव से किया जाएगा। यह मैनुअल कार्यालय आदेश संख्या 1-90/FSSAI/SP(MS&A)/2009 दिनांक 09.01.2017 द्वारा जारी, एफएसएसएआई मैनुअल- मांस और मांस उत्पाद और मछली और मछली उत्पाद में उल्लिखित मछली और मछली उत्पाद के विश्लेषण के तरीकों का स्थान लेता है।

3. चूंकि परीक्षण विधियों के अद्यतन की प्रक्रिया गत्यात्मक है, समय-समय पर होने वाले किसी भी परिवर्तन को अलग से अधिसूचित किया जाएगा। प्रश्न/चिंताएं, यदि कोई हों, ईमेल: sp-sampling@fssai.gov.in, dinesh.k@fssai.gov.in पर अग्रेषित की जा सकती हैं ।

संलग्नक: उपरोक्त अनुसार

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सलाहकार (गुणवत्ता आश्वासन)

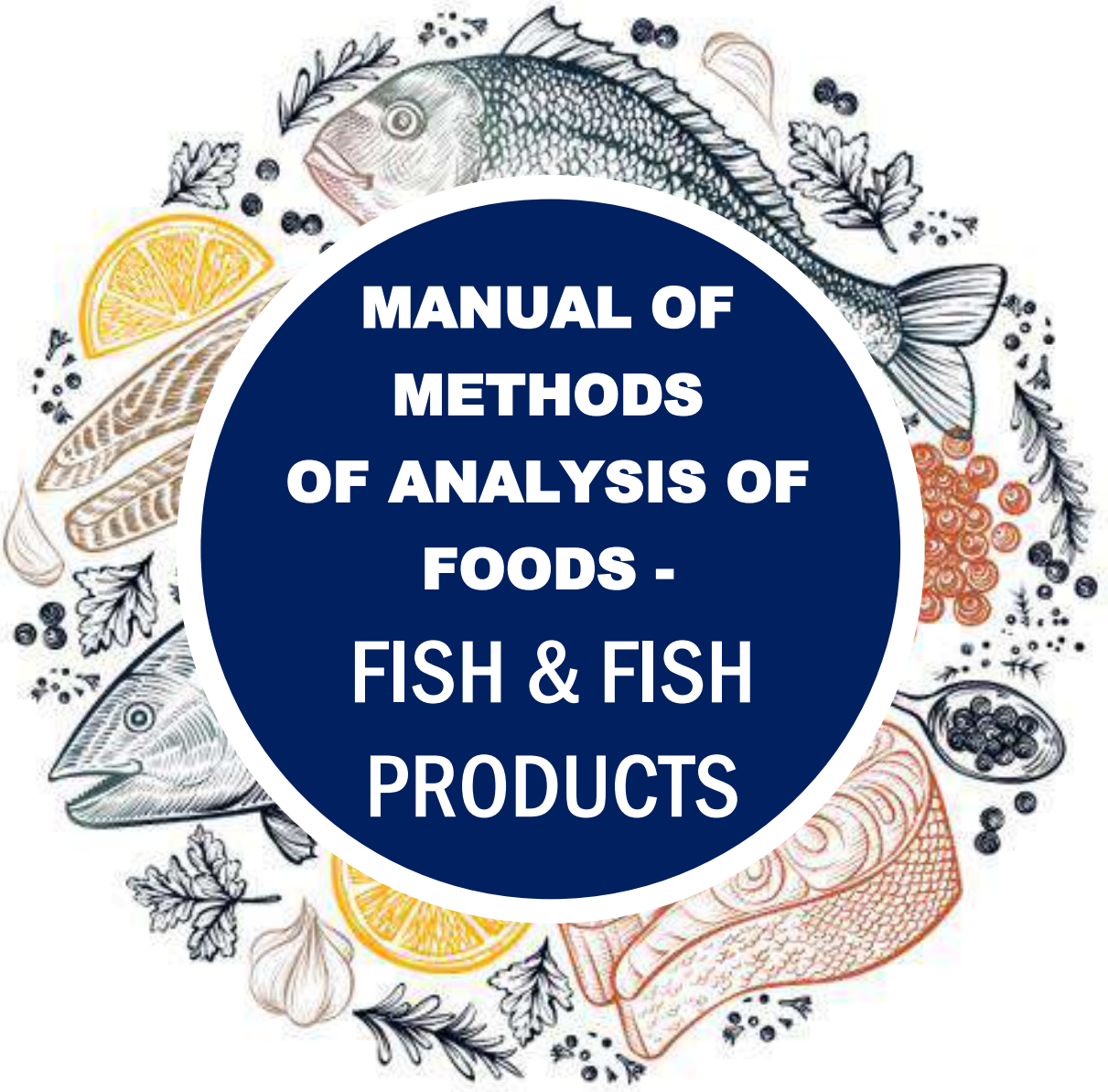
प्रति:

1. सभी एफएसएसएआई अधिसूचित प्रयोगशालाएं
2. सभी राज्य खाद्य परीक्षण प्रयोगशालाएं
3. सीईओ, राष्ट्रीय परीक्षण और अंशशोधन प्रयोगशाला प्रत्यायन बोर्ड

स्वास्थ एवं परिवार कल्याण मंत्रालय
MINISTRY OF HEALTH AND FAMILY WELFARE

सत्यमेव जयते

एफएसएसएआई
fssai
भारतीय खाद्य सुरक्षा और मानक प्राधिकरण
Food Safety and Standards Authority of India
स्वास्थ्य और परिवार कल्याण मंत्रालय
Ministry of Health and Family Welfare



MANUAL OF METHODS OF ANALYSIS OF FOODS - FISH & FISH PRODUCTS

JUNE 2023

जी. कमलावर्धन राव, आई.ए.एस
G. Kamala Vardhana Rao, IAS
सचिव (भारत सरकार) एवं मुख्य कार्यकारी अधिकारी
Secretary(GOI) & Chief Executive Officer

सत्यमेव जयते

आज़ादी का अमृत महोत्सव

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Food Safety and Standards Authority of India
स्वास्थ्य और परिवार कल्याण मंत्रालय
Ministry of Health and Family Welfare

FOREWORD

We are delighted to present the **FSSAI Manual of Methods of Analysis of Foods- Fish & Fish Products**, a comprehensive guide that serves as an invaluable resource for food testing laboratories, researchers & quality control professionals, food technologists, and anyone involved in the analysis of Fish & Fish products.

This manual has been meticulously crafted to offer a wide range of analytical methods specifically tailored for Fish & Fish products. It encompasses various aspects of analysis as per FSSR. In an ever-evolving scientific landscape, it is essential to stay abreast of emerging technologies and methodologies. Therefore, we encourage users of this manual to actively contribute their experiences and expertise. By fostering a collaborative environment, we can continuously refine and expand our understanding of Fish & Fish Products, driving innovation and improvement in the field.

It gives us immense pleasure to release this **FSSAI Manual of Methods of Analysis of Foods- Fish & Fish Products**. The FSSAI laboratories shall use these testing methods only for analyzing samples under the Food Safety and Standards Act, 2006 and Food Safety and Standards Regulations, 2011. This Manual may serve as a catalyst for scientific advancements, quality assurance, and consumer safety, ultimately contributing to the overall well-being and satisfaction of individuals worldwide.

June 2023

Shri G. Kamala Vardhana Rao,
Chief Executive Officer,
Food Safety and Standards Authority of India,
FDA Bhawan, Kotla Road,
New Delhi – 110002

G20 भारत 2023 INDIA

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Eat Right India
सही भोजन, बेहतर जीवन

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Food Safety and Standards Authority of India
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Ministry of Health and Family Welfare

PREFACE

Food safety is assurance that food is acceptable for human consumption according to its intended use. Testing of food to instil confidence amongst consumers that food is safe to eat is important part of the food safety ecosystem. Food testing ecosystem is complex in India and challenges start from sample preparation to final result generation.

Each method in the **FSSAI Manual of Methods of Analysis of Foods- Fish & Fish Products** has been carefully selected based on its scientific rigor, applicability, and relevance to the food testing laboratories, QA/QC Professionals of industry. The procedures are meticulously detailed, providing step-by-step instructions, necessary reagents, and equipment requirements.

We express our sincere gratitude to the numerous experts who have contributed their knowledge, expertise, and insights to the development of this manual especially Dr. Ravi Shankar C.N., Vice-Chancellor, ICAR-CIFE, Mumbai (former Director ICAR-CIFT) for valuable insight. I am thankful to the Chairperson, FSSAI and CEO, FSSAI for their support and constant encouragement without which the work would not have seen the light of day.

Any suggestions/feedback from the stakeholders, which will contribute towards updating the manual from time to time are welcome.

June 2023

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TABLE OF CONTENTS

Note: Test methods given in the manual are standardized/ validated/ taken from national or international methods or recognized specifications, however it would be the responsibility of the respective testing laboratories to verify the performance of these methods onsite and ensure it gives proper results before putting these methods in to use.

	Determination of Foreign Matter- I (Filth)		
Method No.	FSSAI 06.001:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to: • Canned fishery products • Ready –to –eat finfish or shellfish curry in retortable pouches • Pasteurized crab meat • Fish Sauce • Fish Pickle • Surimi		
Caution	1) In preparation of the trap flask, rod of greater length is not desirable because it gives greater displacement of liquid. 2) Isopropanol used should not be cloudy. IPA is acceptable only if 40% mixture with water is clear. 3) In the isolation part, if large number of suspended solids is present, the mixture in the percolator should stand longer to permit separation of oil. 4) During extraction, if filtering action slows, use new filter paper.		
Principle	Filth is basically any objectionable matter contributed by animal contamination such as rodent, insect or bird matter, or any other objectionable matter contributed by unsanitary conditions. The product is digested without affecting the insect exoskeleton or mammalian hair filth contaminants. These oleophilic filth elements are separated from non-oleophilic food product by attraction to oil phase of oil- aqueous mixture. Oil phase is trapped off, filtered and examined microscopically.		
Apparatus/ Instruments	1) **Wildman Trap Flask (2L):** Consists of 2L Erlenmeyer into which is inserted close fitting rubber stopper or wafer stopper supported on a stiff metal rod 5 mm diameter and 10 cm longer than the height of the flask. 2) **Magnetic Stirring bar and stirrer hot plate**: Teflon covered bars 47 mm long × 9 mm od; use with hot plate having independent, continuosly variable heat and speed controls. 3) **Glass Rod (Stirring rod)**: 370 × 10 mm diameter, when specified to prevent compacting of sample in drain opening. 4) **Beakers** 5) **Percolator (2L)**: conforming to the general size and shape: 115 mm id × 400 mm long, 90 mm id at 200 mm down from top, with 8-9 mm bore tip. 6) **Filter paper-** Use smooth, high wet- strength, rapid acting filter paper ruled with oil-, alcohol-, and water proof lines 5mm apart. S&S No. 8 is satisfactory. 7) **Watch Glass** 8) **Wet sieve** 9) **Rubber Policeman/ Spatula** 10) **Widefield Stereoscopic Microscope** 11) **Water bath**		
Materials and Reagents	1) **Mineral Oil** 2) **Tergitol**		

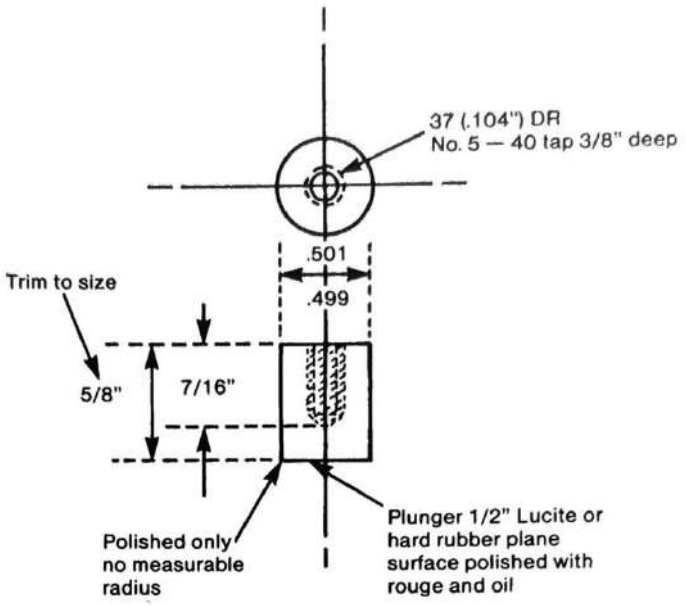
	3) **Igepal® 710** 4) **Igepal® 730** 5) **Flotation liquid** 6) **Heptane –** Commercial n-heptane containing <8% toluene. 7) **Isopropanol (IPA)-** technical or histological grade acceptable 8) **Detergent solution –** 1% or 5% in water 9) **Hydrochloric acid (HCl) (12M)** 10) **Tap water** 11) **Deionized water**
Preparation of Reagents	1) **Detergent**- dissolve 'x' grams of detergent in 100ml of water, to obtain x% of detergent as per requirement. 2) **Mineral Oil**- paraffin oil, white, light, 125/135 Saybolt universal viscosity, specific gravity 0.840-0.860 3) **Tergitol-** i) Niaproof 4 – CAS 139-88-8 ii) Triton X-114.- Octylphenol ethoxylate 4) **Igepal® 710-** Nonyl Phenol 10.5 mole ethoxylate 5) **Igepal® 730**- Nonyl Phenol 15 mole ethoxylate 6) **Flotation liquid-** Mineral Oil and heptane (85+15) 7) **Heptane –** Commercial n-heptane containing <8% toluene. 8) **Isopropanol (IPA)-** technical or histological grade acceptable
Sample Preparation	**1) Canned Crab:** • Transfer the entire contents of ≤ 200g can to 2L trap flask. • Thoroughly wash can (and parchment if present) with tap water and transfer the washings to a flask. • Add approximately 800ml hot (55° - 70°C) tap water. • With magnetic stirring, heat to boiling point. • Add 50ml mineral oil, and stir magnetically for 3 mins while continuing to boil. • Remove flask from heat, fill with hot tap water, and let stand for 30 mins, stirring gently by hand at 10 and 20 mins **2) Fish and Fish products:** • For 225g of test sample, transfer entire contents of can to 1.5 L of beaker and break up the lumps with spatula. Wash can thoroughly with small amount of isopropanol and add washings to beaker. • Add 50ml of HCl and water to make 800ml. With magnetic stirring, heat to boiling point and boil for 29 min (if product foams, add water occasionally). Add 50ml mineral oil and stir magnetically for 5 min and continue boiling. **3) Fish products containing spice, Fish paste and Sauce:** • Weigh 100g test portion into 2L beaker. • Add 800ml 5% HCl (40ml HCl+ 760ml water) and 15 ml Igepal (5ml Igepal 710 and 10 ml Igepal 730) • Cover beaker with watch glass and bring contents to full boil, stirring on magnetic stirrer. • Remove watch glass and boil gently with magnetic stirring on stirrer-hot plate for 60-90 min or until homogenous slurry is obtained (Note: do not let product boil over during digestion procedure)

	4) Canned Shrimp: • For shrimp <2.5cm long, place the entire contents of the can into a 2L beaker containing magnetic stirring bar. • For larger shrimp, skewer on probe and wash each shrimp with hot (55˚-70˚C) water from squeeze bottle over 2L beaker containing the stirring bar • Discard the shrimp, wash can thoroughly, pouring washings into beaker. • Bring water level in beaker to 925ml with hot tap water.
Method of analysis	**Isolation:** 1) **Filth in canned Crab, canned shrimp and Fish & Fish products** • Transfer to the percolator which has its rubber hose fitting clamped shut as close to tubulation opening as possible and containing 200-250 ml hot tap water. Reserve beaker. • Let stand for 3 mins and drain contents to 3 cm of bottom of the oil layer (. Repeat drain and refill steps at 3 mins interval until aqueous phase appears clear. • Finally, slowly drain percolator to minimum volume of aqueous phase without loss of oil phase. • Drain oil layer into reserved beaker. Filter through ruled paper. • Wash percolator with warm water, 1-5% detergent solution, water and isopropanol in sequence, and collect washings in beaker. • Filter onto ruled filter paper and examine microscopically. 2) **Filth in Fish products containing spice, Fish paste and Fish sauce** • Transfer slurry portion wise onto No. 230 plain weave sieve and wet sieve, with forceful stream of hot tap water (55˚-70˚C) from aerator until rinse is clear. Use rubber policeman or spatula to remove residue adhering to sides of beaker. Add 10 ml tergitol anionic 7 (or 25 ml of tergitol, for fish products containing spice), if substantial residue remains on the sieve and let stand for 2-3min. (note: soaking residue in tergitol on sieve for 2-3mins aids in dispersion of clumps). Repeat tergitol procedure twice. • Wet residue on sieve with 40% isopropanol and quantitatively transfer residue to 2 L Wildman trap flask, using 40% isopropanol. (Note: insert No. 10 rubber stopper into flask neck. If stopper extends 1.5-2.0 cm into neck, flask has proper neck diameter, if <1.5cm, flask may allow spillage during trapping procedure). • Dilute to 800 ml with 40% isopropanol and boil gently for 10mins with magnetic stirring. • Remove from heat, add 50ml of mineral oil and stir magnetically for 3min. • Fill flask with 40% IPA and let stand for 30mins with intermittent stirring. • Spin wafer disc or stopper to remove sediment and trap off, rinsing neck of flask with 40% IPA. • Add 35ml mineral oil (50 ml of flotation liquid, in case of fish products with spice).

	• Hand stir sediment with gentle rotary motion. Fill flask with 40% IPA, let stand 20 mins, and trap off as before, rinsing neck with IPA. • Filter onto ruled paper and examine at 30× with stereoscopic microscope.
Calculation with units of expression	Examination under microscope
Reference	[1] JAOAC, **976.27** (16.9.04) [2] JAOAC, **972.38** (16.9.06) [3] JAOAC, **992.10** (16.9.07) [4] JAOAC, **991.37** (16.9.08) [5] JAOAC, **974.32** (16.9.09)
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Foreign Matter- II (Parasites)		
Method No.	FSSAI 06.002:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to: • Quick frozen fish sticks (fish fingers) and fish portions- breaded or in batter. • Ready –to –eat finfish or shellfish curry in retortable pouches.		
Caution	1) The working surface should have a transparency of 45-60%. 2) Overhead illumination (indirect light) in vicinity of candling table should be ≥500 lux.		
Principle	This study determines optimum conditions for detecting parasites in skinned fish fillets by using candling tables under commercial conditions. The best balance of factors was sought for obtaining maximum lighting conditions, reducing operator fatigue, retaining natural fillet color, and having a high contrast between parasites and fish flesh.		
Apparatus/ Instruments	1) **Candling table:** Rigid framework to hold light source below rigid working surface of white, translucent acrylic plastic or other suitable material. Length and width of working surface should be large enough to examine entire test filter, e.g., 30×60 cm sheet, 5-6 mm thick. 2) **Light source:** "Cool white" with color temperature of 4200° K. At least two 20 W fluorescent tubes are recommended. Tubes and their electrical connections should be constructed to prevent overheating of light source. Average light intensity above working surface should be 1500-1800 lux as measured 30 cm above centre of the acrylic sheet. Distribution of illumination should be in a ratio of 3:1:0.1, i.e., brightness directly above light source should be three times greater than that of outer field and brightness of outer limit of visual field should be not more than 0.1 that of inner field.		
Materials and Reagents	-		
Preparation of Reagents	-		
Sample Preparation	1) Test fish samples are to be skinned and cut into thick fillet pieces. 2) Under commercial conditions, large fishes are eviscerated as soon as possible (preferably at sea), well iced during transport, and filleted as soon as practical to reduce the potential for parasites migrating from the intestinal tract into the edible flesh.		
Method of analysis	1) Place skinned fish fillets in single layer on a lighted working surface. 2) Examine visually for parasites.		
Calculation with units of expression	-		
Reference	JAOAC, **985.12** (35.1.38)		
Approved by	Scientific Panel on Methods of Sampling and Analysis		

	Determination of Foreign Matter- III (Shell bits)		
Method No.	FSSAI 06.003:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to: • Canned fishery products. • Ready –to –eat finfish or shellfish curry in retortable pouches. • Frozen clam meat. • Fresh and quick-frozen raw scallop products. • Pasteurized crab meat.		
Caution	The sample is digested in alkaline condition, and filtered with sieve to isolate the shell bits. The shell bits are washed on a pre-weighed filter paper. The foreign matter content as shell bits is expressed as count per Kg sample as well as weight per Kg of sample.		
Principle	Take final weight of the shell bits containing filter paper only when three consecutive measurement has less than 10% relative standard deviation.		
Apparatus/ Instruments	1) Beakers (500 ml) 2) Pipettes 3) Heater 4) No. 12 Sieve 5) No. 60 Sieve 6) Weighing balance 7) Hot air oven 8) Magnetic Stirrer		
Materials and Reagents	1) Sodium Hydroxide (NaOH): 1.5% 2) Alizarin Red S (aq): 1% 3) Deionized water		
Preparation of Reagents	1) Sodium Hydroxide (NaOH) (1.5%): Add 1.5g of NaOH in 100ml of deionized water. 2) Alizarin Red S (aq) (1%): Add 1g of Alizarin Red S indicator in 100ml of deionized water.		
Sample Preparation	The test samples are to be drained of any liquids before analysis.		
Method of analysis	1) Weigh 57g of representative test sample into 400ml beaker (600ml. 2) Add 150ml of 1.5% of NaOH solution and stir to break up lumps. 3) Add 10 drops of 1% aqueous Alizarin Red S indicator. 4) Heat the mixture while stirring, 3 or 4 times at 80˚C for 10 mins until the meat is digested. 5) Pour on No. 12 sieve nested in No. 60 sieve and wash with deionized water. 6) Wash shell from both sieves onto a pre weighed paper, dry at 100˚C in a hot air oven and cool to room temperature. 7) Weigh and count shell.		
Calculation with units of expression	The shell is to be reported as number of pieces and weight/Kg.		
Reference	AOAC 945.75		
Approved by	Scientific Panel on Methods of Sampling and Analysis		

	Determination of Bloom Strength of Gelatin from Fish Processing Waste		
Method No.	FSSAI 06.004:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to Gelatin from fish processing waste as specified in Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011.		
Caution	Check shot hopper on bloom gelometer to assure it is grounded electrically		
Principle	The test determines the weight in grams needed by a specified plunger (normally with a diameter of 0.5 inch) to depress the surface of the gel by 4 mm without breaking it at a specified temperature. The number of grams is called the Bloom value, and most gelatins are between 30 and 300 g Bloom. The higher a Bloom value, the higher the melting and gelling points of a gel, and the shorter its gelling times. This method is most often used on soft gels. To perform the Bloom test on gelatin, a 6.67% gelatin solution is kept for 17–18 hours at 10 °C prior to being tested.		
Apparatus/ Instruments	1) Pipettes 2) Bloom Bottle 3) Water Bath 4) Bloom Gelometer [adjusted for 4 mm depression and to deliver 200 ± 5g shot/5s, using 0.5 in. (12.7 mm) plunger, fig 1]  **Fig 1**		
Materials and Reagents	-		
Preparation of Reagents	-		
Sample Preparation	-		
Method of analysis	• Pipet 105 ml water at 10˚-15˚C into standard bloom bottle, add 7.5 g test portion, and stir.		

	• Let stand 1 hour and then bring to 62 ˚C in 15 min by placing in water bath regulated at 65˚C (test solution may be swirled several times to aid solution). • Finally mix by inversion, let stand 15 min and place in water bath at 10˚ ± 0.1˚C, chill 17 hrs. • Determine jelly strength in Bloom Gelometer, adjusted for 4 mm depression and to deliver 200 ± 5g shot/5s, using 0.5 in. (12.7 mm) plunger and light weight shot receiver (paper or plastic).
Calculation with units of expression	Bloom strength/ Bloom Value = Weight in grams, 'g' required by the plunger to depress the surface of the gel by 4mm.
Reference	JAOAC **948.21**, (38.1.03)
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Acidity of Brine in Canned Fish Products		
Method No.	FSSAI 06.005:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to Canned Fish Products as specified in Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011.		
Caution	1) The strength of the 0.1 N Sodium Hydroxide (NaOH) should be confirmed by titrating against a primary standard (for example Oxalic acid) or it should be freshly prepared before analysis. 2) The titration end point should be carefully noted when only a faint pink colour appears and is stable for 15 s. Dark pink colour indicates over titration. 3) Phenolphthalein indicator solution should be prepared fresh before titration. 4) Calibrated pipettes and burettes should be used.		
Principle	Acidity in the brine is determined in terms of % citric acid content by titration with 0.1 N NaOH, where 1 ml of 0.1NNaOH solution is equivalent to 0.0064g of citric acid. The change in pH is monitored by phenolphthalein indicator that turns slightly pink in basic solution.		
Apparatus/ Instruments	1) Standard flasks (200ml) - for preparation of standard 0.1 N NaOH 2) Erlenmeyer flasks (500ml) – to carry out the titrimetric analysis. 3) Pipettes – to transfer the sample/ analyte. 4) Burette (100ml)- to be filled with the titrant (0.1 N of NaOH). 5) Funnels 6) IS Sieve 200 (Aperture 2.00 mm)/BS Sieve 8/Tyler Sieve 9/ ASA Sieve 10		
Materials and Reagents	1) Phenolphthalein (ACS Reagent grade) 2) NaOH Pallets (≥97% purity) 3) Standard NaOH solution (0.1N) 4) Phenolphthalein Indicator Solution		
Preparation of Reagents	1) Standard NaOH solution (0.1 N)-Dissolve 4g of anhydrous NaOH in 1000ml of water to make 0.1 N standard solution. 2) Phenolphthalein Indicator Solution: - Dissolve 1g of phenolphthalein in 100ml of 95% (w/v) alcohol.		
Sample Preparation	1) Empty the content of the can on a IS Sieve 200 and collect the drained liquid in a clean glass container. 2) Wash the empty can and the residue on the sieve with small volumes of water at least three times and collect the drained liquid in the same container. 3) Transfer the drained liquid in a 1000ml graduated flask and made up the volume with distilled water. Centrifuge the made up liquid for 5 min at 1000 rev/min.		
Method of analysis	1) Take a suitable aliquot of the centrifuged liquid, add about 200ml distilled water and titrate against the0.1 N NaOH solution using phenolphthalein indicator solution till a faint pink color persists for 15 seconds. 2) Note down the volume of 0.1 N NaOH used at endpoint.		

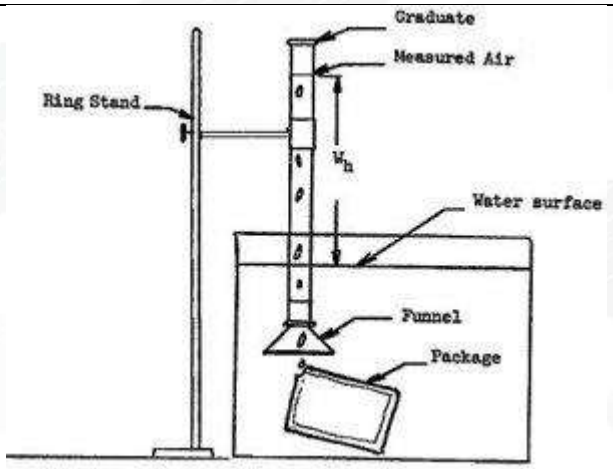
Calculation with units of expression	Calculate the percentage of acidity of the brine in terms of citric acid from the relationship: 1ml of 0.1N NaOH solution is equivalent to 0.0064g of anhydrous citric acid. $$Acidity\ as\ citric\ acid\ (\%, w/v) = (0.0064\ \times v) \times \frac{100}{V}$$ Where v=the titre value in ml, and V=volume of brine aliquot taken in ml
Reference	Indian Standard 2236: 1968
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Drained Weight in Canned Fish Products		
Method No.	FSSAI 06.006:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to Canned Fish Products as specified in Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011.		
Caution	1) The sieve should be dry and clean before weighing. Similarly, the empty can should be dry and clean before weighing 2) The weighing balance should be calibrated and tared to zero before use		
Principle	The weight of the fish product is determined following draining the liquid and expressed as percentage of the water capacity of the can		
Apparatus/ Instruments	1) Weighing balance 2) IS Sieve 200 (Aperture 2.00 mm)/BS Sieve 8/Tyler Sieve 9/ ASA Sieve 10 3) Hot air oven		
Materials and Reagents	-		
Preparation of Reagents	-		
Sample Preparation	Maintain the canned sample at 20-30°C for a minimum 12 h prior to the examination.		
Method of analysis	1) Carefully weigh the clean and dry sieve and empty the contents of the can to the sieve. Allow to drain for five minutes and weigh the sieve with the contents. 2) The difference between the two weight gives the drained weight. For determination of water capacity of the can, cut out the lead without removing or altering the height of the double seam. Wash, dry, and weigh the empty can. Fill the empty can with distilled water at 20°C to 4mm vertical distance below the top level of the container and weigh. 3) The difference in weight between the filled can and the empty can is the water capacity of the can. The drained weight is expressed as percentage of the water capacity of the can.		
Calculation with units of expression	$Drained\ weight\ \ Dw\ (g) = (W_{SC} - W_S)$ Where W_{sc} is the weight of the sieve with the content of the can, and W_s is the weight of the sieve alone $Water\ capacity\ of\ the\ can\ Wc\ (g) = (W_{CW} - W_C)$ Where W_{cw} is the weight of water filled can, and W_c is the weight of the empty can $Drained\ weight\ as\ percentage\ of\ water\ capacity\ \left(\%, \frac{w}{w}\right) = \left(\frac{Dw}{Wc}\right) \times 100$		
Reference	Indian Standard 2236: 1968		
Approved by	Scientific Panel on Methods of Sampling and Analysis		

	Determination of Percentage of Water in Drained Liquid in Canned Fish Products		
Method No.	FSSAI 06.007:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to Canned Fish Products as specified in Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011.		
Caution	All weighing balances used should be well calibrated in a timely fashion and maintained for best sensitivity and accuracy of the readings.		
Principle	Water content in the drained liquid is determined gravimetrically following evaporation of the water in a vacuum oven.		
Apparatus/ Instruments	1) Weighing balance – in milligram scale, well calibrated and maintained for sensitivity. 2) Vacuum oven – for drying purposes, and maintain a constant temperature. 3) IS Sieve 200 (Aperture 2.00 mm)/BS Sieve 8/Tyler Sieve 9/ ASA Sieve 10 4) Petri dish		
Materials and Reagents	-		
Preparation of Reagents	-		
Sample Preparation	Empty the content of the can on a IS Sieve 200 and collect the drained liquid in a clean glass container until drained completely.		
Method of analysis	1) Measure and pour an appropriate aliquot of the drained liquid on a pre-weighed petri dish. 2) Weigh the petri dish with the aliquot 3) Evaporate the aliquot on the petri dish in a vacuum oven, until a constant weight is achieved 4) Weigh the petri dish following complete evaporation of the aliquot		
Calculation with units of expression	$Water\ content\ in\ the\ drained\ liquid\ \left(\%, \frac{w}{w}\right) = \left[\frac{W_{pda} - W_{pdd}}{W_{pda} - W_{pd}}\right] \times 100$ Where W_{pda}= Weight of petridish with aliquot W_{pdd}= Weight of petridish following complete drying of water in the vacuum oven W_{pd}= Weight of empty petridish		
Reference	Indian Standard 2236: 1968		
Approved by	Scientific Panel on Methods of Sampling and Analysis		

	Determination of Vacuum in Cans in Canned Fish Products		
Method No.	FSSAI 06.008:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to Canned Fish Products as specified in Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011.		
Caution	Temperature of the can should be maintained constant at 25°C during the analysis. A calibrated vacuum gauge should be used.		
Principle	A vacuum gauge of piercing type or of an electric recording type is used to determine the vacuum in the can.		
Apparatus/ Instruments	1) Vacuum Gauge – for the measurement of vacuum inside the can. 2) Water bath- for maintenance of optimal temperature.		
Materials and Reagents	-		
Preparation of Reagents	-		
Sample Preparation	The can containing the sample is placed in a water bath and maintained at constant temperature (25°C) for a few hours, till the container temperature is uniform.		
Method of analysis	1) Place the pointed end of the vacuum gauge in the middle of the top plate of the can and press firmly to pierce the can. 2) Note down the vacuum in millimeters of mercury		
Calculation with units of expression	The vacuum level is expressed in form of millimeters of mercury (mmHg).		
Reference	Indian Standard 2236: 1968.		
Approved by	Scientific Panel on Methods of Sampling and Analysis		

	Determination of Residual Air in Retortable Pouch for Ready to Eat Finfish or Shellfish Curry		
Method No.	FSSAI 06.009:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to Ready-to-Eat Finfish or Shellfish Curry in Retortable Pouches as specified in Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011.		
Caution	1) Check the retort pouch for existing leak or breakage. 2) Ensure that the graduated measuring cylinder is fixed in an upright manner.		
Principle	The residual air in the retort pouch is measured by measuring the displaced water inside an inverted graduated measuring cylinder when the residual air in the pouch is squeezed out at the underwater base of the measuring cylinder.		
Apparatus/ Instruments	1) Funnel 2) Graduated cylinder 3) Water bath 4) Glass water tank		
Materials and Reagents	-		
Preparation of Reagents	-		
Sample Preparation	Sample is to be maintained at a uniform temperature(25 °C) prior to the estimation in a water bath.		
Method of analysis	1) Fill a graduated measuring cylinder completely with water and secure a funnel on top with tape. 2) Placing a petri plate at the funnel mouth, slowly invert the graduated measuring cylinder and dip the funnel end completely in a water filled glass tank. 3) Fix the measuring cylinder in an upright manner with a clamp and remove the petri dish slowly and allow an air pocket to form at the base of the cylinder. Following elastration presents a model experimental set up. 4) The test is performed by holding the pouch under water under the funnel attached to a graduated cylinder filled with water. 5) A corner of the pouch is cut open under the funnel and the air is squeezed out. 6) The amount of residual air in the pouch is measured as the water displacement in the cylinder.		

	
Calculation with units of expression	The volumetric measurements of air maybe corrected to atmospheric pressure by Boyle's Law. $$V_1 = [(P_a - W_h)V_m]/P_a$$ Where, V_1- vol of air at atmospheric pressure (ml) P_a- Atmospheric pressure (inches of mercury) W_h- Pressure of water level in graduated cylinder (inches of mercury) V_m- vol of measured air (ml)
Reference	https://inspection.canada.ca/food-safety-for-industry/archived-food-guidance/fish-and-seafood/manuals/flexible-retort-pouch/eng/1350916942104/1350932698250?chap=5
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Bond Strength of Retortable Pouches		
Method No.	FSSAI 06.010:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to Ready-to-Eat Finfish or Shellfish Curry in Retortable Pouches as specified in Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011.		
Caution	1) The tensile testing instrument should be adjusted in such a manner that most test specimen scans fall in the center two thirds of the chart. For this some trial runs must be carried out before actual sample analysis 2) It is important to condition the specimens at least for 40 h before analysis		
Principle	The piles of the laminate test specimen are separated at the edge of the specimen; either mechanically or with the help of a suitable solvents. Further the open ends of the laminates are clamped into a tensile strength machine and the force or energy to separate a 2-inch test portion of the specimen is determined.		
Apparatus/ Instruments	1) Grips— A gripping system that minimizes both slippage and uneven stress distribution is required. Grips lined with thin rubber, crocus cloth or pressure sensitive tape, as well as file-faced or serrated grips have been successfully used for many materials. Air-actuated grips have been found advantageous, particularly in the case of materials that tend to "neck" in the grips, since pressure is maintained at all times. 2) Tensile Strength Testing Machine—A tensile testing machine conforming to the requirements of Test Method D882. 3) Specimen Cutter—In accordance with Test Method D882.		
Materials and Reagents	Solvents like Toluene, ethyl acetate, MEK (2-Butanone) THF (tetrahydrofuran) or other suitable solvent to weaken the bond between layers sufficiently so that delamination may be started.		
Preparation of Apparatus and Calibration	1) Equip the tensile testing machine according to manufacturer's instructions for tensile testing thin films. 2) Set full-scale load so that most test specimen scans fall in the center two thirds of the chart, and draw speed at 28.0 cm/min ± 10% (10 or 12 inches/min are included). A few trial runs may be required. Other draw speeds may be used if it can be shown that they yield the same results as those specified. 3) **Specimen Conditioning**: Store specimens at 23 ± 2°C (73.4 ± 3.6°F) and 50 ± 5% relative humidity for not less than 40 h. a) End-Use Specimen Conditioning: ● Store specimens at the specific end-use temperature and humidity for not less than 40 h. ● Accelerated testing conditions for "wet" materials packaging may be accomplished by placing the test specimens between paper towels saturated with distilled water, sealing in a		

	moisture proof pouch and storing at 23 ± 2°C (73.4 ± 3.6°F) for 40 h. ● Conditioning to simulate other conditions of end use shall be determined by specific application. Normally the conditioning period will be 40 to 96 h. ● In some cases, conditioning will consist of treatment of test specimen prior to actual conditioning. Example: Boilable pouch applications. Test specimens may be immersed in boiling water for a period of time equal to normal end use and then conditioned for testing as specified (or perhaps tested shortly after boiling treatment if desired to simulate performance in use). NOTE 2—At these conditions pouch and contents should be aged to allow time for contents to migrate into seal area. 4) Test Conditions: Conduct tests in the standard laboratory atmosphere of 23 ± 2°C (73.4 ± 3.6°F) and 50 ± 5% relative humidity.
Sample Preparation	1) Sampling must be performed in a manner that will provide the desired information. No single procedure can be given for all situations. Therefore, Practice D1898 should be used as a guide in planning sampling procedures. 2) Test Specimens—Cut strips 1.0 inch (25 mm.) wide ± 5% and about 10 inch (250 mm) long. It is important that the test specimens are cut with clean, uniform edges so as not to affect the test results. 3) Test Unit—Test five specimens in the longitudinal (machine) direction. It may be desirable to test specimens in the transverse (cross-machine) direction for special purposes.
Method of analysis	1) Initiate separation of the plies of the test specimens mechanically if possible. That is, crinkle roughly or apply adhesive tape to both sides, or both, and pull apart. If not, initiate the separation by making a heat seal and pulling it apart. If both the above fail, determine which solvent seems best by trial and error and initiate the separation by immersion of the end of the strip in the solvent for as little time as possible to initiate the separation. Heat the solvent solution only if absolutely necessary. Dry well in room air without heat. 2) Clamp the separated ends of the test specimen in the jaws of the tensile testing machine using an original jaw distance of 25.4 mm (1.0 inch) and make certain the jaws are aligned vertically. 3) The unseparated portion of each test specimen shall be treated in one of the following ways: a) Left loose to move around freely, b) Supported at 90° to the direction of draw by hand, or c) Mechanically supported at 90° to the direction of draw. 4) Activate the tensile testing machine and record the force to separate 3 in. of the test specimen at 280 mm/min ± 10 % (10 or 12 inch/min). Repeat for each test specimen in the test unit.

Calculation with units of expression	1) Disregarding the initial peak, determine the average force to separate the next 2 inch of each test specimen. Express in N·m, g/25.4 mm, or lbf·in. 2) Alternatively, determine the energy to separate this 2-inch segment of each test specimen. Express in J/m or ft·lbf/in.
Reference	ASTM F904-84 Comparison of Bond Strength or Ply Adhesion of similar Laminates Made from Flexible Materials.
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Seal strength of Retortable Pouches		
Method No.	FSSAI 06.011:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to Ready-to-Eat Finfish or Shellfish Curry in Retortable Pouches as specified in Food Safety and Standards (**Food Products Standards and Food Additives) Regulations, 2011**.		
Caution	1) Testing of samples with visual defects or other deviations from normality may or may not be appropriate depending on the purpose of the investigation. Indiscriminate elimination of defects can bias results. 2) The values stated in either SI units or inch pound units are to be regarded separately as a standard. The values stated in each system may not be exact equivalents; therefore, each system shall be used independently of the other. Combining values from the two systems may result in non-conformance with the standard.		
Principle	Sealed test specimens are cut using specimen cutter as per the grip dimensions of the Tensile Strength Testing Machine. The Test specimens are clamped into the machine and seal strength is determined as force or energy required to peel the test specimen.		
Apparatus/ Instruments	1) Tensile Strength Testing Machine— • A testing machine of the constant rate-of-jaw-separation type, equipped with a weighing system that moves a maximum distance of 2 % of the specimen extension within the range being measured. • The machine shall be equipped with a device for recording the tensile load and the amount of separation of the grips. Both of these measuring systems shall be accurate to 62 %. • The rate of separation of the jaws shall be uniform and capable of adjustment from approximately 8 to 12 in. [200 to 300 mm]/min. The gripping system shall be capable of minimizing specimen slippage and applying an even stress distribution to the specimen. • If calculation of average seal strength is required, the testing machine system shall have the capability to calculate its value over a specified range of grip travel programmable by the operator. Preferably, the machine shall have the capability also to plot the curve of force versus grip travel. 2) Specimen Cutter		
Materials and Reagents	-		
Preparation of Reagents	-		
Sample Preparation	1) Cut specimens using specimen cutter to a width of 0.984 in. [25 mm], 0.591 in. [15 mm], or 1.00 in. [25.4 mm] prior to test. 2) Tolerance shall be 60.5 %.		
Method of analysis	1) Calibrate the tensile strength testing machine in accordance with the manufacturer's recommendations. 2) Prepare sealed test specimens for testing by cutting to the dimensions. Edges shall be clean-cut and perpendicular to the		

	direction of seal. Specimen length can be adjusted depending on the grip dimensions of the testing machine. 3) Adhering to one tail-holding technique, clamp each leg of the test specimen in the tensile testing machine. The sealed area the specimen shall be approximately equidistant between the grips. Recommended distance between grips (initial unconstrained specimen length) is: Fin and Hot-Wire Seals • Highly[A] extensible materials 0.39 in. [10 mm] • Less[A] extensible materials 1.0 in. [25 mm] • Lap Seals X + 10 mm[B] [A] - Grip separation distance is recommended to be limited for highly extensible materials (100 + % elongation at seal failure) to minimize interferences. 4) Center the specimen laterally in the grips. Align the specimen in the grips so the seal line is perpendicular to the direction of pull, allowing sufficient slack so the seal is not stressed prior to initiation of the test. 5) A significant difference in measured seal strength has been shown to result, depending on the orientation of a fin-seal tail during the test. The test report should indicate the details of any technique used to control tail orientation. 6) The seal shall be tested at a rate of grip separation of 8 to 12 in./min [200 to 300 mm/min]. 7) For each cycle, report the maximum force encountered as the specimen is stressed to failure and identify the mode of specimen failure. 8) If the test strip peels apart in the seal area, either by adhesive failure, cohesive failure, or delamination, the average peel force may be an important index of performance and should be measured by the testing machine as a part of the test cycle. 9) Follow the machine manufacturer's instructions to select the desired algorithm for calculating average seal strength. 10) If the test strip does not peel significantly in the seal area and failure is largely by breaking, tearing, or elongation of the substrate material, average force to failure may have little significance in describing seal performance and should not be reported in such cases. 11) A plot of force versus grip travel may be useful as an aid in interpretation of results. In those cases, the testing machine should be programmed to generate the plot. 12) Other properties, such as energy to cause seal separation, may be appropriate in cases where grip travel results only in peel. When other failure modes (elongation, break, tear, delamination (when not a designed peel seal separation mode) or other) are present in addition to peel of the seal, energy, and other functions must be interpreted with caution.
Calculation with units of expression	Report the following: 1) Complete identification of material being tested. 2) Equipment and test method or practice used to form seals, if known. 3) Equipment used to test seals.

	4) Ambient conditions during tests; temperature and humidity. 5) Grip separation rate. 6) Initial grip separation distance. 7) Seal width. 8) Machine direction of material in relation to direction of pull may be noted, if known and relevant to the test outcome. 9) Force (strength) values to three significant figures. 10) Technique of holding the tail (Technique A, B, or C) and any special fixtures used to hold specimens. 11) If the seal is made between two different materials, record which material is clamped in each grip. 12) Number of specimens tested and method of sampling. 13) Visual determination of mode of specimen failure. Frequently more than one mode will occur in the course of failure of an individual strip. Record all modes observed. 14) Maximum force encountered as each specimen is stressed to failure, expressed preferably in Newtons/meter or lbf/in. of original specimen width.
Reference	ASTM F88/F88M- 15 Standard Test Method for Seal Strength of Flexible Barrier Materials.
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Tensile Strength of Retortable Pouches		
Method No.	FSSAI 06.012:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to Ready-to-Eat Finfish or Shellfish Curry in Retortable Pouches as specified in Food Safety and Standards (**Food Products Standards and Food Additives) Regulations, 2011**.		
Caution	1) The values stated in either SI units or inch pound units are to be regarded separately as a standard. The values stated in each system may not be exact equivalents; therefore, each system shall be used independently of the other. Combining values from the two systems may result in non-conformance with the standard. 2) Caution needs to be exercised when choosing the type of grips and the type of grip surfaces to use for testing specimen's films composed of high strength LLDPE and VLDPE resins. Test results tend to differ more when comparing these types of specimen's films tested with the grips lined with different materials. 3) Fixed grips are rigidly attached to the fixed and movable members of the testing machine. When this type of grip is used, care must be taken to ensure that the test specimen is inserted and clamped so that the long axis of the test specimen coincides with the direction of pull through the center line of the grip assembly. Self-aligning grips are attached to the fixed and movable members of the testing machine in such a manner that they will move freely into alignment as soon as a load is applied so that the long axis of the test specimen will coincide with the direction of the applied pull through the center line of the grip assembly. The specimens must be aligned as perfectly as possible with the direction of pull so that no rotary motion will cause slippage to occur in the grips; there is a limit to the amount of misalignment self-aligning grips will accommodate. 4) The test specimen shall be held in such a way that slippage relative to the grips is prevented insofar as possible. Grips lined with thin rubber, crocus-cloth, emery cloth, or pressure-sensitive tape as well as file-faced or serrated grips have been successfully used for many materials. The choice of grip surface will depend on the material tested, thickness, etc. Line grips padded on the round face with 0.75-1.00 mm (0.030-0.040 in.) blotting paper or filter paper have been found superior. Air-actuated grips have been found advantageous, particularly in the case of materials that tend to "neck" into the grips, since pressure is maintained at all times. In cases where samples frequently fail at the edge of the grips, it could be advantageous to slightly increase the radius of curvature of the edges where the grips come in contact with the test area of the specimen. 5) The gage of pressure sensitive tape, thin rubber, crocus-cloth, and emery cloth needs to be adequate enough to prevent slipping and premature failures of the test specimens (for example, pressure sensitive tape is used on the surface of the grips: the test specimen		

	can may begin to tear at the edge of the grips during the test if the tape is too thin.). 6) The grit size of crocus-cloth and emery cloth is suggested to be at least 800. The use of these materials helps to prevent test specimens from slipping in the grips. One must be cautious when using these materials so that premature failures of the test specimens do not occur. 7) Utmost care shall be exercised in cutting specimens to prevent nicks and tears that cause premature failures. The edges shall be parallel to within 5 % of the width over the length of the specimen between the grips. 8) Test specimen: The test specimens shall consist of strips of uniform width and thickness at least 50 mm (2 in.) longer than the grip separation used. The nominal width of the specimens shall be not less than 5.0 mm (0.20 in.) or greater than 25.4 mm (1.0 in.). A width-thickness ratio of at least eight shall be used. Narrow specimens magnify effects of edge strains or flaws, or both. 9) Test specimens shall be selected so that thickness is uniform to within 10 % of the thickness over the length of the specimen between the grips in the case of specimens 0.25 mm (0.010 in.) or less in thickness and to within 5 % in the case of specimens greater than 0.25 mm (0.010 in.) in thickness but less than 1.00 mm (0.040 in.) in thickness. 10) If the material is suspected of being anisotropic, two sets of test specimens shall be prepared having their long axes respectively parallel with and normal to the suspected direction of anisotropy 11) Microscopical examination of specimens should be used to detect flaws due to sample or specimen preparation.
Principle	Sealed test specimens are cut using specimen cutter as per the grip dimensions of the Tensile Strength Testing Machine. The Test specimens are clamped into the machine and tensile strength is determined by measuring the specimen extension, indicated by grip separation, extension indicators, or displacement of gage marks. Tensile Strength (nominal) is measured by dividing the maximum load by the original minimum cross-sectional area of the specimen.
Apparatus/ Instruments	1) Testing Machine—A testing machine of the constant rate-of-crosshead-movement type and comprising essentially the following: a) Fixed Member—A fixed or essentially stationary member carrying one grip. b) Movable Member—A movable member carrying a second grip. c) Grips—A set of grips for holding the test specimen between the fixed member and the movable member of the testing machine; grips can be either the fixed or self-aligning type. In either case, the gripping system must minimize both slippage and uneven stress distribution. d) Drive Mechanism—A drive mechanism for imparting to the movable member a uniform, controlled velocity with respect to the stationary member. The velocity shall be regulated as specified in Section.

	e) Load Indicator—A suitable load-indicating mechanism capable of showing the total tensile load carried by the test specimen held by the grips. f) Crosshead Extension Indicator—A suitable extension indicating mechanism capable of showing the amount of change in the separation of the grips, that is, crosshead movement. 2) Extensometer (Optional)—A suitable instrument used for determining the distance between two designated points on the test specimen as the specimen is stretched. The use of this type of instrument is optional and is not required in this test method. This apparatus, if employed, shall be so designed as to minimize stress on the specimen at the contact points of the specimen and the instrument. It is desirable that this instrument automatically record the distance, or any change in it, as a function of the load on the test specimen or of the elapsed time from the start of the test, or both. If only the latter is obtained, load-time data must also be taken. This instrument must be essentially free of inertial lag at the specified speed of testing. a) Modulus of Elasticity and Low-Extension Measurements—Extensometers used for modulus of elasticity and low-extension (less than 20 % elongation) measurements shall, at a minimum, be accurate to 61 % and comply with the requirements set forth in Practice E83 for a Class C instrument b) High-Extension Measurements—Instrumentation and measuring techniques used for high-extension (20 % elongation or greater) measurements shall be accurate to 610 % of the indicated value, or better. 3) Thickness Gauge—A dead-weight dial or digital micrometer. 4) Width-Measuring Devices—Suitable test scales or other width measuring devices capable of measuring 0.25 mm (0.010 in.) or less. 5) Specimen Cutter—Devices that use razor blades have proven especially suitable for materials having an elongation-at-fracture above 10 to 20 %.
Materials and Reagents	--
Preparation of Reagents	--
Sample Preparation	1) Conditioning—Condition the test specimens at 23±2°C (73.4±3.6°F) and 50±10 % relative humidity for not less than 40 h prior to test. 2) In the case of isotropic materials, at least five specimens shall be prepared for testing.
Method of analysis	1) Select a load range such that specimen failure occurs within its upper two thirds. A few trial runs could be necessary to select a proper combination of load range and specimen width. 2) Measure the cross-sectional area of the specimen at several points along its length. Measure the width to an accuracy of 0.25 mm (0.010 in.) or better. Measure the thickness to an accuracy of 0.0025 mm (0.0001 in.) or better for specimens less than 0.25 mm (0.010 in.) in thickness and to an accuracy of 1 % or better for specimens greater than 0.25 mm (0.010 in.) but less than 1.0 mm (0.040 in.) in thickness.

	3) Set the rate of grip separation to give the desired strain rate, based on the initial distance between the grips. Zero the calibrated load weighing system, extension indicator(s) and recording system. 4) In cases where it is desired to measure a test section other than the total length between the grips, mark the ends of the desired test section with a soft, fine wax crayon or with ink. Do not scratch these marks onto the surface since such scratches can act as stress raisers and cause premature specimen failure. 5) Place the test specimen in the grips of the testing machine, taking care to align the long axis of the specimen with an imaginary line joining the points of attachment of the grips to the machine. Tighten the grips evenly and firmly to the degree necessary to minimize slipping of the specimen during test. 6) Start the machine and record load versus extension. a) When the total length between the grips is used as the test area, record load versus grip separation. b) When a specific test area has been marked on the specimen, follow the displacement of the edge boundary lines with respect to each other with dividers or some other suitable device. If a load-extension curve is desired, plot various extensions versus corresponding loads sustained, as measured by the load indicator. c) When an extensometer is used, record load versus extension of the test area measured by the extensometer. 7) If modulus values are being determined, select a load range and chart rate to produce a load-extension curve of between 30 and 60° to the X axis. For maximum accuracy, use the most sensitive load scale for which this condition can be met. The test may be discontinued when the load-extension curve deviates from linearity. 8) In the case of materials being evaluated for secant modulus, the test can be discontinued when the specified extension is reached. 9) If tensile energy to break is being determined, some provision must be made for integration of the stress-strain curve. This can be either an electronic integration during the test or a subsequent determination from the area of the finished stress-strain curve
Calculation with units of expression	1.**Tensile Strength** (nominal) shall be calculated by dividing the maximum load by the original minimum cross-sectional area of the specimen. The result shall be expressed in force per unit area, usually megapascals (or pounds-force per square inch). This value shall be reported to three significant figures. The maximum load can occur at the yield point, the breaking point, or in the area between the yield point and the breaking point. NOTE —When tear failure occurs, so indicate and calculate results based on load and elongation at which tear initiates, as reflected in the load-deformation curve. 2.**Tensile Strength at Break** (nominal) shall be calculated in the same way as the tensile strength except that the load at break shall be used in place of the maximum load.

	3.**Tensile Yield Strength**, where applicable, shall be calculated by dividing the load at the yield point by the original minimum cross-sectional area of the specimen. The result shall be expressed in force per unit area, usually megapascals (or pounds-force per square inch). This value shall be reported to three significant figures. Alternatively, for materials that exhibit Hookean behavior in the initial part of the curve, an offset yield strength shall be obtained. In this case the value shall be given as "yield strength at —% offset." 4.**Tensile Energy to Break**, where applicable, shall be calculated by integrating the energy per unit volume under the stress-strain curve or by integrating the total energy absorbed and dividing it by the volume of the original gage region of the specimen. This shall be done directly during the test by an electronic integrator, or subsequently by computation from the area of the plotted curve. The result shall be expressed in energy per unit volume, usually in megajoules per cubic meter (MJ/m^3) or inch-pounds-force per cubic inch ($in\text{-}lbf/in^3$. This value shall be reported to two significant figures. For each series of tests, the arithmetic mean of all values obtained shall be calculated to the proper number of significant figures. The standard deviation (estimated) shall be calculated as follows and reported to two significant figures: $\{(\sum X^2 - nx^2)/(n-1)\}^{-1/2}$ where: s = estimated standard deviation, X = value of a single observation, n = number of observations, and x = arithmetic mean of the set of observations.
Reference	ASTM D882-18 Standard test method for Tensile Properties of Thin Plastic Sheeting.
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Acidity in Fish Pickle		
Method No.	FSSAI 06.013:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to Fish Pickle as specified in Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011.		
Caution	The solution shall be clear and stored at low temperature (not discolored and shall not gel when about 4°C).		
Principle	This is a form of weak acid- strong base titration. The change in pH is monitored by phenolphthalein indicator that turns slightly pink in basic solution.		
Apparatus/ Instruments	1) Standard flasks (200ml) - for preparation of standard 0.1 N sodium Hydroxide. 2) Erlenmeyer flasks (250ml) – to carry out the titrimetric analysis. 3) Pipettes – to transfer the sample/ analyte. 4) Burette (100ml)- to be filled with the titrant (0.1 N of NaOH). 5) Funnels 6) Whatman filter papers – to obtain clear solutions of titrant and titrand.		
Materials and Reagents	1) Standard Sodium Hydroxide (NaOH) solution (0.1N) 2) Phenolphthalein Indicator Solution		
Preparation of Reagents	1) Standard Sodium Hydroxide solution (0.1 N)-Dissolve 4g of anhydrous NaOH in 1000ml of deionized water. 2) Phenolphthalein Indicator Solution: - Dissolve 1g of phenolphthalein in 100ml of 95% (w/v) alcohol.		
Sample Preparation	The solution to be titrated against the NaOH standard is properly filtered until a clear solution is obtained, free of any suspended solids/ other materials, that can be used for the titration.		
Method of analysis	Take 25-40ml of solution, in a 200ml standard flask add about 20-40ml of water if desired and titrate against the standard Sodium Hydroxide solution using phenolphthalein indicator solution till a faint pink color persists for 15 seconds.		
Calculation with units of expression	Calculate the percentage of acidity in terms of acetic acid from the relationship. 1 ml of 0.1N Sodium Hydroxide solution is equivalent to 0.0060g of acetic acid. $Acidity\ as\ acetic\ acid\ (\%) = \frac{0.0060\ \times volume\ of\ 0.1\ N\ NaOH\ in\ mL\ \times 100}{Volume\ of\ brine\ taken\ in\ ml}$		
Reference	I.S.I Handbook of Food Analysis (Part XII) – 1984, page 50		
Approved by	Scientific Panel on Methods of Sampling and Analysis		

	Determination of Fluid Portion in Fish Pickle		
Method No.	FSSAI 06.014:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to Fish Pickle as specified in Food Safety and Standards (**Food Products Standards and Food Additives) Regulations, 2011**.		
Caution	All weighing balances used should be well calibrated in a timely fashion and maintained for best sensitivity and accuracy of the readings.		
Principle	Basic weighing technique is used and corresponding calculation regarding the wet and dry weights are used to determine water percentage in sample.		
Apparatus/ Instruments	1) Weighing balance – in milligram scale, well calibrated and maintained for sensitivity. 2) Hot air oven – for drying purposes, and maintain a constant temperature. 3) Water bath- maintenance of uniform temperature. 4) Vortex/ shaker – for uniform mixing of the sample.		
Materials and Reagents	-		
Preparation of Reagents	-		
Sample Preparation	1) The sample is maintained at a uniform temperature in a water bath prior to the analysis to be carried out. This could be useful especially if the sample has been frozen over a period of time. 2) The sample is then placed on a shaker to uniformly mix the contents.		
Method of analysis	1) A small incision is made in the pouch/ can that is pre-weighed, containing the sample of interest. 2) The fluid in the container/ pouch is drained and the weight of the drained pouch is taken (this pouch contains only solid portion of the constituents.)		
Calculation with units of expression	$$Fluid\ portion\ in\ the\ pouch\ (\%) = \left[\frac{W_{total} - W_{drained}}{W_{total}}\right] \times 100$$ Where W_{total}– Net weight of pouch containing sample and fluid portion. (in g) $W_{drained}$– weight of the drained pouch (in g)		
Reference	IS 14515		
Approved by	Scientific Panel on Methods of Sampling and Analysis		

	Determination of Alpha Amino Nitrogen in Fish Sauce		
Method No.	FSSAI 06.015:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to Fish Sauce as specified in Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011.		
Caution	Titration needs to carried out carefully, especially near to the end point since pH meter is slow in showing the pH reading.		
Principle	Utilizing the amphoteric nature of amino acids, formaldehyde can be used to fix the basic amino group. After fixing basic amino group with formaldehyde, the carboxylic group shows acidity in the solution. The acidity is titrated with sodium hydroxide to determine formaldehyde ammonia content. Ammoniacal nitrogen is determined by titration with sulfuric acid after releasing the volatile nitrogen into a boric acid solution by distillation. The alpha amino nitrogen content is determined by subtracting the ammoniacal nitrogen content from formaldehyde nitrogen content.		
Apparatus/ Instruments	1) Glasswares: 2) Burettes (50ml) 3) Erlynmeyer flasks (250ml) 4) Calibrated Pipettes 5) Kjeldahl apparatus 6) Centrifuge 7) pH meter		
Materials and Reagents	1) Sodium Hydroxide (NaOH) 0.1M 2) Sulphuric Acid (H_2SO_4)0.05M (99.9%) 3) Formaldehyde solution (Analytical grade) 4) Magnesium Oxide (≥97%,) 5) Boric acid (≥99.5-100.5%) 6) Methyl Red – Bromocresol green indicator		
Preparation of Reagents	1) **Mass (g) required to prepare 0.1 M NaOH =** 0.1 × Mol. Mass of NaOH (40g/mol) ×Vol of solution required (L) 2) **Vol (ml) of concentrated H_2SO_4 required to prepare 0.05M H_2SO_4=** $\frac{0.05\ M \times Vol(ml)\ of\ solution\ required}{19.19\ M}$ 3) **Preparation of 4% Boric acid:** 4g of boric acid crystals dissolved in 100ml MilliQ water.		
Sample Preparation	1) The samples were centrifuged for 15 min at 7700g. 2) The fat layer was separated from the aqueous layer, which was again filtered using a Whatman filter paper No. 4. The filtered liquid obtained was used for analysis.		
Method of analysis	**Formaldehyde nitrogen was determined by the titration method.** 1) 1 ml of sample was mixed with 9 ml of distilled water and titrated to pH 7.0 with 0.1 M NaOH. 2) 10 ml of formaldehyde solution (38% v/v, pH 9.0) were then added to the neutralized samples. Titration was continued to pH 9.0 with 0.1 M NaOH.		

	3) The titration points are determined using a pH meter. **To determine ammonia nitrogen,** 1) 50 ml of 10-fold diluted samples were placed in a Kjeldahl flask containing 100 ml of distilled water and 3 g of MgO. 2) The mixture was distilled to release volatile nitrogen into 50 ml of 4% boric acid containing methyl red-bromocresol green. 3) The distillate was finally titrated with 0.05 M H_2SO_4 until the end-point was obtained.
Calculation with units of expression	**Formaldehyde nitrogen content was calculated as follows:** Formaldehyde nitrogen content (g/L) = ml ($NaOH_{pH7–pH9}$) 0.1 × 14 **Ammonia nitrogen content was calculated as follows:** Ammonia nitrogen content(g/L) = 5.6 × 0.05 × Y; where Y is the volume of H_2SO_4 (ml) **Amino nitrogen content was calculated using the following formula:** Amino nitrogen content(g/L) = (Formaldehyde nitrogen content - Ammonia nitrogen content)
Reference	doi: 10.1016/j.foodchem.2005.06.013
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Total Nitrogen Content in Fish Sauce		
Method No.	FSSAI 06.016:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to Fish Sauce as specified in Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011.		
Caution	1) Use freshly opened H_2SO_4 or add dry P_2O_5 to avoid hydrolysis of nitrites &cyanates. 2) Ratio of salt to acid (w/v) should be 1:1 at the end of digestion for proper temperature control. Digestion maybe incomplete at lower ratio; while Nitrogen maybe lost at higher ratio. Each gram of fat consumes 10ml of H_2SO_4, & each gram of carbohydrate consumes 4ml of H_2SO_4 during digestion. 3) Use boiling chips to avoid bumping		
Principle	The sample is digested by boiling a homogeneous sample in concentrated sulfuric acid. The end result is an $(NH_4)_2SO_4$ solution. Excess amount of an alkali is added to the acid digestion mixture to convert NH_4^+ to NH_3, followed by distillation of the ammonia gas in a receiving standard acid solution. The excess standard acid in the receiving solution is titrated using standard NaOH solution and the amount of nitrogen in a sample can be calculated from the quantified amount of NH_4^+ in the receiving solution.		
Apparatus/ Instruments	1) Kjeldahl flasks of hard, moderately thick, well annealed glass with total capacity ca 500-800ml. 2) Heating device adjusted to bring 250 ml H_2O at 25 °C to rolling boil in about 5 min. 3) Boiling chips-3 to 4 to prevent superheating. 4) Distillation unit- 500-800ml Kjeldahl or other suitable flask, fitted with rubber stopper through which passes lower end of scrubber bulb or trap to prevent mechanical carryover of NaOH during distillation. Upper end of the bulb tube connected to condenser tube by rubber tubing. Trap outlet of condenser in such a way as to ensure complete absorption of NH_3 distilling over into acid in receiver.		
Materials and Reagents	1) Sulfuric acid- 93-98% H_2SO_4, N- free 2) Mercuric oxide or metallic mercury- HgO or Hg, reagent grade N-Free 3) Potassium sulfate (or anhydrous sodium sulfate)- reagent grade, N-free. 4) Salicylic acid – Reagent grade, N-free. 5) Sulfide or thiosulfate, Zinc granules- reagent grade, Zinc dust- Impalpable powder. 6) Methyl red indicator 7) Hydrochloric acid standard solution- 0.5 or 0.1or (sulfuric acid- 0.25 or 0.05M) 8) Sodium Hydroxide standard solution- 0.1M (or other specified concentration).		
Preparation of Reagents	**1) Sulfide or thiosulfate solution** – Dissolve 40g of commercial K_2S in 1L H_2O (Solution of 40g Na_2S or 80g $Na_2S_2O_3$. $5H_2O$ in 1L may be used)		

	2) Sodium Hydroxide- Pellets or solution, nitrate free. For solution dissolve ca 450g solid NaOH in H_2O, cool and dilute to 1L. (Specific gravity of solution should be ≥ 1.36) **3) Methyl red indicator**- Dissolve 1g methyl red in 200ml alcohol. Standardize each standard solution with primary standard & check one against other. Test reagents before use by blank determination with 2g sugar, which ensures partial reduction of any nitrates present. **4) Hydrochloric acid standard solution**. —0.5M, or 0.1M or (sulfuric acid. —0.25M or 0.05M). **5) Sodium hydroxide standard solution**. —0.1M (or other specified concentration) Standardize each standard solution with primary standard and check one against the other. Test reagents before use by blank determination with 2 g sugar, which ensures partial reduction of any nitrates present.
Sample Preparation	-
Method of analysis	1) Place weighed test portion (0.7–2.2 g) in digestion flask. Add 0.7 g HgO or 0.65 g metallic Hg, 15 g powdered K_2SO_4 or anhydrous Na_2SO_4, and 25 ml of H_2SO_4. If test portion >2.2 g is used, increase H_2SO_4 by 10 ml for each g test portion. 2) Place flask in inclined position and heat gently until frothing ceases (if necessary, add small amount of paraffin to reduce frothing); boil briskly until solution clears (Around 2 h needed for test samples containing organic material). 3) Cool, add 200 ml H_2O, cool <25°C, add 25 ml of the sulfide or thiosulfate solution, and mix to precipitate Hg. 4) Add few Zn granules to prevent bumping, tilt flask, and add layer of NaOH without agitation. (For each 10 ml H_2SO_4 used, or its equivalent in diluted H_2SO_4, add 15 g solid NaOH or enough solution to make contents strongly alkaline.) (Thiosulfate or sulfide solution may be mixed with the NaOH solution before addition to flask.) 5) Immediately connect flask to distilling bulb on condenser, and, with tip of condenser immersed in standard acid and 5–7 drops indicator in receiver, rotate flask to mix contents thoroughly; then heat until all NH_3 has distilled (≥150 ml distillate). 6) Remove receiver, wash tip of condenser, and titrate excess standard acid in distillate with standard NaOH solution. Correct for blank determination on reagents.
Calculation with units of expression	When standard HCl is used: **Percent N = [(ml of standard acid × molarity of acid) – (ml of standard NaOH × molarity of NaOH)] × 1.4007/g test portion** When standard H_2SO_4 is used: **Percent N =[(ml standard acid × 2 × molarity acid) – (ml standard NaOH × molarity NaOH)] x 1.4007/g test portion**
Reference	JAOAC 38, 56(1955)
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Water Activity in Edible Fish Powder		
Method No.	FSSAI 06.017:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to Edible Fish Powder as specified in Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011.		
Caution	The sample should not be stored frozen before analysis. In frozen samples ice crystal may form and may interfere with the analysis. The sample which is colder or warmer, needs to be equilibrated at room temperature before analysis can be performed.		
Principle	Water activity, a_w, is ratio of vapor pressure of H_2O in product to vapor pressure of pure H_2O at same temperature. It is numerically equal to 1/100 of relative humidity (RH) generated by product in closed system.The measurement principles are based on the dew-point measurement or on the determination of the change in electrical conductivity of an electrolyte or in the permittivity of a polymer.		
Apparatus/ Instruments	1) Dew point instrument—Equipped to measure temperature to ±0.1°C. 2) Forced-draft cabinet—Constant temperature, set to maintain 25 ± 1°C; capacity ≥0.06 m^3 (2 cu ft); with access port to accommodate instrument sensor leads. Use in conjunction with insulated box. 3) Insulated box with cover—Large enough to hold test container and small enough to fit in forced-draft cabinet; with access port to accommodate instrument sensor leads. Protect test container from short-term temperature fluctuations. 4) Manometric system—Sensitive to pressure differential of ±0.01 mm Hg (1.33 Pa). 5) Test containers—120 or 240 ml (4 or 8 oz) wide-mouth or Mason glass jars with Al- or Teflon-lined screw caps and gaskets. Check integrity of cap seals and sensor leads by any means available, e.g., ability of system to hold vacuum, using Tesla coil. 6) Water bath.—Capable of maintaining temperature constant within 0.1°C at 25 ± 1°C; capacity sufficient to hold measuring chamber of selected apparatus.		
Materials and Reagents	1) Hydrophilic solid—Microcrystalline cellulose, Type PH-101. 2) Reference salts—ACS reagent grade, fine crystal.		

Salt	a_w	Salt	a_w
$MgCl_2$	0.328	$SrCl_2$	0.709
K_2CO_3	0.432	NaCl	0.753
$Mg(NO_3)_2$	0.529	KBr	0.809
NaBr	0.576	KCl	0.843
$CoCl_2$	0.649	K_2SO_4	0.973

Preparation of Reagents	Place selected reference salt in test container to depth of ~4 cm for more soluble salts (lower a_w), to depth of ~1.5 cm for less soluble salts (higher a_w), and to intermediate depth for intermediate salts. Add H_2O in~2 ml increments, stirring well with spatula after each addition, until salt can

	absorb no more H_2O as evidenced by free liquid. However, keep free liquid to a minimum. These salt slushes are ready for use upon completion of mixing, and are usable indefinitely (except for some high aw salts susceptible to bacterial attack), if contained in manner to prevent substantial evaporation losses. Some slushes, eg., NaBr, may solidify gradually by crystal coalescence, with no effect on a_w.
Sample Preparation	-
Method of analysis	**Calibration**: 1) Select ≥5 salts to cover aw range of interest or range of sensor being used. 2) Measure humidity generated by each salt slush in terms of instrument readout. 3) Plot readout against a_w values for selected salts, using cross-section paper scaled for reading to 0.001 a_w unit. 4) Draw best average smooth line through plotted points. 5) Use this calibration line to translate sensor instrument readout of samples to a_w or to check vapor pressure or dew point instruments for proper functioning. **Determination** 1) Place calibration slush or test sample in forced-draft cabinet, or H_2O bath, until temperature is stabilized at 25 ± 1°C. 2) Transfer salt slush or test sample to test container, seal container with sensing device attached, and place in temperature control device. 3) Use volume of sample or slush >1/20 of total volume of sample container plus any associated void volume of sensing system, but not so much as to interfere with operation of system. 4) Record instrument response at 15, 30, 60, and 120 min after test container is placed in temperature control device, or record response on strip chart. 5) Two consecutive readings, at indicated intervals, which vary by lesser than 0.01 a_w are evidence of adequately close approach to equilibrium. 6) Continue reading at 60 min intervals if necessary. Convert last reading to a_w by calculations from physical measurements or by reference to calibration line. 7) Make all measurements within range of calibration points; donotextrapolate calibration line. 8) Make all measurements in same direction of change, and if required by properties of sensor, expose sensor to controlled RH below ambient before starting each measurement.
Calculation with units of expression	$$a_w = \frac{pF(T)}{P_s(T)}$$ where, $\mathbf{a_w}$ – water activity **pF(T)** – The partial water vapour pressure in equilibrium with the product analyzed at the temperature T (kept constant during measurement).

	$P_s(T)$ – is the water vapour saturation pressure in equilibrium with the pure water at the same temperature T.
Reference	ISO 18787:2017
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Acid Insoluble Ash in Salted Fish/Dried fish & Edible Fish Powder		
Method No.	FSSAI 06.018:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to Salted fish/ Dried fish and Edible Fish Powder as specified in Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011.		
Caution	--		
Principle	Acid insoluble ash is determined by dissolving ash in dilute hydrochloric acid (10% m/m). The liquid is then filtered through an ashless filter paper and thoroughly washed with hot water. The filter paper is then ignited in the original dish, cooled and weighed.		
Apparatus/ Instruments	1) Silica crucible 2) Meker burner 3) Ashless filter paper 4) Desiccator 5) Weighing balance 6) Glass wares		
Materials and Reagents	1) Hydrochloric acid (HCl), Purity 37% 2) Silver Nitrate ($AgNO_3$), Purity≥99%		
Preparation of Reagents	1) Approximately 5 N HCl: Add 20 ml of 37% HCl very slowly into 30 ml of water		
Sample Preparation	Dry the test portion in a vacuum oven until no significant change in weight is observed in three consecutive weighing.		
Method of analysis	1) Heat a platinum/porcelain/silica crucible to 600 °C in a muffle furnace for 1 h, cool in a desiccator and weigh. 2) Weigh accurately about 2 g of sample in the porcelain, silica or platinum crucible. Ignite with a Meker burner for about 1 h. 3) Complete the ignition by keeping in a muffle furnace at 600± 20 °C until grey ash results (6 to 8 h). Heat the crucible in muffle furnace at 600 °C for further 30 min, cool and weigh similarly, to confirm completion of ashing, cool 4) Cool and add 25 ml of dilute hydrochloric acid, cover with a watch glass and heat on a water bath for 10 min. Cool and filter through an ashless filter paper. 5) Wash the residues in the filter paper with hot water until the washings are free from chlorides as tested with silver nitrate solution and then place the filter paper and residues back on the crucible dish. 6) Keep it in an electric oven maintained at 135±2 °C for about 3h. Ignite in a muffle furnace at 600±20 °C for 1 h. 7) Cool in a desiccator and weigh. Ignite the dish again for 30 min, cool and weigh. 8) Repeat the process till the difference between two successive weighing is less than one milligram. Note the lowest mass.		

Calculation with units of expression	Acid insoluble ash (on moisture free basis), **Percent by mass =** $\frac{100 \times (M_2 - M)}{(M_1 - M)}$ Where, M_2 = lowest mass in g, of the dish with acid insoluble ash; **M**= mass, in g, of the empty dish M_1 = mass, in g, of the dish with the dried material taken for the test
Reference	IS 14950:2001
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Ash Content in Edible Fish Powder		
Method No.	FSSAI 06.019:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to Edible Fish Powder as specified in Food Safety and Standards (**Food Products Standards and Food Additives) Regulations, 2011**.		
Caution	If the sample contains a large amount of fat, make preliminary ashing at low enough temperature to allow smoking off of the fat without burning.		
Principle	When a known weight of organic matter is ignited to ash, the weight of ash thus obtained is determined gravimetrically and expressed in terms of percentage. Heating is carried out in 2 stages, to remove the water present and finally ashing at 600 °C in a muffle furnace.		
Apparatus/ Instruments	1) Platinum dish 2) Hot air oven 3) Muffle furnace		
Materials and Reagents	-		
Preparation of Reagents	-		
Sample Preparation	Dry the test portion in a hot air oven until no significant change in weight is observed in three consecutive weighing.		
Method of analysis	1) Heat a platinum/silica crucible to 600 °C in a muffle furnace for 1 h, cool in a desiccator and weigh (W_1) 2) Weigh accurately 2 g of the dried sample in to the crucible and take weight of the crucible with sample (W_2) 3) Heat the sample in crucible at low flame by keeping on a clay triangle to char the organic matter. 4) Complete the ashing in a muffle furnace for 6 to 8 h, set at 600 °C, to get white or greyish white ash. 5) Cool the crucible in a desiccator and weigh (W_3) 6) Heat the crucible in muffle furnaceat 600 °Cfor further 30 min, cool and weigh similarly, to confirm completion of ashing, cool.		
Calculation with units of expression	$$\textbf{\textit{Ash content}}\ (\%) = \frac{(\boldsymbol{W_3 - W_1}) \times \mathbf{100}}{(\boldsymbol{W_2 - W_1})}$$ Where $\mathbf{W_1}$-weight of crucible alone $\mathbf{W_2}$-weight of dry sample with crucible $\mathbf{W_3}$-weight of crucible with ash		
Reference	[1] AOAC Official Method 938.08, 21st Edition, 2019, chapter 35 pp 8. [2] JAOAC 21, 85(1938); 23, 589(1940)		
Approved by	Scientific Panel on Methods of Sampling and Analysis		

	Determination of Crude Fat in Edible Fish Powder		
Method No.	FSSAI 06.020:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to Edible Fish Powder as specified in Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011.		
Caution	--		
Principle	Fat from dried sample is extracted using Soxhlet apparatus where fat is extracted repeatedly using petroleum ether. The fat, except phospholipids, is soluble in hot petroleum ether and extracted. The extracted crude fat is quantified gravimetrically.		
Apparatus/ Instruments	1) Soxhlet extraction apparatus 2) Thimble 3) Flat bottom flask 4) Round bottom flask 5) Water Condenser 6) Desiccator 7) Rotary solvent evaporator 8) Weighing balance		
Materials and Reagents	1) Petroleum ether/Diethyl ether		
Preparation of Reagents	--		
Sample Preparation	Dry the test portion in a hot air oven until no significant change in weight is observed in three consecutive weighing.		
Method of analysis	1) Weigh accurately 5-10 g (W_1) of dried sample in to a thimble and keep a cotton plug on top of it. 2) Place the thimble in a Soxhlet apparatus and add approximately 200 mL petroleum ether in to a flat bottom flask, connect in the Soxhlet apparatus and distill for 16 h. In this process, the solvent will be condensed in the attached condenser and recirculate through the thimble, extracting the fat. 3) Cool the apparatus and filter the solvent in to a pre-weighed round bottom flask (W_2). Rinse the flat bottom flask with small amount of petroleum ether and collect the washings in the round bottom flask. 4) Remove the excess solvent using a rotary evaporator and dry the flask in a hot air oven at 80 to 100 °C, cool in a desiccator and weigh. The weight (W_3) should not vary by more than 1 mg, in three consecutive measurement at 30 min interval.		
Calculation with units of expression	$$Fat\ content, \boldsymbol{X}\ (\%) = (\boldsymbol{W_3} - \boldsymbol{W_2}) \times \frac{\boldsymbol{100}}{\boldsymbol{W_1}}$$ Where $\boldsymbol{W_1}$-weight of dry matter taken for extraction; $\boldsymbol{W_2}$-weight of round bottom flask $\boldsymbol{W_3}$-weight of the round bottom flask with fat		

	For conversion of dry weight to wet weight basis: $$\textbf{\textit{Fat content}}\ (\%)\textbf{\textit{wet weight basis}} = \frac{\boldsymbol{X} \times (\mathbf{100} - \textbf{\textit{Moisture content}})}{\mathbf{100}}$$
Reference	AOAC 960.39
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Crude Protein Content in Edible Fish Powder		
Method No.	FSSAI 06.021:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to Edible Fish Powder as specified in Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011.		
Caution	Non protein nitrogen (NPN) if present in the sample may cause overestimation of protein content. If such presence of NPN is known, the amount should be subtracted from total nitrogen content.		
Principle	The nitrogenous compound in the sample are converted in to ammonium sulfate following digestion with concentrated sulfuric acid. The ammonia from the formed ammonium sulfate is liberated upon distillation with excess alkali. The liberated ammonia is absorbed in boric acid solution and titrated with a standardized acid for determination of nitrogen content. The nitrogen content is multiplied by a sample-specific protein factor (6.25 for fish products) to obtain the protein content. Protein + H_2SO_4 ⟶ $(NH4)_2SO_4 + CO_2 + H_2O$ $(NH_4)_2SO_4$ + NaOH ⟶ $Na_2SO_4 + 2\ NH_4OH$ NH_4OH ⟶ $NH_3 + H_2O$ 3 $NH_3 + H_3BO_3$ ⟶ [Ammonium Borate complex]		
Apparatus/ Instruments	1) Burettes 2) Pipettes 3) Erlynmeyer flasks 4) Glass rods 5) Weighing balances 6) Kjeldahl digestion flask 7) Kjeldahl distillation unit		
Materials and Reagents	1) Kjeldahl catalyst 2) Concentrated Sulphuric acid (Nitrogen free, AR grade) 3) NaOH Solution (40%) 4) Standard 0.1 N sodium carbonate solution 5) Standard acid solution (HCl 0.1N or H_2SO_4) 6) Boric acid solution (4%) 7) Tashiro's indicator 8) Methyl orange indicator 9) Phenolphthalein indicator 10) Pumice stones		
Preparation of Reagents	1) Kjeldahl catalyst- Mix 8 part of $K_2SO_4$with 1 part of$CuSO_4$ 2) NaOH Solution (40%)-Dissolve 40 g NaOH pallets in 100 ml distilled water and allow to cool 3) 0.1 N Na_2CO_3 solution-Dissolve 530 mg of Na_2CO_3 in 100 ml of distilled water 4) Standard acid solution (HCl 0.1N or H_2SO_4)- if HCl, 0.1N (3.646g/L) or H_2SO_4, 0.05M or 0.1N (4.9 g/L). Actual strength of the acid was determined by titrating against the 0.1 N Na_2CO_3 primary standard		

	solution, with methyl orange as indicator. The end point is indicated as red. 5) Boric acid solution-Dissolve 40 g boric acid in 500 ml hot distilled water, cool and make up to 1 L. 6) Tashiro's indicator-Stock solution A: 0.2% ethanolic methyl red and Stock solution B: 0.2% ethanolic methylene blue. Mix 100 ml "A" with 50 ml "B". Mix 1 part of the mixture with 1 part of ethanol and 2 part of water and use as working solution.
Sample Preparation	1) Weigh 1 g of prepared sample and transfer to a Kjeldahldigestion flask. 2) Add 7 g of digestion catalyst, 3 to 4 pumice beads to prevent bumping and 20 ml of concentrated H_2SO_4. 3) Heat the flask gently in an inclined position until frothing ceases, then boil briskly for 2 h until a light green colour clear solution is obtained. 4) To the digested and cooled solution add distilled water in small quantities with shaking and cooling till the addition of water does not generate heat. Transfer quantitatively into a 100 ml (V1) standard flask and make up the volume. 5) Similar way prepare an reagent blank, without the sample.
Method of analysis	1) Transfer with pipette a known volume (V2) of the diluted digested solution in to the reaction chamber of the micro-Kjeldahl distillation apparatus. 2) Rinse down with distilled water, add two drops of phenolphthalein indicator and 40% NaOH till the indicator turns pink. 3) The receiver end of the distillation unit should be dipped into 10 ml boric acid solution (4%) containing a drop of Tashiro's indicator. 4) Perform distillation for 4 min and absorb the liberated ammonia in the boric acid solution. Lower the flask, taking care that the receiver tip is not touching the solution, continue heating for another 1 min, wash the tip with distilled water. 5) Determine the amount of ammonia absorbed by titrating with standardized acid solution till the green colour of the solution turns light pink.
Calculation with units of expression	$$\boldsymbol{Nitrogen}\left(\boldsymbol{mg}\frac{\boldsymbol{N}}{\boldsymbol{100g}}\right)=\boldsymbol{14}\times(\boldsymbol{b}-\boldsymbol{a})\times\boldsymbol{N}\times\boldsymbol{V_1}\times\frac{\boldsymbol{100}}{\boldsymbol{V_2}\times\boldsymbol{W}}=\text{"}\boldsymbol{X}\text{"}$$ Where **b**= volume (ml) of standard acid used in sample titration **A**= volume (ml) of standard acid used in reagent blank titration **N**= corrected normality of the standardized acid for titration $\mathbf{V_1}$= made up volume (ml) of the digested solution $\mathbf{V_2}$= volume of diluted digested solution taken for distillation **W**= sample weight 14 is the atomic weight of Nitrogen $$\boldsymbol{Protein\ content}\ (\%)=\frac{\boldsymbol{X}\times\boldsymbol{6.25}}{\boldsymbol{1000}}$$ Where

	6.25 is the nitrogen to protein conversion factor for fish and fish products; 1000 is the factor to covert mg N to g.
Reference	AOAC 928.08
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Total Available Lysine In Edible Fish Powder		
Method No.	FSSAI 06.022:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to Edible Fish Powder as specified in Food Safety and Standards (**Food Products Standards and Food Additives) Regulations, 2011**.		
Caution	--		
Principle	1-Fluoro-2,4- dinitrobenzene (DNFB) reacts with free ε- amino groups in proteins, forming DNFB- ε- amino lysine which is stable to acid hydrolysis. Test portion is acid hydrolyzed and unavailable lysine is determined with amino acid analyzer; total lysine is determined on untreated test portion. Available lysine, which was bound by DNFB, is determined by difference.		
Apparatus/ Instruments	1) Rotary evaporator 2) Weighing balance 3) pH meter 4) HPLC Amino acid analyzer		
Materials and Reagents	1) Sodium bicarbonate ($NaHCO_3$) 2) 1-Fluoro-2,4-dinitrobenzene (DNFB) 3) Hydrochloric acid (35 to 37% purity) 4) Anhydrous ether 5) Stannous Chloride dihydrate ($SnCl_2.2H_20$) 6) Potassium Iodide (KI)		
Preparation of Reagents	1) 10% $NaHCO_3$- Add 10g of anhydrous $NaHCO_3$ into 100ml of deionized water 2) Hydrochloric acid (6M) – Add 250 ml of concentrated Hydrochloric acid (12M) carefully and slowly into 250 ml of deionized water and mix well. Be cautious while handling to prevent bubbling over of acid.		
Sample Preparation	Preparation of protein hydrolysate: (with DFNB) 1) Grind the test sample in a laboratory grinder, and sieve with a 20 mesh size per inch Sieve. Weigh 0.1-1.0g test portion into No. 5/0 crucible. (1.3ml). (Calculate the test portion weight to give final concentration of 0.72- 0.88 mg protein/ml for amino acid analysis) 2) Place test portion or test portion and crucible in 500ml boiling flask and add 4-5 glass beads. Add 10ml freshly prepared 10% $NaHCO_3$ solution (w/v), 10ml alcohol and 0.3ml DNFB. Stopper flask and shake mechanically for more than 3 h. Carefully acidify with 6M HCl (~2ml). Evaporate to oily dryness at 40 °C in vacuum rotary evaporator. Release vacuum very slowly to avoid disturbing residues. Add 50-75ml of anhydrous ether, decant and re- evaporate in rotary evaporator at 40 °C without vacuum. Repeat washing with ether and evaporation for additional 3 times. 3) Add 125ml of 6M HCl. Heat carefully until all CO_2 is released, and boiled under reflux for 18h maintaining constant stream of pre-purified N_2 through tygon capillary tube which comes to about 2.5cm above surface of solution. Cool for 1h and wash down residue in condenser with distilled water. Evaporate to sticky paste in vacuum		

	rotary evaporator at 40 °C. Repeat addition of 100ml of water and evaporation 4 additional times, evaporating to dryness during the last evaporation. Preparation of protein hydrolysate without DFNB: 1) Weigh test portion to give final concentration of 0.18-0.22mg protein/ml for amino acid analysis, in 5/0 crucible and placed into 500ml boiling flask and add 4-5 glass beads. Add 200ml 6M HCl and distil off 100ml H_2O. Wash down residue in condenser with water repeat the addition of water and evaporation cycle 5 additional times, evaporating to dryness during the last evaporation.
Method of analysis	Dilute the dried hydrolysate in suitable HPLC buffer and perform amino acid analysis as per "Method for Determination of Protein Digestibility Corrected Amino Acid Score (PDCAAS): Part 2. Amino Acid Analysis (FSSAI 06.024:2022).
Calculation with units of expression	$$\textbf{\textit{Test portion to use}}\ (\textbf{\textit{mg}}) = \left(\frac{\boldsymbol{C}}{\boldsymbol{P}}\right) \times \mathbf{100}$$ Where, **C** is the final concentration desired (mg/ml) **P** is the % protein in sample Determine the area under the curve for lysine or use integrator and compare areas of test portions with those from calibration standards containing known concentration of lysine (eg. 2.5±0.004 µM/ml 0.1M HCl) **% of available lysine =** % **of lysine of non DFNB treated test portion** − % **of lysine in DFNB treated test portion**
Reference	JAOAC **58**, 599(1975)
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Protein Digestibility Corrected Amino Acid Score (PDCAAS) in Edible Fish Powder: Part 1 Data requirement and calculations		
Method No.	FSSAI 06.023:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to Edible Fish Powder as specified in Food Safety and Standards (**Food Products Standards and Food Additives) Regulations, 2011**.		
Caution	--		
Principle	The calculation of the PDCAAS of a food protein is based on: 1) The food's protein content, usually calculated using the factor 6.25 [or specific AOAC factor listed in the Guidelines), multiplied by the nitrogen (N) content of the food as determined by Kjeldhal method. Where a food contains more than one protein source, the factor 6.25 shall be used to determine the protein content. Where a foodstuff contains only one protein source, the listed shall be used. 2) The food's essential amino acid profile, determined by typical analytical procedures or high-performance liquid chromatography (HPLC). 3) The amino acid scoring pattern based on WHO guidelines. 4) The food's in vitro digestibility, determined using a simulated gastric digestion.		
Apparatus/ Instruments	Burettes Pipettes Erlynmeyer flasks Glass rods Weighing balances Kjeldahl digestion flask Kjeldahl distillation unit		
Materials and Reagents	1) Kjeldahl catalyst 2) Concentrated Sulphuric acid (Nitrogen free, AR grade) 3) NaOH Solution (40%) 4) Standard 0.1 N sodium carbonate solution 5) Standard acid solution (HCl 0.1N or H2SO4) 6) Boric acid solution (4%) 7) Tashiro's indicator 8) Methyl orange indicator 9) Phenolphthalein indicator 10) Pumice stones		
Preparation of Reagents	1) Kjeldahl catalyst- Mix 8 part of K_2SO_4 with 1 part of $CuSO_4$ 2) NaOH Solution (40%)-Dissolve 40 g NaOH pallets in 100 ml distilled water and allow to cool 3) 0.1 N Na_2CO_3 solution-Dissolve 530 mg of Na_2CO_3 in 100 ml of distilled water 4) Standard acid solution (HCl 0.1N or H_2SO_4)- if HCl, 0.1N (3.646g/L) or H_2SO_4, 0.05M or 0.1N (4.9 g/L). Actual strength of the acid was determined by titrating against the 0.1 N Na_2CO_3 primary standard solution, with methyl orange as indicator. The end point is indicated as red.		

	5) Boric acid solution-Dissolve 40 g boric acid in 500 ml hot distilled water, cool and make up to 1 L. 6) Tashiro's indicator-Stock solution A: 0.2% ethanolic methyl red and Stock solution B: 0.2% ethanolic methylene blue. Mix 100 ml "A" with 50 ml "B". Mix 1 part of the mixture with 1 part of ethanol and 2 part of water and use as working solution.
Sample Preparation	1) Weigh 1 g of prepared sample and transfer to a Kjeldahl digestion flask. 2) Add 7 g of digestion catalyst, 3 to 4 pumice beads to prevent bumping and 20 ml of concentrated H_2SO_4 . 3) Heat the flask gently in an inclined position until frothing ceases, then boil briskly for 2 h until a light green colour clear solution is obtained. 4) To the digested and cooled solution add distilled water in small quantities with shaking and cooling till the addition of water does not generate heat. Transfer quantitatively into a 100 ml (V_1) standard flask and make up the volume. 5) Similar way prepare a reagent blank, without the sample.
Method of analysis	1) Analyse for proximate nitrogen (N) of test product following "Method for Determination of Crude Protein Content in Edible Fish Powder (Method No. FSSAI 06.021:2022). 2) Calculate protein content (N x 6.25 or specific AOAC factor). 3) Analyse for essential amino acid (EAA) profile of the food and express as g/100 g protein as described in Method for Determination of Protein Digestibility Corrected Amino Acid Score (PDCAAS): Part 2. Amino Acid Analysis. 4) Convert data to express EAA values to mg/g protein. 5) Determine the amino acid score using the equation. $$\textbf{EAA Score} = \frac{\textbf{mg of EAA in 1 g of test protein}}{\textbf{Acid score mg of EAA in 1 g reference protein}}*$$ 6) Reference protein*=FAO/WHO EAA requirement pattern (mg/g protein for different age groups (See Table below)

Amino Acid (mg/g protein)	Age group (Years)					
	0.5	1 –2	3 –10	11 –14	15 –18	>18
Histidine	20	18	16	16	16	15
Isoleucine	32	31	31	30	30	30
Leucine	66	63	61	60	60	59
Lysine	57	52	48	48	47	45
Methionine plus Cystine	28	26	24	23	23	22
Phenylalanine plus tyrosine	52	46	41	41	40	38
Threonine	31	29	25	25	24	23
Tryptophan	8.5	7.4	6.6	6.5	6.3	6
Valine	43	42	40	40	40	39

	Source: World Health Organization: Protein and amino acid requirements in human nutrition: report of a joint FAO/WHO/UNU expert consultation. In: WHO technical report series, 935(2007)5 7) The amino acid with the lowest EAA score (test protein/reference) is the limiting amino acid. 8) Determine the in- vitro protein digestibility of test products described in 'Method for Determination of Protein Digestibility Corrected Amino Acid Score (PDCAAS): Part 3. In vitro protein digestibility. 9) Calculate PDCAAS of test sample and standard casein.
Calculation with units of expression	**PDCAAS= Lowest EAA score (limiting amino acid) × protein digestibility.** PDCAAS is a number ranging from 0- 1.0 The PDCAAS of Casein should be 1.0
Reference	Rasco, B. (2001). Analyses of Protein Quality. Current Protocols in Food Analytical Chemistry, 00(1), B2.1.1- B2.1.15. doi: 10.1002/0471142913.fab0201s00
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Protein Digestibility Corrected Amino Acid Score (PDCAAS) in Edible Fish Powder: Part 2 Amino Acid Analysis		
Method No.	FSSAI 06.024:2023	**Revision No. & Date**	0.0
Scope	The method describes the determination of amino acid composition of foods. The method described is a precolumn derivatization method followed by RP-HPLC. It is applicable to all foods. ***Other validated methods such as ion exchange chromatography with either pre-or post-column derivatization applicable to foods may also be used to calculate the essential amino acid levels***.		
Caution	--		
Principle	The determination of amino acids in foods involves: 1. Acid hydrolysis of performic acid oxidized protein for the determination of all amino acids except tryptophan. Asparagine and glutamine are converted to aspartic acid and glutamic acid, respectively during acid hydrolysis. Cystine and methionine are converted to cysteic acid and methionine sulfonic acid by oxidation. 2. Separation and quantitation of the released amino acids by ion exchange chromatography (IEC) using cation exchange resins and post-column derivatization (by a commercial amino acid analyzer or HPLC system) or by precolumn derivatization using various reagents followed by reverse phase HPLC. The method described here uses pre column derivatization with Phenylisothiocyanate (PITC). PITC reacts with amino acids to form phenylthiocarbamyl (PTC) derivatives, which can be detected with high sensitivity at 254 nm. A reverse-phase HPLC separation with UV detection is used to analyze the amino acid composition. Phenol: Solutions of phenol are corrosive to the skin and eyes, while phenol vapour can irritate the respiratory tract. Phenol and its solutions are flammable. Use in fume hood. Wear safety goggles and/or a face shield.		
Apparatus/ Instruments	1) Hot air oven maintained at 110±3 °C 2) Vacuum hydrolysis tubes/heat sealable test tubes 3) Centrifugal vacuum concentrator 4) Work station for vapor phase hydrolysis and precolumn derivatization of amino acids 5) HPLC system equipped with a. Pumps for binary gradient separation b. Injection device c. UV/PDA detector set at 254 nm d. Column oven to maintain a temperature of 38 ±1 °C e. Systems software to control operation of HPLC and data integration and calculations. f. PICO-TAG analysis column (15 cm x3.9 mm).		
Materials and Reagents	1) Hydrogen Peroxide (30 %) 2) Formic acid 3) Glacial acetic acid 4) Sodium acetate 5) HPLC Gradient grade acetonitrile		

	6) High-purity calibration Standard amino acid hydrolysate containing 2.5 µMol/mL each in 0.1N HCl, of Asp (D), Glu (E), Ser (S), Gly (G), His (H), Arg (R), Thr (T), Ala (A), Pro (P), Tyr (Y), Val (V), Met (M), Cystine (C), Ile (I), Leu (L), Phe (F), Trp(W) and Lys (K). 7) Phenol (>99% purity). Do not use if colored. 8) L-cystine (>99.5% purity) 9) L-Methionine (> 99.5% purity) 10) Triethylamine (> 99.5% purity) 11) Phenyl isothiocyanate (PITC) (≥99.0%); stored at — 20 °C under nitrogen to prevent breakdown products from forming. Do not use if pale yellow in color 12) Mercaptoethanesulfonic acid Na salt (MESA) 13) Concentrated Hydrochloric acid or Constant boiling Hydrochloric acid (Sequanal Grade)
Preparation of Reagents	1) Performic acid: prepared freshly by adding 1 mL of 30% H2O2 to 9 mL of 88 % formic acid and 50 mg phenol and allowed to stand at 25±3 ˚C for 1 h following which it is cooled to 0 °C. 2) Constant boiling hydrochloric acid (6 N, BP110 ˚C): Dilute concentrated hydrochloric acid 1:1 with distilled water and distill. Collect what distills at 110 ˚C. Store in a dark brown bottle at 5-8 ˚C. Stable for 24 months. Alternatively, ampoules of constant boiling hydrochloric acid (Sequanal grade) are commercially available 3) Acid hydrolysis solution: Constant boiling hydrochloric acid (6 N) containing 0.1% to 1.0% of phenol. Prepare fresh before use. 4) 2.5 M MESA solution: Available commercially 5) Derivatizing reagent: The derivatization reagent is made fresh daily of ethanol-TEA-water-PITC (7+ 1 + 1 + 1). To prepare 300 µLreagent (enough for 12 samples), 210 µL ethanol was mixed thoroughly with 30 µL each PITC, TEA, and water. 6) Solvent A: an aqueous buffer of 0.14M sodium acetate containing 0.5 mL/L TEA and titrated to pH 6.40 with glacial acetic acid: acetonitrile (94:6) 7) Solvent B, 60% acetonitrile in water 8) Sample diluent: Sodium phosphate, pH 7.6, containing 5% acetonitrile.
Sample Preparation	***Method 1: Performic acid oxidation* followed by *acid hydrolysis*** The sample is subjected to performic acid oxidation prior to acid hydrolysis. Acid hydrolysis using hydrochloric acid containing phenol is the most common procedure used for protein hydrolysis preceding amino acid analysis. The addition of phenol to the reaction prevents the halogenation of tyrosine. **1) Performic acid oxidation** a. Weigh sample and standard casein containing 5 mg protein equivalence. If sample is wet, dry. The sample is dried so that water in the sample will not dilute the reagents. b. Add two mL of freshly prepared performic acid to the protein powder/dried sample. c. Allow the reaction to proceed for 4 h at 0 °C. d. Excess reagents are removed by diluting with water and then evaporated under vacuum using a concentrator. e. This is repeated two times and finally dried to completeness. f. Standard casein (equivalent to 5 mg protein), Standard amino acid cystine (1 mg) and methionine (1 mg) are treated similarly.

	2) Acid hydrolysis can be carried out in the liquid phase or vapor phase. **I. Liquid Phase Hydrolysis** a. Add 1.0 mL of the hydrolysis solution per 5 mg of protein. b. Flame seals the tubes in in vacuum or inert atmosphere to prevent oxidation. c. Place tubes in oven set at 110 °C for 24 h. Longer hydrolysis times. (e.g., 48 and 72 hours) are used if there is a concern that the protein is not completely hydrolyzed. d. After hydrolysis dry the test sample in vacuum to remove any acid and process in accordance with either pre column or post column derivatization **II. Vapor Phase Hydrolysis** - This is one of the most common acid hydrolysis procedures, and it is preferred for microanalysis when only small amounts of the sample are available. Contamination of the sample from the acid reagent is also minimized by using vapor phase hydrolysis. a. Place vials containing the dried samples in a vessel that contains an appropriate amount of hydrolysis solution. The hydrolysis solution does not come in contact with the test sample. b. Apply an inert atmosphere or vacuum (less than 200 mm of mercury or 26.7 Pa) to the headspace of the vessel, and heat to about 110 °C for a 24-hour hydrolysis time. c. Acid vapor hydrolyzes the dried sample. Any condensation of the acid in the sample vials is minimized. d. After hydrolysis, dry the test sample in vacuum to remove any residual acid. *Note: These methods result in the destruction of tryptophan* **Method 2: To estimate tryptophan**. Tryptophan oxidation during hydrolysis is decreased by using 2.5 M MESA for hydrolysis. a. Place sample containing about 5 mg of the protein under test in a dried \ hydrolysis tube. b. The hydrolysis tube is placed in a larger tube with about 2 mL of the 2.5 M MESA. c. The larger tube is sealed in vacuum (about 50 mm of mercury or 6.7 Pa) to vaporize the hydrolysis solution. d. The hydrolysis tube is heated to between 170° to 185 °C for about 12.5minutes. e. After hydrolysis, the hydrolysis tube is dried in vacuum for 15 minutes to remove the residual acid. f. The sample is ready for derivatization.
Method of analysis	Precolumn derivatization, a. The calibration standard amino acid hydrolysate containing up to 12.5 nmol of each amino acid, acid hydrolysates of test sample, standard casein, cysteic acid and methionine sulfone were placed in individual 6 x 50 mm tubes enclosed in specially designed vacuum vial with resealable PTFE closure and were dried under vacuum to 50-60 mtorr. b. 20 µL of a freshly prepared redrying solution of methanol-water-TEA (2 + 2 + 1) was added to each tube, vortexed and dried under vacuum. When vacuum reached 50-60 mtorr (6-8 Pa), samples were ready for derivatization. c. The samples were derivatized by adding 20 µL of freshly prepared derivatization reagent to dried samples and sealing the vacuum vials for 20 min at room temperature.

d. The excess of reagents are then removed under vacuum using the workstation. When the vacuum reached 50-60 mtorr, the samples are ready for analysis by RP-HPLC.

RP-HPLC separation and detection of amino acids in the hydrolysate.

a. The derivatized sample was dissolved in 0.2 mL of sample diluent.

b. 5 µL of calibration standard is injected, followed by cysteic acid and methionine sulfone standard.

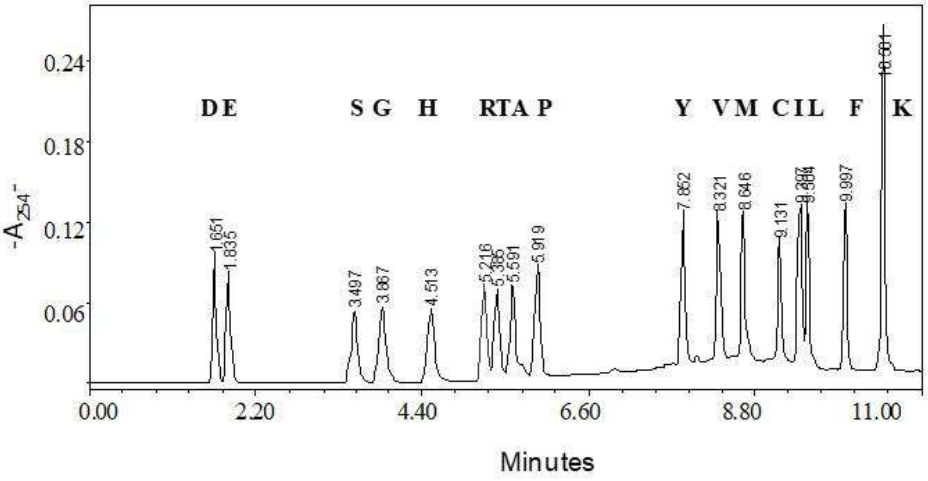
c. Samples are injected in volumes ranging from 5-50 µL.

d. HPLC conditions

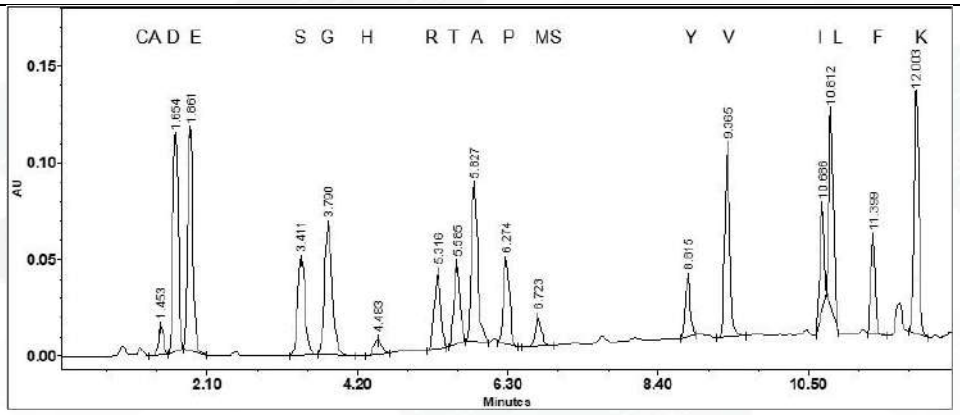
- Column: PICO-TAG analysis column (15 cm x 3.9 mm). or equivalent
- Column oven temperature:38 ±1℃
- Flow rate: 1 mL/min
- Detection: 254 nm
- Elution: Gradient

Time (min)	Flow rate (ml/min)	%A	%B	Gradient
0.01	1.0	100	0	
10.01	1.0	54	46	Convex
11.00	1.0	0	100	Linear
13.00	1.0	0	100	Linear
14.00	1.0	100	0	Linear
25.00	1.0	100	0	

Calibration standards, casein hydrolysate and sample are injected in duplicate.



A typical elution profile of the calibration standard **(**312.5 pmol of each amino acid)**. For single letter code refer 'Materials and reagents'**

	 A typical RP-HPLC elution profile of the hydrolysate of a performic acid oxidized protein. CA=Cysteic acid and MS=Methionine sulfone
Calculation with units of expression	Using the area under the curve obtained from the chromatogram the g% g/100g protein is calculated for each individual amino acid as follow: $$g\ of\ Asp = \frac{Area\ of\ Asp\ in\ sample}{Area\ of\ Asp\ in\ standard} \times C \times MW$$ Where **C** = Concentration of standard injected **MW** = Molecular weight of amino acid Calculate the '**g**' of all the amino acids individually Sum the total mass of all the amino acid Calculate the g% for each amino acid e.g. Asp $$\mathbf{g/100g}\ protein = \frac{g\ of\ Asp}{Sum\ total\ of\ all\ amino\ acids} \times 100$$ For each of the essential amino acids this value is converted into mg/g protein and used in the calculation of EAA score shown in Part 1. mg/g protein =g/100 g × 0.1
Reference	Davidson, I. (2003). Hydrolysis of Samples for Amino Acid Analysis. Protein Sequencing Protocols, 111–122. doi:10.1385/1-59259-342-9:111 Bidlingmeyer, B. A., Cohen, S. A., Tarvin, T. L., 1984. Rapid analysis of amino acids using precolumn derivatization. *J. Chromatogr*. 336, 93-104 Bidlingmeyer, B. A., Cohen, S. A., Tarvin, T. L., & Frost, B. (1987). *A New, Rapid, High-Sensitivity Analysis of Amino Acids in Food Type Samples. Journal of AOAC INTERNATIONAL, 70(2), 241–247.*
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Protein Digestibility Corrected Amino Acid Score (PDCAAS) In Edible Fish Powder: Part 3 In- Vitro Protein Digestibility		
Method No.	FSSAI 06.025:2023	**Revision No. & Date**	0.0
Scope	The method describes, an in vitro enzyme digestion method that has a very high correlation to the rat digestion model and uses casein standard as a completely digestible control. The method is applicable in all types of food products and protein concentrates. The range of this method is from 0 to 1 for in vitro digestibility.		
Caution	--		
Principle	Food samples are digested with pepsin at pH 2.0 followed by digestion with trypsin and chymotrypsin in a neutral buffer to simulate the physiological conditions of gastric and intestinal digestion, respectively (1, 2). (1) Proteins —(pepsin; pH 2.0, 37°C)→ proteins + peptides + amino acids (2) Proteins —(trypsin + chymotrypsin; pH 7.4, 37°C)→ proteins + peptides + amino acids Undigested proteins are removed by precipitation with trichloroaceticacid. The soluble nitrogen made available in the supernatant by the digestion is determined by Kjeldhal method. This digestibility score (D), in conjunction with the essential amino acid analysis of the sample, is used to calculate the PDCAAS.		
Apparatus/ Instruments	1) Micro-pipettes (20 µL, 200 µL and 1 mL) 2) Timer 3) pH Meter 4) Analytical balance (capable of weighing to +/- 0.0001 g) 5) Heated water bath (capable of 95 °C) 6) Shaking incubator (capable of 37 °C) 7) Refrigerated Centrifuge		
Materials and Reagents	1) Pepsin (from porcine gastric mucosa) 2) Porcine pancreatic Trypsin (Type IX) 3) Bovine Chymotrypsin (Type II) 4) Concentrated HCl 5) Sodium hydroxide 6) Trichloroacetic acid 7) TRIZMA Base		
Preparation of Reagents	1) Hydrochloric acid (0.06 N, pH 2.0): Place approx. 900 mL of distilled water in a 1 L beaker. Add 5 mL of concentrated HCl~ (12N) while stirring. Adjust the pH to 2.0 with2 N NaOH. Transfer to a 1 L volumetric flask and bring to volume (1 L) with distilled water. Transfer to a suitable sealed container. Store for up to 1 year at 23±2 ˚C. 2) Trichloroacetic acid (40% w/v): Add 40 g of trichloroacetic acid to approx 80 mL of distilled water and dissolve by stirring. Make to volume (100 mL) with distilled water. Store for up to 1 year at room temperature. Note: Ice-cold solution is preferred for precipitation 3) Hydrochloric acid (0.001 N, pH 3.0): Place approx 550 mL of distilled water in a 500 mL beaker. Add 8.3 mL of 0.06 N HCl while stirring. Adjust		

	the pH to 3.0with 0.1 N HCl/NaOH. Transfer to a 500 mL volumetric flask and bring to volume (500 mL) with distilled water. Transfer a suitable sealed container. Store for up to 1 year at 23±2 ˚C. 4) Tris Buffer (1.0 M, pH 7.4): Place 150 mL of distilled water in a beaker. Add 30.29 g of Tris base while stirring. Slowly add 15 mL 12 N HCl. Adjust the pH to 7.4 with 1 N HCl and transfer to a 250 mL volumetric flask. Bring to volume (250 mL) with distilled water and mix. Sterile filter buffer and transfer to a sealed container. Store for up to 4months at 23±2 ˚C. 5) Pepsin Solution (1 mg/mL) – Weigh 1 mg of Pepsin per sample into a suitably sized centrifuge tube. Add 2additional milligrams so that there is extra for pipetting. Add 1 mL of the 0.06 N HCl per mg of Pepsin to the centrifuge tube. Lightly vortex to mix. *Note: Make fresh daily, use within 30 min.* 6) Trypsin/Chymotrypsin Solution (5 mg/mL) – Weigh 1 mg (~15000 U) of Trypsin and 1 mg (~150 U) of Chymotrypsin (per sample into a suitably sized centrifuge tube. Add 2 additional mg of each enzyme so that there is extra for pipetting. 7) Add200 µL of the 0.001 N HCl per sample (plus an additional 400µL) to the centrifuge tube. Lightly vortex to mix. *Note: Make fresh daily, use within 30 min.*
Sample Preparation	1) Ground, frozen samples should be stored below -10 °C and thoroughly homogenized prior to weighing. 2) Refrigerated samples should be weighed cold and returned to the fridge or freezer as soon as possible. 3) Do not allow refrigerated or frozen samples to warm to room temperature before weighing. Weigh —as is to ensure integrity of the matrix. 4) Liquid samples should be thawed under a stream of nitrogen prior to weighing. 5) Solid samples should be ground to a fine powder
Method of analysis	1) Accurately weigh 0.5 g of milled sample and casein control sample (in triplicate) into a 50 mL Beckman centrifuge tube or conical flask. 2) Ensure that all of the sample drops to the bottom of the tube/does not stick to neck of flask. 3) Add 19 mL of HCl (0.06 N) and cap the tube. Mix thoroughly by vortex and incubate for 30 min at 37 °C in a shaking incubator set at 300 rpm. 4) Add 1 mL of pepsin solution to each sample and stopper. Mix thoroughly by vortex and incubate for 60 min at37 °C in a shaking incubator set at 300 rpm. 5) After the pepsin incubation is complete, remove samples, cool and adjust the pH to 7.4 by the addition of 2 mL of 1.0 M Tris buffer, pH 7.4. 6) Cap the tubes and mix each sample thoroughly by vortex. 7) Add 200 µL of Trypsin/Chymotrypsin mixture to each sample, 8) Mix thoroughly by vortex and incubate for 4-5 h at 37 °C shaking incubator set at 300 rpm. 9) At the end of incubation place the samples in a boiling water bath for 10 min to inactivate enzymes. 10) Remove all samples from the boiling water bath and mix thoroughly by vortex. 11) Allow the samples to cool to 23± 3 ˚C for at least20 min

	12) Add ice cold 40% TCA (~2.2 mL) solution to obtain a final TCA concentration of 10%, cap and mix thoroughly by vortex. 13) Incubate the samples at 4 °C overnight (at least 16 h).
Calculation with units of expression	In vitro digestibility (D) is expressed as a %: $$\mathbf{D}(\%) = \frac{\mathbf{N} - \mathbf{n}}{\mathbf{N}} \times \mathbf{100}$$ Where: N= Total nitrogen(g/100g) n= Soluble nitrogen(g/100g)
Reference	Plank, D. W. (2017). US Pat 9,738,920. ―In vitro method for estimating in vivo protein digestibility‖. The State of Food and Agriculture, Food and Agriculture Organization of the United Nations, 2011.
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of pH in Fish and Fish Products		
Method No.	FSSAI 06.026:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to Fish Pickle and Fish Sauce as specified in Food Safety and Standards (**Food Products Standards and Food Additives) Regulations, 2011**.		
Caution	1) The pH meter used for detection should be calibrated regularly and well maintained. 2) The calculations should be made sensitive according to the altitude of the place where the experiment takes place. 3) The temperature during the measurement should be maintained at a constant value. 4) The sample should be homogenized before measurement.		
Principle	pH is the measurement of H+ ion activity; It measures active acidity. pH may be determined by measuring the electrode potential between glass and reference electrodes; pH meter is standardized using standard pH buffers.		
Apparatus/ Instruments	1) Sensitive pH meter. 2) Water bath – to monitor and ensure stable temperature throughout the sample		
Materials and Reagents	-		
Preparation of Reagents	-		
Sample Preparation	**Class 1 - Fish Sauce** Mix the sample constituted by the whole product thoroughly, using a stirrer or a spatula. **Class 4 - Fish Pickle** After opening the container, transfer the liquid phase of the laboratory sample to a separating funnel. Collect the aqueous phase and eliminate the oil phase. In applicable cases, combine the aqueous phase with the solid part of the laboratory sample, homogenize a representative aliquot to give a moist homogeneous mixture. Place the homogenized sample in a water bath at room temperature for a few hours. Mix the sample using a vortex before the measurement. Note: The class 1 and class 4 products are specified according to ISO method.		
Method of analysis	1) After the sample preparation is done, immerse or embed the electrode and ensure that there is adequate contact between probe and sample. 2) Read when the meter reading is stable. 3) Do three separate measurements on the test sample - the extreme readings should not differ by more than 0.15 pH units. 4) Take as the result the arithmetic mean of the three readings.		

Calculation with units of expression	$$\mathbf{pH} = \frac{(\mathbf{pH1}+\mathbf{pH2}+\mathbf{pH3})}{\mathbf{3}}$$ where, $\mathbf{pH_1}$ – pH of the first reading $\mathbf{pH_2}$ – pH of the second reading $\mathbf{pH_3}$ – pH of the third reading **pH** – the final pH value that is the arithmetic mean.
Reference	ISO 11289: 1993(E)
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Moisture content in Fish & Fish Products		
Method No.	FSSAI 06.027:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to Edible Fish Powder and Freeze Dried Shrimp as specified in Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011.		
Caution	All the instruments used should be calibrated in a timely fashion & maintained well for accuracy and sensitivity.		
Principle	The principle of the thermogravimetric method of moisture content determination is defined as the weight loss of mass that occurs as the material is heated. The sample weight is taken prior to heating and again after reaching a steady-state mass subsequent to drying.		
Apparatus/ Instruments	1) Weighing balance 2) Hot air oven 3) Silica crucible		
Materials and Reagents	-		
Preparation of Reagents	-		
Sample Preparation	Cut the large pieces into small sizes and mix. Grind the pieces as finely as possible preferably using an electric grinder so that a homogenous sample is obtained. Keep the material in an airtight container in order to prevent the loss of moisture during subsequent handling. Use this material for testing.		
Method of analysis	Weigh about 5g of the prepared sample into tared silica crucible or dish. Dry the dish in an air oven 100±1˚C for 6h. Cool in a desiccator and weigh.		
Calculation with units of expression	$\textbf{Moisture}(^{W}/_{W}\%) = \frac{\mathbf{M1 \times 100}}{\mathbf{M2}}$ Where, $\mathbf{M_1}$= loss of mass in g in sample $\mathbf{M_2}$= mass in g of sample taken for test		
Reference	JAOAC 930.15ami		
Approved by	Scientific Panel on Methods of Sampling and Analysis		

	Determination of Salt Content in Fish & Fish Products		
Method No.	FSSAI 06.028:2023	**Revision No. & Date**	0.0
Scope	This is a titrimetric analysis that can be applicable to: ● Salted fish/dried salted fish ● Canned Fishery Products ● Ready–to-Eat Finfish or Shell Fish Curry in Retortable Pouches ● Fish Pickle ● Sturgeon Caviar ● Fish sauce		
Caution	Standards solutions should be freshly prepared and standardized		
Principle	This method determines the chloride ion concentration of a solution by titration with silver nitrate. As the silver nitrate solution is slowly added, a precipitate of silver chloride forms. The end point of the titration occurs when all the chloride ions are precipitated.		
Apparatus/ Instruments	1) Weighing balance 2) Pipettes 3) Burettes 4) Erlenmeyer flasks 5) Standard flasks 6) Beaker 7) Hotplate /Sand bath		
Materials and Reagents	1) Silver nitrate standard solution (0.1M) 2) Ammonium thiocyanate standard solution (0.1M) 3) Ferric Indicator		
Preparation of Reagents	1) Silver nitrate standard solution (0.1M) – Prepare 0.1M $AgNO_3$ & standardize against 0.1M NaCl containing 5.844g of pure dry NaCl/L. 2) Ammonium thiocyanate standard solution (0.1M) – Prepare 0.1M and standardize against 0.1M $AgNO_3$. 3) Ferric Indicator – Saturated solution of $FeNH_4(SO_4)_2.12\ H_2O$		
Sample Preparation	1) Shellfish meats- Weigh 10g meats, liquid, or mixed meats and liquid into 250ml Erlenmeyer or beaker. 2) Other fish products- Use suitable size test portion, depending on NaCl content.		
Method of analysis	1) Add known volume 0.1M $AgNO_3$ solution, more than enough to precipitate all Cl as AgCl & then add 20ml of HNO_3, boil gently on hot plate or sand bath until all solids except AgCl dissolve (usually 15 min). 2) Cool, add 50ml water & 5ml indicator & titrate with 0.1M NH_4SCN solution until becomes permanent light brown. 3) Subtract mL 0.1M NH_4SCN used from the volume of 0.1M $AgNO_3$ added and calculate difference as NaCl.		
Calculation with units of expression	With 10g test portion each mL 0.1N $AgNO_3$= 0.0058% NaCl		
Reference	JAOAC **20**. 410(1937), **23**. 589(1940)		

	CAS-7647-14-5 (sodium chloride)
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Histamine in Fish & Fish Products		
Method No.	FSSAI 06.029:2023	**Revision No. & Date**	0.0
Scope	High-performance liquid chromatography (HPLC) method to analyze histamine in following categories of fish and fishery products intended for human consumption – ● Raw/ chilled/ frozen finfish ● Thermally processed fishery products ● Smoked fishery products ● Fish mince/ surimi analogues ● Battered and breaded fishery products ● Other ready to eat fishery products ● Other value-added fishery products ● Other fish-based products ● Dried/ salted and dried fishery products ● Fermented fishery products ● Fish pickle		
Caution	1) The instruments used are required to be calibrated and maintained in a timely fashion for better sensitivity and accuracy. 2) To avoid matrix effect and bias (Method of analysis), carry out calibration line on same matrix (histamine free) as the sample analyzed.		
Principle	This method enables the separation of histamine among biogenic amines in fish and fishery products. The sample is extracted by mixing with perchloric acid. Pre-column derivatization is performed using dansyl chloride. The biogenic amines and the components in the solution are separated by HPLC using UV detection. Histamine concentration is calculated from the peak area ratio of histamine and internal standard with a calibration curve.		
Apparatus/ Instruments	1) Grinder 2) Balances (precisions 0.1 g & 0.001 g) 3) Crusher/ Homogenizer 4) Refrigerated centrifuge (capable of centrifugal force of 8000 g) 5) Centrifuge tubes (plastic with closing caps) 6) Pipettes (ranges 20 µl to 200 µl & 100 µl to 1000 µl) 7) Tubes (temperature resistant glass with caps) 8) Vortex 9) Water bath ((60 ˚C ± 1˚C) with dark cover or equivalent) 10) Refrigerator (5 ˚C ± 3 ˚C) 11) Freezer (capable of temperatures < -18 ˚C) 12) Nitrogen evaporator 13) Needles (20 G 0.9 mm disposable) 14) Filters (0.2 µm disposable, PTFE/ PP) 15) Syringes (2 ml, disposable) 16) LC system (pump, refrigerated autosampler, column oven (25 ˚C ± 2 ˚C)), UV detector λ = 254 nm 17) LC Column (C18 5 µm 100 Å (250 mm x 4.6 mm) or equivalent) 18) Glass autosampler vial (2 ml with insert (200 µl) & cap)		

Materials and reagents	Use only reagents of recognized analytical grade & water complying with grade 1 of ISO 3696, unless otherwise specified. Solvents shall be of quality for HPLC analysis, unless otherwise specified. 1) Acetone 2) Acetonitrile 3) Toluene 4) Water (HPLC) grade 5) Water (distilled or equivalent) 6) Nitrogen gas 7) Perchloric acid, c($HClO_4$) = 0.2 mol/l 8) Saturated sodium carbonate solution 9) Dansyl chloride solution, p($C_{12}H_{12}ClNO_2S$) = 7.5 mg/ml 10) L- proline solution, p($C_5H_9NO_2$) = 100 mg/ml 11) Histamine stock solution, p($C_5H_9N_3$) = 12.5 mg/ml 12) Internal standard (IS) 1,7 –diaminoheptane stock solution, p($C_7H_{18}N_2$) = 6.4 mg/ml
Preparation of reagents	1) *Perchloricacid*, ($HClO_4$) = 0.2 mol/l 2) Dilute 19.5 ml of $HClO_4$ (65%) or 17.2 ml of $HClO_4$ (70%) to 1000 ml of water. The solution is stable for six months if stored at room temperature (15 ˚C to 25 ˚C) 3) *Saturated sodium carbonate solution*: Dissolve 110 g of sodium carbonate in about 150 ml of water. The solution is stable for 3 months if stored at 5 ˚C ± 3 ˚C 4) *Dansyl chloride solution*, p($C_{12}H_{12}ClNO_2S$) = 7.5 mg/ml 5) Dissolve 0.375 g of dansyl chloride in 50 ml of acetone. The solution is stable for 3 weeks if stored in dark at a temperature less than -18 ˚C. 6) *L-proline solution*, p($C_5H_9NO_2$) = 100 mg/ml. Dissolve 1 g of L-proline in 10 ml of water. The solution is stable for 3 weeks if stored at a temperature of around 5 ˚C ± 3 ˚C 7) *Histamine stock solution*, p ($C_5H_9N_3$) = 12.5 mg/ml. Dissolve 1.034 g of histamine hydrochloride in 50 ml of water. The solution is stable for 1 year if stored at < -18 ˚C 8) *Internal standard* (IS) 1, 7diaminoheptane stock solution, p($C_7H_{18}N_2$) = 6.4 mg/ml. Dissolve 0.320 g of 1, 7diaminoheptane in 50 ml of water. The solution is stable for three weeks if kept at a temperature of 5 ˚C ± 3 ˚C
Sample preparation	1) Homogenize the sample by grinding in a mixer. 2) Transfer a test portion consisting of 5 g ± 0.1 g of homogenate to a centrifuge tube. 3) If the matrix is complex or difficult to obtain in histamine free condition (e.g. fishmeal, fish sauce, etc.) the spiking can be performed directly using standard addition method.
Method of analysis	***Extraction*:** 1) Add 10 ml of perchloric acid & 100 µl of 1, 7diaminoheptane to 5 g of fish (sample) in the centrifuge tube and mix. 2) After complete homogenization, centrifuge at 8000 g for 5 min at 4 ˚C.

***Derivatization*:**

1) Transfer 100 µl of the supernatant into a tube; add 300 µl of sodium carbonate solution and 400 µl of dansyl chloride solution.
2) Vortex and incubate for 5 min in the dark at 60 ˚C.
3) Cool the tube under the tap water and add 100 µl of L- proline solution.
4) Vortex and place the tube in the dark for 15 min. Supernatant can be stored at <-18 ˚C for one week).

***Purification*:**

1) Add 500 µl of toluene and vortex. Manipulation can be stopped at this step with storage at <-18 ˚C for a week maximum.
2) Transfer as much as possible of the upper organic phase into a new tube and dry it in the fume hood with a stream of nitrogen.

(Note 3: The organic phase toluene contains the derivatized histamine and not the "non organic" (aqueous) phase. The organic phase can easily be recovered by freezing theaqueous phase (<-18 ˚C for 30 min minimum). In addition, freezing can improve the quality of the upper phase.)

3) Re-suspend the dry tube with 200 µl of acetonitrile/water (60/40 volume fraction) and vortex. Filter the solution in a glass autosampler vial and fill the autosampler.

LC conditions:

- Injection volume: 20 µl
- Column oven: 25 ± 2 ˚C
- Tray temperature: 5 ± 2 ˚C
- Flow rate: 1 ml/min
- Mobile phase: Acetonitrile/ water

Gradient-

Time (min)	Water (%)	Acetonitrile (%)
0	40	60
11	25	75
11.1	5	95
20	5	95
20.1	40	60
30	40	60

Range of standard sample:

4) Standard samples should be prepared by supplementing histamine stock solution to sample homogenates from a histamine free matrix.

Concentration (mg/Kg)	Volume of histamine stock solution (µl)
0	0
25	10
50	20

<table>
<tr><td></td><td>
<table>
<tr><td>100</td><td>40</td></tr>
<tr><td>250</td><td>100</td></tr>
<tr><td>500</td><td>200</td></tr>
</table>
5) After adding the specified volume to histamine free samples, proceed to the extraction and remaining procedure for histamine estimation.
</td></tr>
<tr><td>Calculation with units of expression</td><td>Perform a calibration function by linear regression analysis, using histamine standard samples and an internal standard with following formula:
$$f\,(C_{HS}) = \frac{A_{HS}}{A_{IS}} \times C_{HS}$$
where,
C_{HS} is concentration of histamine in the standard sample (mg/ Kg)
A_{HS} is area of the histamine standard peak
A_{IS} is area of the internal standard peak

Histamine quantification: Calculate the concentration of histamine in the sample by following regression equation:
$$C_H = \frac{\frac{A_H}{A_I} \times \frac{5}{m}}{a}$$
where,
$\mathbf{C_H}$ is measured concentration of histamine in sample (mg/ Kg)
$\mathbf{A_H}$ is area of the histamine peak
$\mathbf{A_{IS}}$ is area of the internal standard peak
$\mathbf{a}$ is slope of the calibration line
$\mathbf{m}$ is the mass of the sample taken

The mass, m, usually corresponds to 5 g, but if the sample concentration is outside the range of standard sample, conduct a new analysis with smaller test portion in order to be in linear range regarding representativity of the sample.</td></tr>
<tr><td>Reference</td><td>ISO 19343:2017, Microbiology of the food chain- Detection and quantification of histamine in fish and fishery products- HPLC method</td></tr>
<tr><td>Approved by</td><td>Scientific Panel on Methods of Sampling and Analysis</td></tr>
</table>

	Determination of free formaldehyde in fish		
Method No.	FSSAI 06.030:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to Raw/ chilled/ frozen finfish as specified in Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011		
Caution	1) The DNPH must be recrystallize to get pure DNPH crystals. Purity of DNPH affects the derivatization reaction performance. 2) Appropriately dilute the extract before injection into GC-MS/MS. Higher concentration injection will lead to carry over and contamination of GC column.		
Principle	Free formaldehyde in fish is extracted by aqueous extraction and derivatized with 2,4-dinitrophenylhydrazine (DNPH). The derivatized formaldehyde is identified and quantified using GC-MS/MS in MRM mode.		
Apparatus/ Instruments	1) Laboratory Tissue Grinder 2) Balances (precisions 0.1 g & 0.001 g) 3) Refrigerated centrifuge (capable of centrifugal force of 8000 g) 4) Centrifuge tubes (plastic with closing caps) 5) Pipettes (ranges 20 µl to 200 µl, 100 µl to 1000 µl, 5000 µl & 10000 µl) 6) Vortex mixer 7) pH paper/pH meter 8) Shaking Incubator (40 ˚C ± 1˚C) with dark cover or equivalent) 9) Refrigerator (5 ˚C ± 3 ˚C) 10) Freezer (capable of temperatures < -18 ˚C) 11) Needles (20 G 0.9 mm disposable) 12) Filters (0.2 µm disposable, PTFE/ PP) 13) Syringes (2 ml, disposable) 14) Glass autosampler vial (2 ml with insert (200 µl) & cap) 15) GC Capillary Column (DB-5MS 30m, 0.250mm, 0.25µm or equivalent; temperature 60^0 c to 325^0 c) 16) Gas Chromatograph – tandem mass spectrometer.		
Materials and reagents	1) Formaldehyde in water (CRM) 2) Formaldehyde d_2(Internal standard) 3) Deionized Water 4) Sodium Hydroxide (1.0 N) 5) Glacial Acetic Acid 6) 2,4 Dinitrophenyl hydrazine 7) Acetonitrile 8) Orthophosphoric Acid (85% in water) 9) Ethyl Acetate (HPLC grade) 10) Dispersive cleanup kit (Mg SO_4, C18, PSA)		
Preparation of reagents	*1)* NaOH-1N: 100 g in 1000 ml of water *2)* Acetate buffer : Dilute 64.3 ml of 0.1 N NaOH and 5.7 ml glacial Acetic acid to 900 ml with organic free reagent water. Dilute to 1 liter with organic-free reagent water. Adjust the pH to 4.93±0.02 if needed		

<table>
<tr><td></td><td>3) Recrystallisation of 2,4 dinitrophenyl hydrazine: DNPH should be recrystallized prior to use by dissolving 10g of 2,4 DNPH in 100ml hot analytical grade acetonitrile to form saturated solution. After complete dissolution, the solution was cooled to room temperature, capped in brown bottle and stored overnight at 4^{0}c for crystallization. The solvent is decanted and the crystals were collected after drying under gentle stream of nitrogen.
4) DNPH working solution: 150 mg of 2,4 DNPH Crystals were accurately weighed, dissolved in 49.5 ml of acetonitrile and mixed with 0.5 ml of orthophosphoric acid (85%)
5) Formaldehyde in water CRM solution = 55.3 mg/l
6) Internal standard (IS) formaldehyde D2= 1000 mg/l-Dissolve 10 mg of formaldehyde D2 in 10 ml of HPLC/GC grade Ethyl acetate. The solution is stable for one year if kept at a temperature of 5 ± 3 °C</td></tr>
<tr><td>Sample preparation</td><td>1)Homogenize the sample by grinding in a laboratory grinder mixer.
2)Transfer a test portion consisting of 2 g ± 0.1 g of homogenate to a centrifuge tube.
3)Add 40 ml of Acetate buffer in 2 g of fish (sample) in the centrifuge tube and mix and adjust pH 5 using pH paper, then sonicate for 30 min.
4)After complete homogenization, centrifuge at 8000 rpm for 10 min at 4 °C.
5)Collect 10 ml of supernatant in a graduated centrifuge tube, then add type-1 water to made up to 20 ml, adjust the pH to 5 with orthophosphoric acid.
6) Derivatization: Add 6 ml of 2,4 DNPH in the centrifuge tube, vortex and place in a shaking incubator for 1 h, at 150 rpm in the dark at 40 °C.
7) After derivatization extract with 10 ml of HPLC/GC grade ethyl acetate, vortex for 10 mins then centrifuge for 8000 rpm for 10 mins. Repeat the ethyl acetate extraction steps twice and pull all the supernatant.
8)Clean up: Add 2 ml of the pulled ethyl acetate extract to the dispersive clean up tube containing (150 mg $MgSO_4$, 25 mg C18 and 25 mg PSA) and vortex for 2 min then centrifuge at 12000 rpm for 10 min.
9)After centrifugation, filter the supernatant with 0.22µm (PTFE) syringe filter.
10)Dilute the samples appropriately with ethyl acetate as per the sensitivity of the instrument used and spiking concentration of matrix fortified standards. Add equal volume of internal standard solution to each vial before injection.</td></tr>
<tr><td>Method of analysis</td><td>Detection and estimation by GC- MS/MS:
GC conditions:
● Injection volume: 1 µl (constant temperature splitless, preferably in a PTV injector)
● GC Oven Programs
<table><tr><td>Rate(^{0}c/min)</td><td>Temperature(^{0}c)</td><td>Hold Time(min)</td></tr><tr><td>0</td><td>150.0</td><td>3.00</td></tr><tr><td>25.0</td><td>290.0</td><td>3.00</td></tr><tr><td>15.0</td><td>310.0</td><td>1.00</td></tr></table></td></tr>
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Injection mode: PTV, CT Splitless
Carrier mode : Constant flow
Inlet
Temperature : 290 °C
Split flow : 50.0 ml/min
Split less time : 1.00 min
Carrier gas flow: 1.200 ml/min
MRM Conditions:

<table>
<tr><th>Compound</th><th>Precursor ion</th><th>Product ion</th><th>Collision energy</th></tr>
<tr><td>Formaldehyde 1</td><td>210</td><td>78</td><td>10</td></tr>
<tr><td>Formaldehyde 2</td><td>210</td><td>122</td><td>10</td></tr>
<tr><td>Formaldehyde D3</td><td>213</td><td>125.1</td><td>5</td></tr>
</table>
Matrix fortified calibration samples: Weigh 2 g blank tissue each in six 50 ml centrifuge tubes. Prepare two sets of six tubes. Spike the tubes at 0, 1, 2, 4, 8, and 16 ppm level for one set and at 0, 10, 20, 40, 80, 160 ppm level in another set. The different range of calibration is required to cover fresh fish where formaldehyde content might be low, and for high concentration ranges for deliberately adulterated samples. Prepare the samples in the same way as mentioned in the sample preparation protocol and use them as matrix fortified calibration standards. Multiplication with dilution factor won't be necessary unless different dilution is used for a particular sample.
</td></tr>
<tr><td>Calculation with units of expression</td><td>Area ratio of Formaldehyde quantifier ion to formaldehyde internal standard is plotted against different calibration concentration and a linear regression equation is formed. The concentration in sample is calculated through the instrument software using the calibration curve. One transition is used as quantifier transition and the other transition is used as qualifier transition. Other than quantifier and qualifier transitions, ion ratio is considered for unambiguous identification.</td></tr>
<tr><td>Reference</td><td>EPA METHOD 8315A</td></tr>
<tr><td>Approved by</td><td>Scientific Panel on Methods of Sampling and Analysis</td></tr>
</table>

	Determination of Paralytic Shellfish Poison (PSP) in Molluscs		
Method No.	FSSAI 06.031:2023	**Revision No. & Date**	0.0
Scope	PSTs are a group of neurotoxic alkaloids, which are structurally related to the parent compound saxitoxin (STX). Toxins included in the analysis were; GTX1-5, dcGTX2&3, dcSTX, dcNEO, C1&2, NEO and STX. This method is specifically used for detection in: • Live and raw bivalve molluscs • Frozen clam meat		
Caution	1) All standards should be handled carefully, avoiding contact with eyes and should not be ingested. 2) All instruments used should be calibrated on a routine basis for sensitivity and accuracy.		
Principle	Toxins are extracted from 2 g fish sample homogenate with a single dispersive extraction using 18 mL 1% HAc without any further dilution to volume. The extract is centrifuged to allow transferring of the supernatant, and 1 mL is pipetted to a polypropylene tube. The extract is then cleaned up through amorphous polymer graphitized carbon SPE cartridge and diluted with acetonitrile (MeCN). The diluted extract is then analyzed by HILIC-MS/MS.		
Apparatus/ Instruments	1) Hilic coloumn: HILIC-Z column (150mm × 2.1 mm × 2.7 µm) 2) Chromatography System: Infinity 2 binary pump equipped with a thermostatically controlled autosampler and a column oven (set at 30°C). 3) Detection system: triple quadrupole mass spectrometer was used for the detection		
Materials and Reagents	• Certified reference toxin (STX) was obtained from National Research Council Canada (NRCC, Halifax, Nova Scotia, Canada) or any other source with same specification. • Acetonitrile (HPLC and LCMS grade) • (MeCN) • LC-MS grade water • Acetic acid (HAc) (99%) • Ammonium formate (97%) • Formic acid (97%) • Ammonium acetate (99%) • Hydrogen peroxide (99%) • Periodic acid (99%) • Methanol (LCMS/HPLC grade)		
Preparation of Reagents	1) Mobile phase A: 0.6% of 25% ammonium hydroxide (in water) + 0.015% Formic acid 2) Mobile phase B**:** 90% Acetonitrile (in water) + 0.01% Formic acid **Preparation of Standards:** 1) Prepare matrix standards using previously analyzed PST negative material, and subject to de-salting clean up. 2) Dilute 1mL of carbon SPE cleaned extract in 3 mL of MeCN to create the matrix solvent.		

	3) Generate matrix curves using six calibration standards analyzed in duplicate, average the responses and calculate subsequent slopes.

S. No	Analyte	Calibration range (nmol/L)
1	C1	4.6-183.2
2	C2	1.4-55.0
3	dcGTX2	4.0-161.4
4	dcGTX3	1.2-47.8
5	GTX2	4.1-164.2
6	GTX3	1.7-69.6
7	GTX1	2.3-91.5
8	GTX4	0.7-28.8
9	GTX5	2.3-93.6
10	dcSTX	2.7-106.9
11	dcNEO	1.2-48.6
12	STX	2.7-107
13	NEO	2.6-104.2

Sample Preparation

- Freeze and store the materials at –20°C until analysis.
- Add 18mL of 1% HAc to 2.0 g of homogenized tissue, vortex and mix for 90 seconds, and place into boiling water for 5 min prior to cooling in running cold water.
- After centrifugation (4500 rpm; 10 min; 20°C), decant the extracts into a clean 15 mL centrifuge tube and subject to further centrifugation (at same conditions detailed previously) to separate fats and proteins from the aqueous extracts of all matrices.
- Take this from the extract and avoid the fat layer separated during centrifugation, and subject it to desalting carbon SPE.

SPE cleanup:

- Condition a 250 mg/3 mL cartridge with3 mL 20% MeCN + 1% HAc, followed by 3 mL 0.025% NH_3 using an approximate flow rate of 6 mL/min as a guide.
- Elute both to the level of the top frit and discard to waste. Add 400 µL sample extract to the cartridge and elute to the top of the frit, using an approximate flow rate of 3 mL/min and discarding to waste.
- Wash the cartridge with 700 µL water and elute to dryness, discard the eluent to waste, with an approximate flow rate of 3 mL/min. Add 2 mL 20% MeCN + 1% HAc, elute it to dryness and collect the eluent in a clean polypropylene tube using an approximate flow rate of 3 mL/min. Mix eluent on a vortex mixer.
- Diluted 100 µL of post carbon SPE eluants in 300 µL acetonitrile and analyze using HILIC-MS/MS.

Method of analysis

1) Optimize the quantitative ESI *m/z* transitions and collision energies for use with the HILIC-Z column on the Agilent 6495.
2) Prepare the analytical standards at six concentration levels in 80% MeCN with 0.25% Hac.
3) Assess the linearity using six calibration standard levels analyzed in duplicate.
4) Prepare the solvent standards. Prepare the matrix standards using previously analyzed PSP negative material and subject to de-salting clean up.
5) Clean 1mL of extract using carbon SPE and dilute using 3 mL of MeCN to create the matrix solvent.
6) Generate matrix curves using six calibration standards analyzed in duplicate, average the responses and calculate the subsequent slopes.

Chromatographic Gradient:

Column temperature: 30 ℃

Flow rate: 2 ml/min

Chromatographic Gradient (%)		
Time (min)	A	B
0	10	90
0.5	10	90
5.5	25	75
6	50	50
7.5	50	50
8	10	90
11	10	90

MS Parameters:

Analyte	Polarity	1' MRM transition	2' MRM transition	Cone Voltage (V)	Collision Energy (eV)
dcSTX	+ve	257.1>126.1	257.1>222.0	10	19; 22
dcNEO	+ve	273.1>126.1	273.1>225.1	10	20; 18
STX	+ve	300.1>204.1	300.1>138.0	10	23; 30
NEO	+ve	316.1>126.0	316.1>220.1	10	26; 23
dcGTX3	+ve	353.1>255.1		10	18
GTX3	+ve		396.1>298.1	10	17
GTX4	+ve	412.1>314.1		10	18
GTX5	+ve	380.1>300.1		10	16
C2	+ve	396.1>298.0		18	20
dcGTX2	-ve	351.1>164.0	351.1>333.1	10	30;17
dcGTX3	-ve		351.1>333.1	10	17
GTX2	-ve	394.1>351.1	394.1>333.1	10	16
GTX3	-ve	394.1>333.1		10	22
GTX1	-ve	410.1>367.1	410.1>349.1	10	15;22
GTX4	-ve		410.1>367.1	10	15
GTX5	-ve		378.1>122.0	10	25
C1	-ve	474.1>122.0	474.1>351.0	10	30;25
C2	-ve		474.1>122.0	10	30

Calculation with units of expression	The concentration of each of the analytes can be calculated from the graph using the equation: **y=mx+C** (as obtained from the graph), where, **y** – Signal/Area given by the standards. **x** – Known concentration of the standard used for calibration. **m** – Slope of the curve **C** – Intercept The result is calculated based on the calibration curve and then multiplied by the dilution factor (if dilution is conducted during preparation).
Reference	Karl J. Dean, Robert G. Hatfield and Andrew D. Turner (2021), "Performance Characteristics of refined LC-FLD and HILIC-MS/MS methods for the Determination of Paralytic Shellfish Toxins in Shrimp, Whelk and Crab".
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Okadaic Acid (DSP) and Azaspiracid (AZP) in Molluscs		
Method No.	FSSAI 06.032:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to the determination of the lipophilic marine biotoxins [(direct quantitative determination of okadaic acid (OA) and Azaspiracid 1 (AZP or AZA 1). Assuming an equal response factor, the procedure was validated by using OA for the indirect quantification of dinophysistoxin 1 (DTX1) and dinophysistoxin 2 (DTX2)] & AZA 1 was used for the indirect quantification of AZA 2 & AZA 3 in different molluscan shellfish matrices: • Live and raw bivalve molluscs • Frozen clam meat		
Caution	1) All standards should be handled carefully, avoiding contact with eyes and should not be ingested. 2) All instruments used should be calibrated on a routine basis for sensitivity and accuracy. 3) Use only reagents of recognized analytical grade		
Principle	The method is based on the extraction of OA & AZA group toxins with 100% methanol from homogenized tissue. Extracts are then filtered and directly analyzed by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) in order to investigate the presence of free OA, free DTX1, free DTX2, AZA 1, AZA 2 and AZA 3. To determine the total content of OA group toxins, an alkaline hydrolysis is necessary from methanolic extract prior to LC-MS/MS analysis with the aim of converting any acylated esters of OA and/or DTXs to the parent OA and/or DTX1 or DTX2 toxins. After hydrolysis, extracts are filtered and analyzed by LC-MS/MS. Chromatographic separation is performed by gradient elution.		
Apparatus/ Instruments	• Analytical balance, accuracy to the nearest 0.1 mg • Balance, accuracy to the nearest 0.01 g • High-speed blender or homogenizer • Shaker (e.g. Vortex) • Ultra Dispensor/ Homogenizer • Centrifuge, up to 2000 g • Heat block or water bath, at 76ºC • Instruments for sample preparation, knives, spatulas, scissors, stainless steel sieve, plastic jars • Volumetric flask, 20 ml, 100 ml, 250 ml, 500 ml and 1000 ml • Adjustable automatic pipettes and graduated cylinders • 50 ml polypropylene centrifuge tubes • Syringe or membrane filter (pore size 0.45 µm) • HPLC autosampler vials • Syringe for filter system • Syringe or membrane filter (pore size 0.2 µm) • Analytical reverse phase HPLC column: Examples for pH range between 2 and 8 (acidic conditions): BDS-Hypersil C8, 50 mm (length) x 2 mm (diameter), 3 µm particle size. Examples for both acidic conditions and alkaline conditions (pH range: 1-12): X-Bridge C18, 50 mm (length) x 2.1 mm (diameter), 2.5 µm particle size.		

	Acquity UPLC® BEH C18, 50 mm (length) x 2.1 mm (diameter), 1.7 µm particle size. X-Bridge C18, 150 mm (length) x 3 mm (diameter), 5 µm or 3.5 µm particle size. X-Bridge C18, 150 mm (length) x 2.0 mm (diameter), 3.5 µm particle size. **NOTE**: These are the columns that support chromatographic separation of the analytes. Each laboratory can decide the suitable column and optimize the gradient system accordingly. • Liquid chromatograph, system able to analyze in gradient mode • Mass spectrometer, equipped with an ESI interface and able to analyze in tandem MS/MS
Materials and Reagents	1) Water - Ultrapure 2) Acetonitrile, HPLC grade or Hypergrade for LCMS 3) Methanol, HPLC grade 4) Formic acid (98-100% purity) 5) Ammonium formate (≥99% purity) 6) Hydrochloric acid (37% purity) 7) Hydrochloric acid 2.5 M 8) Sodium hydroxide (≥99% purity) 9) Sodium hydroxide 2.5 M 10) Ammonia (25%) 11) Ammonium hydrogen carbonate (bicarbonate; ≥98% purity) 12) Ammonium hydroxide solution (>25 % or greater purity) 13) Okadaic acid (CRM-OA-c). 14) Azaspiracid 1 (CRM AZA1). Standard solution of azaspiracid 1 in methanol. 15) Azaspiracid 2 (CRM AZA2). Standard solution of azaspiracid 2 in methanol. 16) Azaspiracid 3 (CRM AZA3). Standard solution of azaspiracid 3 in methanol
Preparation of Reagents	1) Hydrochloric acid 2.5 M: Add 20 ml hydrochloric acid to a 100 ml volumetric flask and make up to the mark with water. This solution is stored at room temperature and can be used for 3 months. 2) Sodium Hydroxide 2.5 M: Dissolve 10 g sodium in 75 ml water in a 100 ml volumetric flask and made up to the mark with water. This solution is store at room temperature and can be used for 3 months. 3) Okadaic acid (CRM-OA-c): Standard solution of okadaic acid in methanol 4) Azaspiracid 1 (CRM AZA1): Standard solution of azaspiracid 1 in methanol. 5) Azaspiracid 2 (CRM AZA2): Standard solution of azaspiracid 2 in methanol. 6) Azaspiracid 3 (CRM AZA3): Standard solution of azaspiracid 3 in methanol 7) Stock standard solution: • Ampoules containing OA toxins & AZA 1 used are supplied with a certified concentration (14.3 µg/ml for OA & 1.24 µg/ml for AZA 1).

<table>
<tr><td></td><td>
14 µl of OA & 161 µl the reference standard is diluted with methanol to the 1000 ml to get a stock standard solution.
8) Working standard solutions:
A certain volume of the toxin stock standard solution is diluted with methanol to the volume to prepare multitoxin working standard solutions for the calibration curve.
These solutions can be used for 1 week, being stored in a freezer (< -20ºC) when not in use.
A longer storage time is allowed if the stability has been proven in the laboratory.
<table>
<tr><td>Stock standard solution (µl)</td><td>Solvent (µl)</td><td>OA & AZA1 concentration (ng/ml)</td><td>Calibration standard</td></tr>
<tr><td>15</td><td>985</td><td>3</td><td>Std 1</td></tr>
<tr><td>30</td><td>970</td><td>6</td><td>Std 2</td></tr>
<tr><td>50</td><td>950</td><td>10</td><td>Std 3</td></tr>
<tr><td>100</td><td>900</td><td>20</td><td>Std 4</td></tr>
<tr><td>150</td><td>850</td><td>30</td><td>Std 5</td></tr>
<tr><td>200</td><td>800</td><td>40</td><td>Std 6</td></tr>
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</td></tr>
<tr><td>Sample Preparation</td><td>
Raw samples have to be thoroughly cleaned outside of the shellfish with fresh water. Open by cutting adductor.
Rinse inside with fresh water to remove sand and foreign material. Remove meat from shell by separating adductor muscles and tissue connecting at hinge. Do not use heat or anesthetics before opening the shell.
After removal from shellfish, drain tissues in a sieve to remove salt water.
For representative sampling, at least 100-150 g of pooled tissue should be homogenized in a blender or homogenizer.
Sub-samples from this homogenate can be taken immediately after blending, while still well mixed, or after mixing again.
Sample Extraction:
Accurately weigh 2.00 g ± 0.05 g of tissue homogenate into a centrifuge tube.
Add 9.0 ml of 100% methanol and homogenize the sample via vortex mixing for 3 min at maximum speed level.
Centrifuge at 2000 g or higher for 10 min at approx. 20ºC and transfer the supernatant to a 20 ml volumetric flask. Repeat the extraction of the residual tissue pellet with another 9.0 ml of methanol 100% and homogenize for 1 min.
After centrifugation (at 2000 g or higher for 10 min and approx. 20ºC), transfer and combine the supernatant with the first extract and make up the extract to 20 ml with 100% methanol.
</td></tr>
<tr><td>Method of analysis</td><td>
Free OA and AZA group toxins analysis:
The determination of free OA & AZA toxins is performed after filtering an aliquot of the methanolic extract through a dry
</td></tr>
</table>

methanol-compatible 0.45 µm or 0.2 µm syringe filter and injecting between 5 µl and 20 µl, depending on sensitivity of instrument, onto LC-MS system.

Hydrolysis:

- In order to detect and quantify the total content of OA/DTX toxins an alkaline hydrolysis is required before LC-MS/MS analysis.
- In a test tube, add 313 µl of NaOH 2.5 M to 2.5 ml of methanolic extract, homogenize using a vortex mixer for 0.5 minutes and heat the mixture using a heating block or water bath set at 76 ºC for 40 minutes.
- Cool to room temperature, neutralize with 313 µl of HCl 2.5M and homogenize in vortex for 0.5 minutes.
- Filter this extract through a dry methanol-compatible 0.45 µm or 0.2 µm syringe filter and inject 5 µl -20 µl onto the LC column.
- Cleanup is to be used, if necessary, to eliminate matrix effects. Possible options: liquid-liquid partitioning, SPE, etc. If this approach is used, the recovery of this step must be individually evaluated and reported by the laboratory.

Chromatographic conditions:

(i)

Column	BDS- Hypersil C8, 50mm (length) x 2mm (diameter), 3µ particle size		
Flow	0.2 ml/min		
Injection volume	5-10 µl (depending on MS sensitivity)		
Column temp	25-40 ˚C		
Gradient	Time	%A	%B
	0	70	30
	8	10	90
	11	10	90
	11.5	70	30
	17	70	30

(ii)

Column	X-Bridge C18, 50 mm (length) x 2.1 mm (diameter), 2.5 µm particle size		
Flow	0.3 ml/min		
Injection volume	5-20 µl (depending on MS sensitivity)		
Column temp	25 ˚C		
Gradient	Time	%A	%B
	0	90	10
	4	20	80
	6	20	80
	6.5	90	10
	9	90	10

MS Parameters:

MS Parameters	OA & AZA Group
Curtain Gas (CUR)	20 psi
Collision Gas (CAD)	Medium
Voltage (IS)	4500 V
Temperature (TEM)	650 ˚C
Gas 1 (GS1)	40 psi
Gas 2 (GS2)	60psi

Fragmentation Conditions:

Compound	ESI	Q1	Q3	Mseg	DP (v)	EP (v)	CEP (v)	CE (v)	CXP (v)
OA	-ve	803.5	255.0	125	-120	-10	-28	-62	-2
OA	-ve	803.5	113.0	125	-120	-10	-28	-60	-2
DTX-2	-ve	803.5	255.0	125	-120	-10	-28	-62	-2
DTX-2	-ve	803.5	113.0	125	-120	-10	-28	-60	-2
DTX-1	-ve	817.5	255.0	125	-120	-10	-28	-62	-2
DTX-1	-ve	817.5	113.0	125	-120	-10	-28	-60	-2
AZA-1	+ve	842.5	824.5	35	81	4.5	64	55	6
AZA-1	+ve	842.5	806.5	35	81	4.5	64	55	6
AZA-2	+ve	856.5	838.5	35	81	4.5	76*	55	8
AZA-2	+ve	856.5	820.5	35	81	4.5	76*	55	8
AZA-3	+ve	828.5	810.5	35	81	4.5	68*	55	6
AZA-3	+ve	828.5	792.5	35	81	4.5	68*	55	6

Calculation with units of expression

The concentration of each of the analytes can be calculated from the graph using the equation:

y=mx+C (as obtained from the graph),

where,

y – Signal/Area given by the standards.

x – Known concentration of the standard used for calibration.

m – Slope of the curve

C – Intercept

The result is calculated based on the calibration curve and then multiplied by the dilution factor (if dilution is conducted during preparation).

Reference	LC-MS/MS (EU-Harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS).
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Domoic Acid (ASP) in Molluscs		
Method No.	FSSAI 06.033:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to Live and raw bivalve molluscs and Frozen clam meat as specified in Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011.		
Caution	1) Extract and analyze the test portion on the same day. Refrigerate test samples and extracts when not being handled. 2) Domoic acid in acidic extracts slowly decomposes if left at room temperature.		
Principle	Domoic acid is extracted from homogenized mussel tissue by boiling for 5 mins with 0.1M HCl. Mixture is cooled and centrifuged and an aliquot of the supernatant is diluted, filtered and analyzed by isocratic LC and UV detection at 242 nm with mobile phase of CH_3-CN-H_2O acidified to pH ca 2.5.		
Apparatus/ Instruments	a) Liquid Chromatograph: With injection valve, solvent delivery system, recording integrator, variable wavelength UV detector. b) LC column: Stainless steel, 15 cm x 4.6mm id, packed with reversed phase C18, 5µm material. Column brand does not affect analysis if CH_3CN concentration is adjusted in mobile phase. c) Membrane filters: Disposable, plastic-sealed mini-filters with Leur – Lok hub, 0.45 µm (3cm diameter), attached to 5ml glass or disposable plastic syringe. [Millex HV (Millipore corp.) meets these specifications.] d) Centrifuge: High speed, with timer. Capable of 3000 rpm using 100ml centrifuge tubes.		
Materials and Reagents	• Acetonitrile (CH_3CN): LC Grade • Hydrochloric acid (HCl): 0.1M • Mobile Phase • Domoic acid standard solution: 1.09 ng/µL		
Preparation of Reagents	1) **Mobile Phase:** Add 2ml of 8.5% Aqueous H_3PO_4 to 873 ml Deionized Water and vortex mix & check to ensure pH is 2.4. Add 125 ml CH_3CN, mix and degas. Perform preliminary analysis of Domoic Acid Standard and adjust CH_3CN concentration as necessary to give Domoic Acid Retention Time Ca 8min (K', Ca 6) under method condition. 2) **Domoic Acid Standard Solution:** 1.09 Ng/µl. Aqueous Solution of Domoic. Refrigerate when not in use. Warm to room temperature before use.		
Sample Preparation	1) Clams, oysters and mussels: • Thoroughly clean outside of shellfish with fresh water. Open by cutting adductor muscles. • Rinse inside with fresh water to remove sand or other foreign material. Remove meat from shell by separating adductor muscles and tissue connecting at hinge. • Do not use heat or anaesthetize before opening shell, and do not cut or damage body of mollusk at this stage. • Collect ca 100-150g meats in a glazed dish.		

	• As soon as possible transfer meats to No. 10 sieve without layering, and let it drain for 5min. • Pick out the pieces of shell and discard drainings. • Grind in household- type grinder with 1/8 in– ¼ in. (3-6 mm) holes or in blender until homogenous. 2) Scallops: • Separate edible portion (adductor muscle) and apply test to this portion alone. Drain and grind as has been done for Clams, Oysters and Mussels. 3) Canned Shellfish: • Prepare by blending
Method of analysis	1) Inject replicate 20 µl portions of domoic acid standard solution into LC system until peaks (measured as height or area) for three consecutive injections do not vary by more than 3%. 2) Ensure baseline resolution of L- tryptophan from domoic acid; adjust mobile phase composition accordingly. 3) Make alternate, duplicate injections of test solution from D and standards. 4) Determine recoveries of domoic acid at 20 µg/g level.
Calculation with units of expression	Calculate results as follows: **Domoic acid, µg/g =** $\frac{R}{R'} \times \frac{W'}{W}$ Where, **R**: Average peak heights or areas of test solutions **R'**: Average peak heights or areas of standards **W**: Weights injected of test portion (mg) **W'**: Weights injected of Standard (ng)
Reference	JAOAC **49** (1999), Official method 991.26, p. 91
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Brevetoxins in Molluscs		
Method No.	FSSAI 06.034:2023	**Revision No. & Date**	0.0
Scope	Neurotoxic shellfish poisoning (NSP) toxins (i.e. brevetoxins) are a group of marine biotoxins produced by the marine dinoflagellate, Kareniabrevis(formerly known as *Gymnodiniumbreve*and *Ptychodiscusbrevis*) . The phytoplanktonic toxins can be accumulated in filter feeding shellfish and other seafood.Toxins included in the analysis were PbTx-1, PbTx-2 and PbTx-3;. This method is specifically used for detection in: • Live and raw bivalve molluscs • Frozen clammeat		
Caution	1) All standards should be handled carefully, avoiding contact with eyes and should not be ingested. 2) All instruments used should be calibrated on a routine basis for sensitivity and accuracy. 3) Prepare matrix matched calibration standards freshly before use.		
Principle	An analytical method using LC-MS/MS for the determination of neurotoxic shellfish poisoning (NSP) toxins (brevetoxins) in shellfish using solvent extraction, SPE clean-up, chromatographic separation and mass spectrometric detection [multiple reaction monitoring (MRM)].		
Apparatus/ Instruments	1) SPE cartridge - Strata-X (33 mm, 3 mL, 60 mg) 2) HPLC - Nanospace SI-2 HPLC system 3) Column specifications - XB-C18 100 Å column (100mm 2.1mm i. d., 2.6 mm) which was maintained at 35 ˚C. 4) Mass spectrometric system - Triple Quad 4500 LC-MS/MS system equipped with an electrospray ionization (ESI) source.		
Materials and Reagents	1) Standard of PbTx-1 2) Standards of PbTx-2 and PbTx-3, 3) Acetonitrile (HPLCgrade). 4) Methanol (HPLCgrade). 5) Formicacid (_98%) 6) Water (LCMS Grade)		
Preparation of Reagents	**Calibration:** 1) Dissolve each of the Brevetoxin standard in methanol. This gives stock solutions of 10,000 ng/mL for PbTx-1, PbTx-2 and PbTx-3, respectively. 2) Prepare the mixed stock solutions by mixing appropriate volumes of the individual stock solutions. 3) The concentration of the mixed stock solution is 1000 ng/mL for all the toxins. Store the stock solutions and mixed stock solution at -20˚C in darkness. 4) Prepare matrix matched calibration standards by the addition of known amounts of mixed stock solution to appropriate volumes of the extracts which had not been contaminated with Brevetoxins. 5) Prepare the blank matrix extracts in the same way as described in preparation.		
Sample Preparation	1) Poole the edible portions, homogenize and store in the freezer (-20˚C) until analysis.		

2) Weigh an aliquot of 5g of homogenized sample into a 50 mL graduated polyethylene tube and extract with 5mL of 80% methanol. Vortex the solution for 1 min.
3) After extraction, heat the suspension in a water bath at 60˚C for 20 min and centrifuge the hot solution at 3000 rpm for 20 min.
4) Decant the supernatant into a 50 mL tube and store in the freezer (-20˚C) for 1 h. Filter the supernatant through a 0.45 mm nylon syringe filter.
5) The filtered crude extract (about 4.5 mL) is used for the solid phase extraction (SPE) clean-up.

Solid phase extraction:

1) Purify the crude extract on a SPE cartridge prior to LC-MS/MS analysis.
2) Condition the SPE cartridge previously with 3mL of 25% methanol.
3) Pass 3mL of crude extract through the SPE cartridge and then, wash the cartridge with 4mL of 25% methanol.
4) Elute the components in the cartridge using 3mL of acetonitrile.
5) The resulting solution is passed through a 0.22 mm nylon syringe filter for LC-MS/MS analysis.

Method of analysis

Perform the Chromatographic analysis using the following conditions.

1) Column Conditions:

- Injection volume: 10ml
- Column temperature: 35˚C
- Mobile phase A: 0.1% formic acid/ water, v/v
- Mobile phase B: 100% acetonitrile
- Flow rate: 200 mL/min

2) Gradient:

Time (min)	%A	%B
0.0	60	40
2.0	10	90
5.0	10	90
6.0	40	60
6.1	60	40
10.0	60	40

3) Optimized MS/MS conditions:

- ESI: +ve, multiple ion monitoring (MRM)
- Ion spray (IS) voltage: 5500 V
- Curtain gas: 20 psi
- Nebulizer gas (GS1): 50 psi
- Heating gas (GS2): 50 psi
- Source temperature: 450 ˚C
- Nebulizer and collision gas: Nitrogen

4) MRM transitions:

Toxins	M.W (g/mol)	Precursor Ion (m/z)	Product Ion(m/z)	DP (V)	EP (V)	CP (V)	CXP (V)
PbTx-1	866	$[M+H]^+$ 867	**849***	140	10	21	20
			831	140	10	25	18
PbTx-2	894	$[M+H]^+$ 895	**877***	161	10	23	10
			859	161	10	31	10
PbTx-3	896	$[M+H]^+$897	**725***	130	10	31	6

	<table><tr><td></td><td></td><td></td><td>879</td><td>130</td><td>10</td><td>22</td><td>6</td></tr></table> *denotes the Quantification ion.
Calculation with units of expression	Calculate the concentration of each analyte from the graph using the equation: **y=mx+C** (as obtained from the graph), where, **y** – Signal/Area given by the standards. **x** – Known concentration of the standard used for calibration. **m** – Slope of the curve **C** – Intercept Calculate the result based on the calibration curve and then multiply by the dilution factor (if dilution is conducted during preparation).
Reference	Choonshik Shin, Jeong-Yun Hwang, Jin-Hong Yoon, Sheen-Hee Kim, Gil-Jin Kang, "Simultaneous determination of neurotoxic shellfish toxins (brevetoxins) in commercial shellfish by liquid chromatography tandem mass spectrometry, Food Control, Vol 91, 2018, pg 365-371, ISN 0956-7135
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Polyaromatic Hydrocarbons in Fish & Fish Products		
Method No.	FSSAI 06.035:2023	**Revision No. & Date**	0.0
Scope	Applicable for the determination of the PAHs (acenaphthylene, acenaphthene, anthracene, benz[*a*]anthracene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*g,h,i*]perylene, benzo[*k*]fluoroanthene, chrysene, dibenz[*a,h*]anthracene, fluoranthene, fluorene, indeno [1,2,3-*cd*] pyrene, naphthalene, phenanthrene and pyrene) in: • Live and raw bivalve molluscs • Frozen clammeat • Raw/ chilled/ frozen finfish The maximum residue limit has been specified only for benzopyrene in: • Smoked fishery products		
Caution	1) Poly aromatic hydrocarbons metabolizes through photo oxidation. Hence the standard solution and the samples should be protected from direct exposure to light.		
Principle	Method uses a single-step ethyl acetate extraction and salting out liquid-liquid partitioning from water in the sample with Magnesium sulphate ($MgSO_4$) and Sodium chloride (NaCl). Cleanup is done to remove co-extracted fat with Silica SPE cartridge. The cleaned extracts are then analyzed by GC-MS/MS in multiple reaction monitoring method (MRM).		
Apparatus/Instruments	1) Polypropylene centrifuge tubes- 50ml 2) Polypropylene centrifuge tubes – 15ml 3) Vortex 4) Centrifuge 5) Gas chromatograph – tandem mass spectrometer		
Materials and Reagents	1) Ethyl acetate 2) Magnesium Sulphate ($MgSO_4$, analytical grade) 3) Sodium Chloride (NaCl, analytical grade) 4) Dichloromethane 5) Iso-octane 6) Hexane		
Preparation of Reagents	1) **Silica gel SPE column**- containing 1g silica gel. Any commercially available silica gel SPE cartridge can be used as long as it provides adequate fat cleanup and meets requirements for low background contamination specified by laboratory qualification requirements. The concentration of all analytes in the reagents had to be below the concentration in the lowest calibration level standard (equivalent to 5ng/g naphthalene in the sample) are still acceptable if the source of contamination could be eliminated. Silica gel SPE columns can be prepared in-house using the following procedure. Activate silica by heating at 180 °C for 5h and deactivate by adding 5% deionized water, shaking for 3h, store in a desiccator for 16h before use(silica gel prepared and stored as directed can be used for 14 days). Place a piece of deactivated		

<table>
<tr><td></td><td>glass wool in a Pasteur pipette (5ml), add 1g activated silica gel (Silica gel 60, 0.063-0.2mm, 70-230 mesh or equivalent) and top it with 0.2g muffled anhydrous Na_2SO_4.
2) Anhydrous magnesium sulphate ($MgSO_4$) - ≥99.0% powder heated (muffled) at 600C for 7h, and then store in a desiccator before use ($MgSO_4$ prepared and stored as recommended can be used for 1 month). Note: A pre weighed (commercially available) mixture of 2g sodium chloride and 4g anhydrous magnesium sulphate (muffled) in pouches or tubes can be used.</td></tr>
<tr><td>Sample Preparation</td><td>Extraction and cleanup:
1) Take 10 g homogenized tissue in a 50 ml centrifuge tube. Add 10 ml distilled water to it and vortex for 1 min.
2) Add 10 ml of ethyl acetate and vortex for 1 min.
3) Add 6 g of $MgSO_4$, 2 g of NaCl and vortex for 3 min. Centrifuge at 4000 rpm for 5 min.
4) Collect the supernatant in 15 ml tube & cool at -20 ˚C for 30 min.
5) Collect 2 ml supernatant, add 50 µl isooctane as keeper and evaporate in a nitrogen evaporator system
6) Reconstitute the residue in 1 ml hexane and load in a silica SPE cartridge previously equilibrated with 4 ml Hexane:DCM (3:1) and 3 ml hexane.
7) Elute the SPE cartridge with 10 ml Hexane: DCM (3:1).
8) Add 2 ml of ethyl acetate and 200 µl isooctane as keeper and evaporate in nitrogen evaporator system.
9) Reconstitute the residue in 1 ml ethyl acetate.
10) Filter through PTFE syringe filter and vial for analysis.</td></tr>
<tr><td>Method of analysis</td><td>Detection and estimation by GC- MS/MS:
GC conditions:
● Injection volume: 5 µl (Large volume, preferably in a PTV injector)
● GC Oven Programs

<table>
<tr><th>Rate(^{0}c/min)</th><th>Temperature(^{0}c)</th><th>Hold Time(min)</th></tr>
<tr><td>0</td><td>60.0</td><td>2.00</td></tr>
<tr><td>25.0</td><td>150.0</td><td>0.00</td></tr>
<tr><td>3.0</td><td>200.0</td><td>0.00</td></tr>
<tr><td>8.0</td><td>290.0</td><td>4.00</td></tr>
<tr><td>8.0</td><td>310.0</td><td>1.00</td></tr>
</table>
● Injection mode: PTV - Large volume
● Carrier mode : Constant flow

● Inlet
Temperature: 50°C
Split flow : 50.0ml/min
Split less time: 2.00 min
● Carrier mode: Programmed pressure
<table>
<tr><th>Rate (kPa/min)</th><th>Pressure (kPa)</th><th>Hold Time (min)</th></tr>
<tr><td></td><td>110.00</td><td>2.00</td></tr>
</table>
</td></tr>
</table>

5.00	130.00	35.00	

PTV PROGRAMME

	Rate (°C/s)	Temperature(°C)	Time (min)	Flow (ml/min)
Injection			0.01	20.0
Evap.	14.5	90	0.08	30.0
Transfer	5.00	300	25.00	
Cleaning	14.5	330	10.00	80.0

MRM Conditions:

Name	**Parent ion**	**Product Mass**	**Collision Energy**
Acenaphthalene	151.9	125.8	24
Acenaphthalene	151.9	150	28
Acenaphthene	152.8	152.2	18
Acenaphthene	154.1	153.1	16
Fluorene	165	163	30
Fluorene	166.1	165.1	16
Phenanthrene	178	150.9	28
Phenanthrene	178	151.6	22
Anthracene	178	151	32
Anthracene	178	151.7	20
Fluoranthene	202	200	25
Fluoranthene	202	202	20
Pyrene	202.1	200	36
Pyrene	203.3	201	36
Benz(a)anthracene	225.9	224.1	34
Benz(a)anthracene	228	226	28
Chrysene	225.9	200	28
Chrysene	229.2	227.1	30
Benzo(b)fluoranthene	126.1	113	12
Benzo(b)fluoranthene	252.1	250.1	32
Benzo(k)fluoranthene	250	248	32
Benzo(k)fluoranthene	252.1	250	34
Benzo(a)pyrene	250	248	36
Benzo(a)pyrene	252.1	250	34
Indeno(1,2,3cd)pyrene	276.2	276.2	10
Indeno(1,2,3cd)pyrene	277.2	275.1	35
Dibenzo(a,h)anthracene	278.2	276.1	30
Dibenzo(a,h)anthracene	278.2	278.2	10
Benzo(g,h,i)perylene	276.1	274.1	38
Benzo(g,h,i)perylene	276.1	274.6	18

Calculation with units of expression

The analyte concentrations in the final extract (c_{PAH}, µg/L) are determined from the equation:

$$c_{PAH} = \frac{[\frac{S_{PAH}}{S_{13C-PAH}}] - b}{a}$$

Where,

	a is the slope of the calibration curve ***b*** is the y- intercept. The concentration of PAHs in the sample (C, µg/kg) is then calculated: $$\mathbf{C} = \frac{C_{PAH}}{C_{13C-PAH}} \times \frac{X_{13C-PAH}}{m}$$ Where, $\mathbf{C_{13C\text{-}PAH}}$is the concentration of the corresponding **^{13}C- PAH** in calibration standard solutions (in µg/L); **X ^{13}C-PAH**. A calibration curve was obtained and the curve was extrapolated to find the unknown concentrations
Reference	[1] J. AOAC *Int.***81**, 1011(1998) [2] J. AOAC *Int.***83**, 933(2000) [3] AOAC 2014.08: 2016 [4] AOAC 2014.08: 2019
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Polychlorinated Biphenyls (Sum of PCB28, PCB52, PCB101, PCB138, PCB153 and PCB180) in Fish & Fish Products		
Method No.	FSSAI 09.036:2021	**Revision No. & Date**	0.0
Scope	The method is applicable to: • Raw/ chilled/ frozen finfish • Live and raw bivalve molluscs • Frozen clam meat • Smoked fishery products		
Caution	1) The GC-MS/MS is an extremely sensitive instrument which needs to be handled with safety and precaution, following all the protocols associated with it. 2) All the injection volumes and concentration limits are to be maintained for the stable working of the instrument. 3) Routine calibrations along with checkups are to be performed for accurate and sensitive detection and analysis of the compounds.		
Principle	The QuEChERS (quick, easy, cheap, effective, rugged and safe) method uses a single step buffered acetonitrile (MeCN) extraction and salting out liquid-liquid partitioning from water in the sample with $MgSO_4$ and Sodium acetate. Cleanup is done to remove organic acids, excess water, and other components with a combination of primary secondary amine Z-Sep Florisil, C_{18}, $CaCl_2$ and $MgSO_4$. The extracts are then analyzed by Gas Chromatography – tandem mass spectrometry.		
Apparatus/ Instruments	1) Polypropylene centrifuge tubes -50 ml 2) Polypropylene centrifuge – 15 ml 3) Vortex 4) Centrifuge 5) Gas Chromatograph – tandem mass spectrometer		
Materials and Reagents	1) Acetic acid 2) Anhydrous Magnesium Sulphate ($MgSO_4$) 3) Sodium Acetate (CH_3COONa) 4) Acetonitrile (HPLC grade) 5) C_{18} 6) Z- Sep$^+$ 7) Anhydrous Calcium Chloride ($CaCl_2$) 8) Ethyl acetate		
Preparation of Reagents	Prepare standards of PCBs (PCB28, PCB52, PCB101, PCB138, PCB153 and PCB180) for calibration in a range of 5ppb, 10ppb, 20ppb, 40ppb, 80ppb all in ng/ml. All the analytes were prepared in these same concentrations together.		
Sample Preparation	1) Received samples are coded, filed and immediately stored in -20 °C freezer until further processing 2) Within three days of receiving the sample. 3) Analysis to be done in duplicate. 4) Take 5 g of homogenized tissue in a 50 ml centrifuge tube. 5) Add 10 ml of distilled water to it and vortex for 1 min.		

	6) Add 15 ml of Acetonitrile (1% of acetic acid) and Vortex for 1 min. 7) Add 6 g of MgSO4, 2 g of sodium acetate and vortex for 3 min 8) Centrifuge at 4000 rpm for 5 min. 9) The collected supernatant is kept at -20 °C for 30 min. 10) Take 1 ml of cooled acetonitrile supernatant from this and add 150 mg $CaCl_2$, vortex for 3 min and centrifuge at 10000 rpm for 10 min. 11) Take 1.5 ml of supernatant and add to another Eppendorf tube containing 50 mg Z-Sep, 150mg C_{18}, 150 mg $CaCl_2$ and 150 mg $MgSO_4$. 12) Vortex for 1 min, followed by centrifuge at 10000 rpm for 10 min. 13) Collect 1 ml of supernatant and evaporate using Nitrogen evaporator after adding 100 µl toluene. 14) Filter 1 ml with PTFE syringe filter and vial for GC- MS/MS analysis.
Method of analysis	Detection and estimation is done by GC- MS/MS: 1) Inject suitable aliquots into a gas chromatograph operated normally. Measure peaks (height area). If necessary, dilute sample to give residue concentration ca that of standard solution. 2) Inject aliquot of PCB standard solution (in same solvent as extract) and again measure peaks. *GC conditions:* Injection volume: 5 µl ((Large volume, preferably in a PTV injector) ● GC Oven Programs

Rate(^{0}C/min)	Temperature(^{0}C)	Hold Time(min)
0	60.0	2.00
25.0	150.0	0.00
3.0	200.0	0.00
8.0	290.0	4.00
8.0	310.0	1.00

- Injection mode: PTV - Large volume
- Carrier mode : Constant flow
- Inlet

  Temperature: 50°C

  Split flow : 50.0ml/min

  Split less time: 2.00 min
- Carrier mode: Programmed pressure

Rate (kPa/min)	Pressure (kPa)	Hold Time (min)
	110.00	2.00
5.00	130.00	35.00

- PTV PROGRAMME

	Rate (°C/s)	Temperature(°C)	Time (min)	Flow (ml/min)
Injection			0.01	20.0
Evap.	14.5	90	0.08	30.0
Transfer	5.00	300	25.00	
Cleaning	14.5	330	10.00	80.0

MRM Conditions:

Name	**Parent ion**	**Product Mass**	**Collision Energy**
PCB 101	254	184	30
PCB 101	325.9	254	20
PCB 101	325.9	255.9	25
PCB 138	360	287.9	25
PCB 138	360	289.8	25
PCB 138	360	324.9	10
PCB 153	357.84	287.88	25
PCB 153	359.9	289.7	30
PCB 153	361.9	289.9	35
PCB 180	323.9	253.8	30
PCB 180	391.81	321.84	25
PCB 180	393.9	323.8	30
PCB 28	256	150.1	50
PCB 28	256	151.1	25
PCB 28	256	186	40
PCB 52	292	220	25
PCB 52	292	257	25
PCB 52	292	222	10

Calculation with units of expression

Stock: PCB mixture (PCB28, PCB52, PCB101, PCB138, PCB153 and PCB180) 100 µg/ml.

Matrix based calibration standards: 2, 4, 8, 16, 32, and 64 ng/ml.

$$\textbf{Each residue, ppm (µg/ml)} = \frac{C_S \times A \times V_S \times V_D}{V_E}$$

Where,

$\mathbf{C_s}$ = Concentration of standard(µg/ml)

A = peak size of analyte

$\mathbf{V_S}$ = Volume of standard

$\mathbf{V_E}$= Volume of extract

$\mathbf{V_D}$= Dilution volume/ 1.0g of test portion

Reference	AOAC -2007.01: 2016
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Methyl Mercury in Fish & Fish Products		
Method No.	FSSAI 06.037:2023	**Revision No. & Date**	0.0
Scope	This method is applicable to: • All Fish & Fish Products		
Caution	5) Methylmercury in extraction solution decomposes over time. To ensure accurate quantification of methylmercury, extracts must be analyzed within 8 h of preparation. 6) To assist homogenization of the analytical sample, reagent water ≤20% of the mass of seafood may be added, if its addition provides a more visually homogenous and easier to-manipulate material. If reagent water is added to assist homogenization, record to 4 significant figures the weights of edible portion and reagent water that are combined to prepare the analytical sample and apply mass correction factor (MCF) in calculation of concentration of analyte in analytical portion. Reserve a portion of reagent water used for homogenization to prepare method blanks. 7) Baseline resolution between inorganic and methylmercury peaks should be verified and that peaks are not tailing excessively before start of analysis. Verify that peak area standard deviation is less than 5%. 8) Absence of instrument carryover should be verified.		
Principle	This method describes procedures for analysis of methyl mercury and total mercury in fish and fishery products using high performance liquid chromatography (HPLC) and inductively couple plasma-mass spectrometry (ICP-MS). Total mercury in this method is calculated as the sum of inorganic and methylmercury determined in analytical solution. Other matrices may be analyzed by these procedures if performance is verified in the matrix of interest, at the concentration levels of interest.		
Apparatus/ Instruments	7) Inductively coupled plasma-mass spectrometer—Capable of measuring mass-to-charge (m/z) ratio 202 in time resolved (chromatographic) mode. Equipped with Mist nebulizer, and quartz, Scott-type, double-pass spray chamber maintained at 2 °C. Instrument should electronically interface with or can be configured to remote start by standard HPLC instruments for integrated operation. HPLC-ICPMS of any vendor with equivalent feature is suitable for use. 8) High performance liquid chromatograph 9) HPLC analytical column— C-18, 250 x 4.6 mm, 5 µm particle size or equivalent. 10) Glass vials for extracting analytical samples—Amber, borosilicate glass vials, 60 mL capacity, with screw caps. 11) Heated water bath—Capable of temperature control with sufficient water and thermal capacity to allow immersion of extraction vials to cap level and maintain water temperature at 60 ± 4 °C for 120 minutes. 12) Syringe for filtering extracts—Disposable, general use and non-sterile. 13) Syringe filters for filtering extracts—Disposable, 0.45 µm polypropylene membrane with polypropylene housing.		
Materials and Reagents	5) Reagent water—Water that meets specifications for Type I water 6) Methylmercury (II) chloride—CH_3HgCl crystals, purity ≥ 95% 7) Mercury (II) chloride—$HgCl_2$ crystals, ACS grade		

	8) L-cysteine hydrochloride monohydrate (L-cysteine.HCl.H_2O)—Purity > 98.5% 9) L-cysteine (free base)—Purity ≥ 99.8%. 10) Extraction solution, [aqueous 1% (w/v)] 11) Cysteine solution [aqueous 10% (w/v)] 12) Mobile phase, aqueous 0.1% (w/v) 13) Methylmercury stock solution. 14) Inorganic Hg stock solution 15) Multi-analyte intermediate solution 16) Multi-analyte working standard solution. 17) Check solution 18) Independent check solution (ICS)
Preparation of Reagents	3) **Extraction solution, aqueous 1% (w/v) L-cysteine.HCl.H_2O—** Dissolve 10 ± 0.1 g L-cysteine.HCl.H_2O crystals in 1000 ± 10 mL reagent water. 4) **Cysteine solution (aqueous, 10%) (for preparation of standard solutions), (w/v)**: Dissolve 5 ± 0.05 g L-cysteine.HCl.H_2O crystals in 50 ± 0.5 mL reagent water. 5) **Mobile phase [aqueous 0.1% (w/v)]**: Dissolve 0.5 ± 0.01 g L-cysteine and 0.5 ± 0.01 g L-cysteine.HCl.H_2O in 500 ± 5 mL reagent water. 6) **Methylmercury stock solution, [(CH_3HgCl in H_2O that may contain up to 20% (v/v) methanol), (Hg=1000 mg/L)]:** Tare 100-mL volumetric flask on analytical balance in chemical fume hood. Weigh 0.1252 g CH_3HgCl (FW=251.08) in flask with stopper in place. Add ≤ 20 mL methanol and swirl stoppered flask to dissolve CH_3HgCl. Dilute to 100.0 mL with reagent water. Discard solution in which inorganic Hg is > 3% of the theoretical methylmercury concentration. 7) **Inorganic Hg stock solution [$HgCl_2$ in 0.1% (v/v) HCl, Hg = 2000 mg/L]**: Tare 50-mL polypropylene centrifuge tube. Weigh 0.1354 g HgCl2 (M.W = 271.50) in tube. Add 5.0 ± 0.1 mL 1% (v/v) HCl and swirl to dissolve. Dilute to 50.0 ± 0.5 mL with reagent water. 8) **Multi-analyte intermediate solution, Hg due to CH_3HgCl = 1000 µg/L and Hg due to $HgCl_2$ = 1000 µg/L in 0.02% (w/v) L cysteine.HCl.H_2O**: Mix approximately 40 mL reagent water and 0.1 mL 10% (w/v) L-cysteine.HCl.H_2O in 50-mL polypropylene tube. Add 50.0 µL methylmercury stock solution and 25.0 µL inorganic Hg stock solution. Dilute to 50.0 ± 0.5 mL with reagent water. 9) **Multi-analyte working standard solution, [Hg due to CH_3HgCl = 1 µg/L and Hg due to $HgCl_2$ = 1 µg/L in 1% (w/v) L-cysteine.HCl.H_2O]**: Mix approximately 40 mL reagent water and 5.0 ± 0.05 mL 10% (w/v) L-cysteine.HCl.H_2O in 50-mL polypropylene tube. Add 50.0 µL multi-analyte intermediate solution. Dilute to 50.0 ± 0.5 mL with reagent water. Mix and immediately transfer a portion to glass HPLC autosampler vial(s) for storage before use.

<table>
<tr><td></td><td>10) Check solution:
Use multi-analyte working standard solution for the check solution.
11) Independent check solution (ICS):
Prepare independent inorganic and methylmercury stock solutions, and independent multi-analyte intermediate and working standard solutions according to steps (4) – (7) from a different starting material than that used to prepare the primary stock solutions. Use of a commercial source material with a different lot number is acceptable, but a source material from a different manufacturer is preferred.</td></tr>
<tr><td>Sample Preparation</td><td>4) Weigh analytical portion into 60-mL amber glass extraction vial and determine mass of analytical portion. Generally, weigh 0.5 ± 0.1 g edible portion of fish and fish product. Use 0.2 ± 0.01 g for reference materials.
5) Add 50.0 ± 0.5 mL extraction solution (aqueous 1% (w/v) L-cysteine.HCl.H_2O) to extraction vials, cap tightly, and shake vigorously by hand.
6) Heat extract vials for 120 ± 5 min in water bath at 60 ± 4 °C. Shake each vial vigorously by hand after 60 minutes of heating and again after 120 minutes of heating.
7) Remove extraction vials from water bath and allow cooling to room temperature.
8) Filter a portion of extract through 0.45 µm filter directly into HPLC auto sampler vial.</td></tr>
<tr><td>Method of analysis</td><td>HPLC column: 5µ C18, 100Å, 250 × 4.60 mm
HPLC conditions:
<table><tr><th colspan="3">Inlet</th></tr><tr><td colspan="2">Mobile Phase</td><td>0.1% L-Cysteine mixture</td></tr><tr><td colspan="2">Flow Rate (mL/min)</td><td>1.5</td></tr><tr><td colspan="2">Flow type</td><td>Isocratic</td></tr><tr><td colspan="2">Injection Volume (µL)</td><td>20</td></tr><tr><td colspan="2">Run time (mins)</td><td>6.6</td></tr><tr><td rowspan="2">Retention time (min. approx.)</td><td>Inorganic Hg</td><td>2 min</td></tr><tr><td>Methyl Hg</td><td>3.96 min</td></tr></table>
Instrument: ICP-MS
Mass: 201.9706 (Hg)

ICP-MS Conditions:
<table><tr><th>Condition</th><th>Setting</th></tr><tr><td>R F Power (W)</td><td>1550</td></tr><tr><td>Plasma Gas Flow Rate (L/Min)</td><td>15</td></tr><tr><td>Auxiliary Gas flow rate (L/min)</td><td>0.9</td></tr></table></td></tr>
</table>

Nebulizer Gas Flow Rate (L/min)	1.2
Sampling Depth (mm)	8
Peristaltic Pump Speed (rps)	0.2
Spray Chamber Temperature (°C)	2
Isotope (mass-to-charge ratio)	202
Integration time (sec/point)	1
Total acquisition time (sec)	300
Reaction/ collision cell mode	OFF
Dwell time (s)	0.1
Tolerance	10.0

1. Tune the instrument using the above-mentioned parameters and condition using several water & solvent blanks.
2. Plot a standard curve in the concentration range 1, 2, 5, 10, 20 and 40 µg/kg using the working standard solution prepared previously.

Note: Any vendor instrument can be used and instrument specific parameters have to be optimized by the implementing lab.

Calculation with units of expression

Calculation of Response factor of analyte, RF (cps-s/µg/L)

$$RF = \frac{A_{std-ave} - A_{es-ave}}{C_{std}}$$

Where,

$A_{std\text{-}ave}$ = average peak area of n > 2 injections of standard solution(s) (cps-s). $A_{es\text{-}ave}$ = average peak area of n > 2 injections of extraction solution (cps-s) (0 if no peak is detected).

C_{std} = analyte concentration (µg/L) in standard solution(s).

Calculation of concentration of analyte (inorganic mercury or methyl mercury) in analytical solution, S (µg/L):

$$S = \frac{A_{as} - A_{es-ave}}{RF}$$

Where,

A_{as} = peak area of analyte in analytical solution (cps-s).

$A_{es\text{-}ave}$ = average peak area of analyte in extraction solution (cps-s) (0 if no peak is detected).

RF = response factor of analyte (cps-s per µg/L).

Calculation of concentration of total Hg in analytical solution, S_T (µg/L):

$$S_T = S_{inorg} + S_{methyl}$$

Where,

S_{inorg} = concentration of inorganic Hg in analytical solution (µg/L).

S_{methyl} = concentration of methyl Hg in analytical solution (µg/L).

Calculation of the concentration (mass fraction) of analyte in the analytical portion according to the formula:

$$Concentration\ (\mu g/kg) = [(S_T \times DF) - MBK_L] \times \frac{V}{m \times MCF}$$

	Where, S_T = concentration of analyte (S or total Hg, ST) in analytical solution (or diluted analytical solution) (µg/L). MBK_L = laboratory method blanks (MBK) (µg/L). Average of two method blanks. V = volume (L) of analytical solution (0.050 L). m = mass of analytical portion (kg). DF = dilution factor (1 if analytical solution not diluted). MCF = mass correction factor (1 if water or other solvent not added to aid homogenization). Round calculated concentration to at most 3 significant figures. Concentration may be converted to other convenient units (e.g., mg/kg, ng/kg).
Reference	[1] ASTM International (2006) ASTM D 1193-06, "Standard Specification for Reagent Water". [2] Hight, S. C., and Cheng, J. (2006) Determination of Methylmercury and Estimation of Total Mercury in Seafood Using High Performance Liquid Chromatography (HPLC) and Inductively Coupled Plasma-Mass Spectrometry (ICP-MS): Method Development and Validation, Anal. Chim. Acta 567, 160-172. [3] Cheng, J., and Hight, S. C. (2008) USFDA Elemental Analysis Manual: Food and related products: High Performance Liquid Chromatographic-Inductively Coupled Plasma-Mass Spectrometric Determination of Methylmercury and Total Mercury in Seafood.
Approved by	Scientific Panel on Methods of Sampling and Analysis

ANNEXURE I

Fish Products to which the methods are specified are mentioned here.

2.6.1 Frozen shrimp

2.6.4 Frozen finfish

2.6.5 Frozen Fish Fillets

2.6.8 Canned Fishery Products

2.6.4.1 Finfish (sardine and other clupeoids, tuna and bonito, Mackerel, Seer fish, Pomfret)

2.6.4.2 Crustacean (Shrimp/Prawn, Crab)

2.6.4.3 Molluscs (Mussels, Squid)

2.6.9 Frozen Cephalopods

2.6.10 Smoked Fish Products

2.6.11 Ready –to-Eat Finfish or Shell Fish Curry in Retortable Pouches

2.6.12 Sardine Oil

2.6.13 Edible Fish Powder

2.6.14 Fish Pickles

2.6.15 Frozen Minced Fish Meat

2.6.16 Freeze Dried Shrimp/Prawns

2.6.17 Frozen Clam Meat

RAPID ANALYTICAL FOOD TESTING (RAFT) KIT/ EQUIPMENT

Alternate Rapid kits/equipments may be used to get quick results for screening and surveillance purposes, provided the kit/equipment is approved by FSSAI. Details of the rapid food testing kit/equipment approved by FSSAI are available at https://www.fssai.gov.in/cms/raft.php.

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Food Safety and Standards Authority of India
स्वास्थ्य और परिवार कल्याण मंत्रालय
Ministry of Health and Family Welfare

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