



CD/K/514:2010
ICS 67.120.30

EAST AFRICAN STANDARD

Frozen tuna loins — Specification



EAST AFRICAN COMMUNITY

Foreword

Development of the East African Standards has been necessitated by the need for harmonizing requirements governing quality of products and services in East Africa. It is envisaged that through harmonized standardization, trade barriers which are encountered when goods and services are exchanged within the Community will be removed.

In order to meet the above objectives, the EAC Partner States have enacted an East African Standardization, Quality Assurance, Metrology and Test Act, 2006 (EAC SQMT Act, 2006) to make provisions for ensuring standardization, quality assurance, metrology and testing of products produced or originating in a third country and traded in the Community in order to facilitate industrial development and trade as well as helping to protect the health and safety of society and the environment in the Community.

East African Standards are formulated in accordance with the procedures established by the East African Standards Committee. The East African Standards Committee is established under the provisions of Article 4 of the EAC SQMT Act, 2006. The Committee is composed of representatives of the National Standards Bodies in Partner States, together with the representatives from the private sectors and consumer organizations. Draft East African Standards are circulated to stakeholders through the National Standards Bodies in the Partner States. The comments received are discussed and incorporated before finalization of standards, in accordance with the procedures of the Community.

Article 15(1) of the EAC SQMT Act, 2006 provides that "Within six months of the declaration of an East African Standard, the Partner States shall adopt, without deviation from the approved text of the standard, the East African Standard as a national standard and withdraw any existing national standard with similar scope and purpose".

East African Standards are subject to review, to keep pace with technological advances. Users of the East African Standards are therefore expected to ensure that they always have the latest versions of the standards they are implementing.

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Introduction

Freezing of fish, as a method of preservation, is recommended highly. The brine freezing system used on tuna purse seiners is the most common method of freezing. A new freezing method for tuna, known on the market under the name “Confreeze” and “mini confreeze system” has a few distinct advantages over the old brine freezing method, allowing the tuna to be frozen $-37\text{ }^{\circ}\text{C}$ and to be held at $-18\text{ }^{\circ}\text{C}$. Tuna is vacuum-packed in plastic bags before freezing and protected by insulation. The quality of tuna frozen by the confreeze method should be better than tuna frozen by the old brine system.

The fish shall be received fresh in iced fish boxes or frozen then given pre-treatment such as weighing, grading, nobbing (heads, viscera, tails, waste water removed) washing, brining, washing then packaged fresh or pre-cooked using steam or air, then frozen at $-18\text{ }^{\circ}\text{C}$.

In the preparation of this East African Standard, the following sources were consulted extensively:

KS 1642:2000, *Specification for frozen tuna loins*

Codex Alimentarius website: http://www.codexalimentarius.net/mrls/vetdrugs/jsp/vetd_q-e.jsp

USDA Foreign Agricultural Service website: <http://www.mrlidatabase.com>

USDA Agricultural Marketing Service website: <http://www.ams.usda.gov/AMSV1.0/Standards>

European Union: http://ec.europa.eu/enterprise/sectors/pharmaceuticals/veterinary-use/maximum-residue-limits/index_en.htm

Assistance derived from these sources is hereby acknowledged.

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Frozen tuna loins — Specification

1 Scope

This East African Standard prescribes the quality requirements, sampling method and methods of test for frozen tuna.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

CAC/GL 21, *Principles for the establishment and application of microbiological criteria for foods*

CAC/RCP 1, *Recommended international code of practice — General principles of food hygiene*

CAC/GL 30, *Principles and guidelines for the conduct of microbiological risk assessment*

CAC/GL 31, *Guidelines for the sensory evaluation of fish and shellfish in laboratories*

CD-K-572-2010, *Fish and fisheries products — Methods of sampling*

CAC/RCP 52[CD/K/521:2010], *Code of practice for fish and fishery products*

EAS 35, *Edible salt — Specification*

EAS 12, *Drinking (potable water) — Specification*

EAS 38, *Labelling of prepackaged foods — Specification*

EAS 41, *Fruits, vegetables and derived products — Sampling and methods of test*

EAS 103, *Schedule for permitted food additives*

EAS 123, *Distilled water — Specification*

ISO 4831, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection and enumeration of coliforms — Most probable number technique*

ISO 4832, *Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coliforms — Colony-count technique*

ISO 4833, *Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of microorganisms — Colony-count technique at 30 degrees C*

ISO 6579, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection of Salmonella spp.*

ISO 6887-1, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions*

ISO 6887-2, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 2: Specific rules for the preparation of meat and meat products*

ISO 6887-3, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 3: Specific rules for the preparation of fish and fishery products*

ISO 6888-1, *Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coagulase-positive staphylococci (Staphylococcus aureus and other species) — Part 1: Technique using Baird-Parker agar medium*

ISO 6888-2, *Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coagulase-positive staphylococci (Staphylococcus aureus and other species) — Part 2: Technique using rabbit plasma fibrinogen agar medium*

ISO 6888-3, *Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coagulase-positive staphylococci (Staphylococcus aureus and other species) — Part 3: Detection and MPN technique for low numbers*

ISO 7251, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection and enumeration of presumptive Escherichia coli — Most probable number technique*

ISO 7937, *Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of Clostridium perfringens — Colony-count technique*

ISO 13720, *Meat and meat products — Enumeration of Pseudomonas spp.*

ISO 16050, *Foodstuffs — Determination of aflatoxin B₁, and the total content of aflatoxin B₁, B₂, G₁ and G₂ in cereals, nuts and derived products — High performance liquid chromatographic method*

ISO 16654, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection of Escherichia coli O157*

ISO 21567, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection of Shigella spp.*

ISO/TS 21872-1, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection of potentially enteropathogenic Vibrio spp. — Part 1: Detection of Vibrio parahaemolyticus and Vibrio cholerae*

ISO/TS 21872-2, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection of potentially enteropathogenic Vibrio spp. — Part 2: Detection of species other than Vibrio parahaemolyticus and Vibrio cholerae*

ISO 11290-1, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection and enumeration of Listeria monocytogenes — Part 1: Detection method*

ISO 11290-2, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection and enumeration of Listeria monocytogenes — Part 2: Enumeration method*

3 Description

Tuna are members of the scombridae family and are related to mackerel, bonito and bill fish among others. Frozen tuna are the products consisting of the flesh of any of the appropriate species listed below.

Common Name	Scientific Name
Northern bluefin tuna	<i>Thunnus thynnus</i>
Southern bluefin	<i>Thunnus macoyyii</i>
Albacore	<i>Thunnus germa</i>
Blackfin tuna	<i>Thunnus atlanticus</i>

Oriental	<i>Thunnus orientalis</i> (<i>Thunnus thynnus</i>)
Bigeye	<i>Thunnus obesus</i> or <i>parathunnus mebachi</i>
Yellow-fin	<i>Thunnus albacares</i> (<i>Neothunnus rarus</i>)
Longtail	<i>Thunnus tongol</i> (<i>Neothunnus rarus</i>)
Skipjack	<i>katsuwonus pelamis</i>
Little thunny	<i>Euthynnus alleteratus</i>
Black skipjack	<i>Euthynnus lineatus</i>
Kawakawa	<i>Euthynnus affinis (yaito)</i>
Bullet tuna	<i>Auxis rochei</i>
Frigate tuna	<i>Auxis thazard</i>
Atlantic bonito	<i>Sarda sarda</i>
Pacific bonito	<i>Sarda chiliensis</i>

4 Quality requirements for raw tuna

4.1 The frozen tuna shall be prepared from clean, wholesome and sound fish belonging to one of the species listed in 2. The Tuna shall be either fresh or frozen and be suitable for human consumption.

4.2 Sampling shall be done in accordance with Annex A.

5 Presentation

5.1 The frozen tuna shall be presented in any of the following forms:

5.1.1 Pre-cooked packs shall be prepared from cooked fish without skin (Tuna loins or fillets).

5.1.2 Not pre-cooked solid pack shall be prepared directly from raw fish, which may be presented as "skin-on".

5.1.3 "Whole" shall include tuna weighing minimum of 2 kg.

5.2 Form of pack

The fish shall be packed in the following forms:

5.2.1 **Frozen Fish Blocks or Solid** — Fish shall be cut into transverse segments to which no free fragments are added in packs of 450 g or less of net contents. Such segments are cut into lengths suitable for packing into one layer. In packs of more than 450g net contents, such segments shall be cut into lengths suitable for packing in one or more layers of equal thickness.

5.2.2 **Chunks** — Shall be a mixture of pieces of raw or cooked fish with dimensions of not less than 1.2 cm in each direction and in which the original muscle structure is retained.

5.2.3 **Flakes** — Shall be a mixture of particles of cooked fish in which the muscular structure of the flesh is retained.

5.2.4 **Grated or shredded** shall be a mixture of particles of cooked fish that have been reduced to a uniform size and in which particles are discrete and do not comprise a paste.

6 Quality requirements for final product

6.1 The frozen tuna shall be practically free from skin (except when presented as "skin-on" pack), scales, prominent blood streaks, blood-clots, bones, bruises, and red muscles known as red meat.

6.2 The colour shall be a good characteristic of pre-cooked tuna white pink and light green.

6.3 Frozen tuna in its thawed state shall be consistent with freshly cooked Tuna. It shall be free of a mushy, waterlogged or honey comb texture.

6.4 Frozen tuna flavour and odour in its thawed state shall be consistent with freshly pre-cooked of good condition. It shall be free of all ammonia, oxidized, putrid, decomposed or otherwise non-typical tuna fresh flavours/odours.

6.5 For the double-cleaning quality, flakes arising from cleaning belly pieces and broken pieces shall be separated from other products.

6.6 For the single-cleaning quality, flakes arising from cleaning belly pieces and broken pieces shall not be included in the package.

6.7 Extra precautions such as continuous collection on cleaning table and immediate freezing shall be taken to prevent oxidation of flakes.

6.8 All frozen tuna shall be stored in a suitable clean cold storage, at a temperature of -18 °C or less.

6.9 At all times during transport a temperature below 0 °C shall be maintained.

6.10 Frozen tuna shall have a maximum of 2.7 per cent salt in one individual product.

7 Hygiene requirements

The product covered by the provisions of this standard shall be prepared and handled in accordance with CAC/RCP 1, CAC/RCP 52[CD/K/521:2010] and the relevant public health regulations.

8 Microbiological limits

The fresh and cooked tuna shall comply with the microbiological limits prescribed in Table 1.

Table 1 — Microbiological limits

S/No.	Characteristic	Fresh Max. limit	Cooked Max. limit	Method of test
(i)	Total Plate Count	10 ⁷ /g	10 ³ /g	ISO 4833
(ii)	Coliforms	100/g	20/g	ISO 4832
(iii)	<i>E. Coli</i>	100/g	Nil	ISO 7251
(iv)	<i>Salmonella</i>	Nil/g	Nil	ISO 6579
(v)	<i>Staphylococcus aureus</i>	100/g	Nil	ISO 6888
(ix)	<i>Faecal coliforms</i>	100/g	Nil	ISO 4832
(vii)	<i>Vibro parahemolyticus</i>	Nil	Nil	Annex C

9 Chemical limits

The frozen tuna shall comply with the chemical limits indicated in Table 2.

Table 2 — Chemical limits

S/No.	Characteristic	Maximum limit	Method of test
(i)	Histamine	50 ppm	Annex B
(ii)	Mercury	1 ppm	Annex D
(iii)	T.V.B (Total Volatile Base)	35 mg/100 g	Annex F
(iv)	Lead	0.5 ppm	Annex E
(v)	Cadmium	0.1 ppm	Annex E

10 Packaging and Labelling

10.1 Packaging

10.1.1 Frozen tuna shall be packed in food grade containers which will safeguard the hygienic, nutritional, technological and organoleptic qualities of the products.

The containers, including packaging material, shall be made of substances which are safe and suitable for their intended use. They shall not impart any toxic substance or undesirable odour or flavour to the product.

10.1.2 The frozen tuna shall be packed inside a polythene pouch of suitable size or wrapped with a polythene film either before or after freezing.

10.2 Labelling

The containers shall be labelled in accordance with EAS 38 and shall include the following:

- (a) Name of product;
- (b) Name and physical address of the manufacturer;
- (c) Batch/Code Number;
- (d) Net content in g or kg;
- (e) Presentation of the product;
- (f) Manufacturing date;
- (g) Expiry date;
- (h) Instructions of use;
- (i) Condition of storage; and
- (j) Country of origin.



Frozen Albacore



Frozen Albacore

Draft for comments

Asian Standard



Frozen tuna loins



Frozen tuna loins

Draft for comment

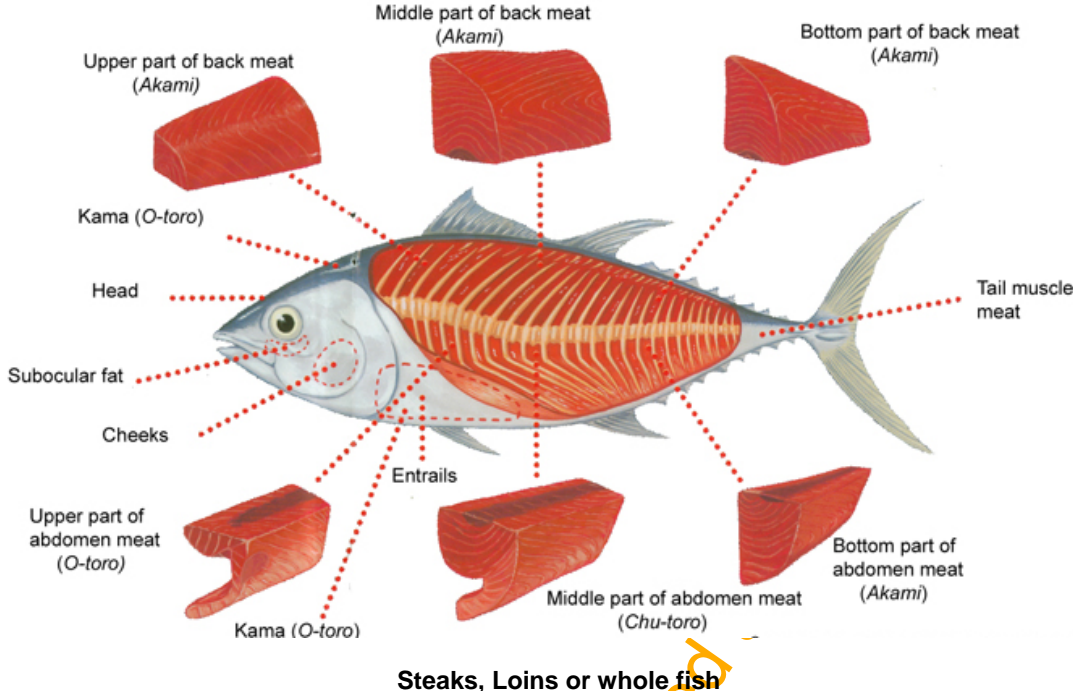


Frozen tuna loins



Frozen tuna loin steaks

ward



Steaks, Loins or whole fish

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Annex A
(normative)

Sampling of frozen tuna products

A.1 General requirements for sampling

A.1.1 The sampling instrument shall be dry and sterile.

A.1.2 The sample shall be protected against adventitious contamination.

A.1.3 The samples shall be stored in such a manner that there is no deterioration of the frozen fish.

A.2 Scale of sampling

A.2.1 Lot — Samples shall be tested from each lot for ascertaining compliance of the fish to the requirements of the specification.

NOTE *Lot* means a single consignment of the material packed on the same day and of the same grades.

A.2.2 The containers shall be selected at random. In order to ensure the randomness of selection, a random number table shall be used.

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Annex B (normative)

Determination of histamine

B.1 Introduction

B.1.1 Principle — Histamine is extracted with methanol and derivatized with o-phthalaldehyde (OPT) to generate the fluorescent product. This method is used to determine the histamine content in raw, pre-cooked, and canned tuna.

B.1.2 Interference — All methods of histamine determination are overwhelmed with interfering substances which have to be removed in order to accurately measure the histamine present. The two naturally occurring substances that cause the most interference are histamine and spermidine since they also react with OPT to form fluorescent products. However, spermidine, the major contaminant in extracts can be separated from histamine on cellulose phosphate cation-exchange columns. There is also variability due to the pH and temperature sensitivity of the o-phthalaldehyde-histamine fluorophor. Because of the ubiquity of interfering fluorophors, all reagents used must be of the highest obtainable purity. Exposure of any of the materials involved to rubber or silicones may produce erratic results. It is recommended that polyethylene labware be used in place of glass, due to an observed loss of fluorescence. All labware should be acid-washed and rinsed in distilled water prior to use. New solution must be prepared after four to seven days, due to an observed increase in blank readings.

B.1.3 Summary of method — The histamine-containing material are homogenized and extracted with methanol. The extract can then be passed through an anion exchange column to remove any remaining interfering substances. The elutant is reacted with the OPT reagent and allowed to stand for 4 minutes. The mixture is acidified with H_3PO_4 and the corresponding fluorescence is read on a calibrated instrument.

B.2 Material required

TD-360 Min-Fluorometer with U.V. optical configuration of (P/N 36000-010) 10 mm x 10 mm Methacrylate fluorescence cuvettes (P/N 7000-959).

B.2.1 Labware — All re-usable labware (glass, polyethylene, Teflon etc.) should be cleaned by soaking in laboratory grade detergent and water for 4 h, rinsed with tap water, deionized water, and methanol. It is recommended that polyethylene ware be used due to absorbency observed when using glass.

B.2.1.1 Assorted Class A calibrated pipettes

B.2.1.2 Graduated cylinder — 100 ml.

B.2.1.3 Assorted Volumetric Flasks — For preparing dilution standards.

B.2.2 Chromatographic Columns (Kontes M.K 422250).

B.3 Reagents and standards

B.3.1 Ion Exchange Resin — Sigma 1 x 8-200, chloride form 100-200 mesh: or BioRad AG1- x 8, 50-100 mesh, chloride form, Cat. No. 140-1431, or equivalent.

B.3.2 Ion Sodium Hydroxide — Dissolve 40 g NaOH in 1 Litre of distilled water.

B.3.3 2.0N Sodium Hydroxide — Dissolve 80 g NaOH in 1 Litre of distilled water.

B.3.4 Histamine Dihydrochloride — MCB X 0440 or J.T. Baker 1-N330.

B.3.5 1.0N Hydrochloric Acid — Add 83 ml concentrated HCL to about 500 ml distilled water. Cool and bring to 1-litre volume with distilled water.

B.3.6 0.1N Hydrochloric Acid — Add 100-mL 1N HCl to about 500-mL distilled water. Cool and bring to 1-Litre volume with distilled water.

B3.7 Methanol Reagent Grade

B3.8 0.1 % o-phthalaldehydol (OPT reagent) — Phthalic dicarboxaldehyde (Aldrich, Milwaukee, WI), or o-phthaldialdehyde (Sigma, St. Louis, MO) $C_6H_4(CHO)_2$. F.W, 134.13. Dissolve 0.10 g OPT in 100-mL methanol. Store in an amber bottle and refrigerate when not in use. Prepare fresh weekly.

B.3.9 3.57N Phosphoric Acid — Add 121.8 ml of 85 % H_3PO_4 to about 500-mL distilled water. Bring to 1-litre volume with distilled water.

B.3.10 Histamine Standard Solution A, 1 mg Hm/ml — Weigh 0.1656 g of histamine dihydrochloride into 100-ml volumetric flask. Dissolve in, and dilute to volume with 0.1N HCl.

B.3.11 Histamine Standard Solution B, 10 µg Hm/ml — Dilute 1.0 ml Solution A to 100 ml with 0.1N HCL.

B.3.12 Histamine Standard Solution A1 (This is our control solution) — Dilute 1.0 ml Solution A to 100 ml with methanol.

B.3.13 Histamine Standard Solution C, 0.1 mg Hm/ml — Dilute 1.0 ml Solution B to 100 ml with 0.1N HCl.

B.3.14 Histamine Standard Solution D, 0.2 M 0.2 M Hm/ml — Dilute 2.0 ml Solution B to 100 ml with 0.1 N HCl.

B.3.15 Histamine Standard Solution E, 0.3 mg Hm/ml — Dilute 3.0 ml Solution B to 100 ml with 0.1N HCl.

NOTE Prepare Solution A and B monthly. Prepare Solutions C, D, E, and A1 weekly. Refrigerate solutions when not in use.

B.4 Preparation

B.4.1 Resin preparation

B.4.1.1 Place 20 g of ion exchange resin in a beaker.

B.4.1.2 Add 2 N sodium hydroxide to the resin in a ratio of 15 ml per gram of resin.

B.4.1.3 Mix well and allow the resin to settle for a minimum of 15 minutes, but no more than 30 minutes. Decant liquid and repeat with additional 2 N sodium hydroxide.

B.4.1.4 Wash resin thoroughly with distilled water to remove traces of the sodium hydroxide until pH is less than or equal to 8.5.

B.4.1.5 Slurry resin with distilled water and transfer to a funnel containing a fluted filter paper. Thoroughly wash with distilled water.

B.4.1.6 Transfer resin to a suitable container and make sure the distilled water level is above the resin level at all times.

B.4.2 Column preparation

B.4.2.1 Slurry sufficient prepared resin into each column to form a bed 8 cm in height. Maintain a liquid level above the top of the resin at all times.

B.4.2.2 Refill columns with fresh resin at least twice per week.

B.5 Instrument set-up

B.5.1 Check that light source and filter holder are installed in your TD-360 Mini-Fluorometer. Turn on the instrument and allow to warm-up. For additional assistance, refer to your TD-360 Operating Manual.

B.5.2 Blank with a reagent blank — Calibrate instrument with the prepared histamine standard Solution E. Enter standard value of 3 000 mg/lm. Remember later to divide all reading by 10 000 to get mg Hm/ml of sample.

B.5.3 Analyze Histamine Standard Solutions C and D like you would a sample. You now have a standard curve for your samples.

B.6 Procedure

B.6.1 Sample preparation

B.6.1.1 Blend fish in a warring blender with an equal weight of deionized water to produce a 1:1 slurry.

B.6.1.2 Transfer 10.0 g of the slurry to a 150-ml beaker. Add 40.0 ml of methanol and mix thoroughly.

B.6.1.3 Using Whatman No.1 filter paper, or equivalent, filter the mixture into a suitable container. If the filtrate is to be saved for later analysis, refrigerate in a closed container.

NOTE Evaporation of methanol from the filtrate can cause erroneous results.

B.6.2 Histamine Elusion

B.6.2.1 Pass 15-20-ml distilled water through the exchange column and discard.

B.6.2.2 Place a 50-ml volumetric flask containing 5 ml in HCl at the column outlet.

B.6.2.3 Pipette 1.0 ml of filtrate (methanol extract) onto the resin bed with 5.10 ml distilled water.

B.6.2.4 Immediately initiate column flow. Flow should be maintained at a rate grater than 3 ml/min.

B.6.2.5 When liquid level is slightly above the resin, add about 5-ml distilled water and allow it to flow through the resin. Repeat with distilled water in larger increments until total water through column is about 40 ml.

B.6.2.6 Discontinue Column Flow

B.6.2.7 Remove volumetric flask and bring to 50-ml volume with distilled water. Store column effluent in the refrigerator if necessary to postpone determination for more than 2 h.

B.6.3 Controls and blanks

B.6.3.1 At the beginning of a set of analysis, and again at the end, pass 1 ml of Solution A1 through one of the columns and proceed through the procedure as though it were a fish extract. Fluorescence readings should be very similar to Solution D reading. If readings are not within 20 per cent of Solution D, all analysis performed at the same time are suspect and should be repeated.

B.6.4 Histamine determination

B.6.4.1 Into separate 25-ml glass stoppered flask, pipette 5.0 ml of 0.1 HCl (Blank); Solutions C, D and E: and each diluted column effluent.

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B.6.4.2 Add 10 ml 0.1N HCl to each flask.

B.6.4.3 Add 3 ml in NaOH. Mix thoroughly.

B.6.4.4 Within 5 minutes, add 1 ml OPT solution and mix thoroughly.

B.6.4.5 After exactly 4 minutes, add 3 ml 3.57 N H₃PO₄ and mix immediately.

B.6.4.6 Let solutions stand for 15-20 minutes and then determine the fluorescence intensities on the TD-360 Min-fluorometer. If a sample reading is greater than that of Solution E, dilute 25 ml of the column effluent to 100 ml with 0.1N HCl and proceed from B.6.4.1.

CAUTION! Fish with high salt content may cause problems with the resin necessitating more frequent changing of columns.

B.6.4.7 If sample dilution was necessary in B.6.4.6, multiply the obtained result by 4.

B.6.4.8 After all readings are obtained, divide all results by 10, 1 000 to get histamine concentration in mg Hm/ml

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Annex C (normative)

Determination of *Vibrio parahaemolyticus* in fish

C.1 Introduction

C1.1 Principle

Analytical Scheme for culturing *vibrio parahaemolyticus* as an indication of toxin in seafood.

C.2.1 Enrichment, Isolation and Enumeration.

C.1.3 Procedure — Weigh 50 g of seafood sample into a blender. Obtain surface tissues, gills and gut of fish. Sample the entire interior of selfish. For crustaceans, such as shrimp, use the entire animal if possible; if it is too large, select the central portion, including gills and gut.

C.1.4 Add 450 ml of 3 per cent NaCl dilution water and blend for one minute at 8 000 rpm. This constitutes the 1:10 dilution.

C.1.5 Prepare 1:100, 1:1,000; 1, 10,000 dilution or higher if necessary.

C.1.6 Inoculate 3x10 ml portion of the 1:10 dilution into 10 ml of glucose salt teepol broth (GSTB) – 2 x concentration. This represents the 1 gm portion. Similarly, inoculate 3 x 1 ml portions of the 1:100, 1:1 000 and 1:10,000 dilutions into 10 ml of single strength.

C.1.7 Incubate the tubes overnight (18 hours or less) at 37 °C.

C.1.8 *Vibrio Parahaemolyticus* appears as round, green or bluish colonies, 2 to 3 mm in diameter. Interfering, competitive, *vibrio alginolyticus* colonies are large and yellow.

C.1.9 When these blue green colonies are finally identified biochemically as V-parahaemolyticus (see C.2), refer to the original positive dilutions on GSTB.

C.2 Biochemical identification of isolates

Pick two or more typical or suspicious colonies with a needle to:

C.2.1 TSI agar slants/Acid butt.

- (a) Steak the slant, stab the butt, and incubate overnight at 37 °C.
- (b) V-parahaemolyticus produces an alkaline (red) slant and an acid (yellow) butt but no gas or H₂S in TSI.

C.2.2 Trypticase soy broth – 3 per cent NaCl. and trypticase soy agar – 3 %.

- (a) Inoculate both media and incubate overnight at 37 °C. These cultures provide inocula for other tests as well as material for the gram stain and for microscopic examination.
- (b) V-parahaemolyticus is a gram negative, pleomorphic organism exhibiting curved or straight rods with polar flagella.

C.3 Motility test medium

- (a) Inoculate a tube of motility test medium by stabbing the column of the medium to depth of approximately 5 mm. Incubate for 18 h at 37 °C. A circular outgrowth from the line of stab

constitutes a positive test, V-parahaemolyticus is motile.

(b) **Preliminary screening data**

Only motile, gram negative rods which produce an acid butt and an alkaline slant on TSI and do not form H₂S or gas are examined further. The identifying characteristics of V-parahaemolyticus are shown in Table C.1.

Table C.1 — Identifying characteristics *Vibrio parahaemolyticus*

Tests	Reactions
Grain stain	Gram negative
Morphology	Curved/straight rod
Motility	+
TSI	K/A*.H ₂ S(-) GAS(-)
Hugh-Liefson glucose	+
Medium	-
Oxidase	+
Arginine dihydroloase	+
Lysine decarboxylase	+
Galatin	+
Halophilism (NaCl)	60 %, 8 % (+) 0 %, 10 %(-)
Growth at 42 °C	+
Voges-Proskaner	-
Indole	+
Cellulose	-
Sucrose	-
Maltose	+
Mannitol	+
Trehalose	+

Annex D
(normative)

Determination of total mercury using flameless atomic absorption spectrophotometry (AAS)

D.1 Principle

All the mercury present in food is converted to the inorganic form by wet oxidation. The mercury is then reduced to metallic stage using stannous chloride and is released from the solution as vapour with a stream of air. The mercury vapour in the airstream is determined by flameless atomic absorption-spectrophotometry.

D.1.1 Apparatus

- (a) Flowmeter — calibrated from 0.5 litres/mm
- (b) Drechsel Bottle A — 125-mL capacity with sintered head of No. 1 porosity
- (c) Tube B — containing a plug of cotton wool
- (d) Tube C — containing Mallcosorb (Mallinckrodt Chemical) 30-50 mesh (prevents acid vapours reaching meter and pump).
- (e) Pump — Charles Austin Copex Mark II
- (f) Mercury Vapour — Concentration Meter Hendry Type 1177 C.

D.1.2 Reagents

NOTE A fresh reagent blank should be performed when any reagent is renewed. Any metal in the blank is usually derived from the acid.

- (a) Sulphuric acid — "Lead-Free" grade.
- (b) Nitric acid
- (c) Water — Deionised
- (d) Stannous Chloride — 20 per cent m/v in 6 M hydrochloric acid.
- (e) Potassium Permanganate — 60 per cent m/v aqueous solution.

D.1.3 Standard mercury solution

D.1.3.1 Dissolve 0.25g mercury in 5-ml nitric acid and dilute 500 ml with water (0.5 mg/ml)

D.1.3.2 Pipette 5 ml of solution 1 into a 250-ml volumetric flask and add about 100-ml water, 1-ml potassium permanganate solution (6 per cent) and 1 ml 9 M sulphuric.

Dilute to the mark with water (10 µg/ml). Renew weekly.

D.1.3.3 Pipette 2 ml of solution 2 into a 200-ml volumetric flask and add 100-mL water and 1 ml of each of the permanganate and acid reagents as before.

Dilute to the mark with water (0.1 µg/ml). This solution must be freshly prepared.

D.1.4 Procedure

D.1.4.1 Wet oxides 5.25 g of sample, taking special precautions to trap volatile mercury.

When the oxidation is complete, filter the combined solution + condensate through a No. 541 Whatman Paper into a 100 ml of 250-ml volumetric flask, rinse the filter and make up the mark with water in A. Switch on the pump and adjust the value until the flowmeter records 1.5 litres/min. Switch the amplifier to x 5 and the recorder to 10 m V. Set the full-scale adjustment on the meter and recheck the zero setting

Remove A, with the pump still operating, and reject the contents. Pipette 10 ml of sample digest into the vessel and add 40 ml deionised water and 5-ml stannous chloride solution. Replace vessel A into the air-train. The liberated vapour is then drawn along through the mercury meter. Observe the resulting trace on the recorder chart. If the height of the peak exceeds 50 divisions (half the scale), repeat with a smaller volume of digest solution. If the peak is less than 10 divisions, either increase the recorder sensitivity or take a large volume of digest.

For very low levels use the 2-mv recorder setting and take 50-ml digest, but always adjust the volume in vessel A to 50 ml before the addition of the stannous chloride. When all the mercury has been displaced and the meter has regained its zero level remove vessel A and pipette 2 ml of standard mercury solution 3 into the contents. Replace the vessel, compare the height of the sample peak with that given by the standard and assess the amount of mercury in the sample aliquot. Using this calculated amount as a guide, add the correct amount of standard mercury solution to vessel A.

On replacing the vessel in the air-train, the resultant peak should be of very similar height to that given by the sample. Repeat the sequence with a fresh portion of sample solution, add the correct amount of standard solution as soon as the mercury originally present has been displaced from the meter. Also perform a reagent blank by carrying out a complete analysis without the addition of the sample. Calculate the mercury content of the sample from the peak heights by direct proportion.

Annex E (normative)

Determination of lead and cadmium using atomic absorption spectroscopy method

E.1 Principle

E.1.1 Lead and cadmium are extracted from a solution of the ash of the sample 0.5 M with respect to HCl by diethyl ammonium diethylcarbodithioate in methyl isobutyl ketone. Standards are treated in the same way and both sample and standard extracts are sprayed in the flame of an atomic absorption spectrophotometer.

E.1.2 Apparatus

Before use, all items of glassware and silica dishes should be immersed in 5 per cent v/v hydrochloric acid (reagent grade) for several hours, and then rinsed with double-distilled water.

- (a) Lipped silica dishes — volume approx. 30 ml.
- (b) Graduated 5-mL and 1-ml — pipettes.
- (c) 25-ml volumetric flasks — with plastic stoppers.
- (d) Atomic absorption spectrophotometer.

The operating conditions for lead and cadmium, using an Atospek H 1170, (Hilger & Watts) are shown below.

	Lead	Cadmium
Lamp current (Ma)	6	5
Wavelength (nm)	217.0	228.9
Slit width (μm)	100	45
Burner height (mm)	9	9
Acetylene (litre min ⁻¹ at 5 psig)	0.75	0.7
Air (litres min ⁻¹ at 30 psig)	2	2
Scale expansion	1.0	0

- (e) Recorder (e.g. Servoscribe Flat-bed)

E.2 Reagents

- (a) *Water* — Double-distilled, using silica-sheathed elements.
- (b) *Nitric acid (S.G/ 1.42)* — Low-in-lead quality.
- (c) Hydrochloric acid (S.G.1.17) — Low-in-lead quality
- (d) Diethylammonium diethylcarbodithioate (DDCD) — A per cent w/v solution in methyl isobutyl keton: The solution may be kept for several weeks without deterioration.
- (e) Methyl isobutyl keton (MIBK) solvent — A saturated solution of water in MIBK.
- (f) Ascorbic acid — A freshly prepared, 10 per cent w/v aqueous solution.
- (g) Standard solution of lead and cadmium: A 2 per cent w/v solution of hydrochloric acid containing 10 mg/kg of lead and 2 mg/kg of cadmium.

E.3 Method

E.3.1 Dry ash (see Note 1) 10 g of homogenised sample (weighed to nearest 0.1 g) at 450-500 °C in a silica dish until the ash is grey or white. Moisten with diluted nitric acid/water (1 + 9) and briefly re-ash if necessary. Treat the ash with 5 ml of water followed by 5 ml of hydrochloric acid and evaporate to dryness on a steambath. Add 1.0 ml of hydrochloric acid and 3-5 ml of water, stirring with a glass

rod, filter through a small No. 4 paper into a 25-ml volumetric flask and make to the mark with dish and rod risings. Prepare aqueous standards in 25-ml calibrated flasks using 0.010, 0.20, 0.50 and 1.00 ml of the standard lead/cadmium solution. To each flask add 1.0 ml of hydrochloric acid and make up to the mark with water (see Note 2).

NOTE 1 Char milk powders, cereals and dehydrated foods over a Bunsen burner before ashing. A dish larger than the 30-ml size is preferable for these materials.

NOTE 2 The samples and standards may be left at this stage for several days, but once the extraction process has been started, the analysis should be completed as soon as possible.

NOTE 3 See the determination of lead by dithizone for precautions to be taken in ashing.

E.4 Discussion

E.4.1 Extraction of lead and cadmium from 0.5 M HCl by DDCD in MIBK is a convenient alternative to the procedure given under the dithizone method. If an atomic absorption spectrophotometer is not available, the lead and cadmium may be extracted with DDCD/MIBK, using a somewhat larger volume and repeating the extraction with fresh DDCD to ensure complete extraction. The organic phase must then be evaporated without loss by spattering and digested in the minimum amount of acid and the determination completed with dithizone. Calcium, magnesium and phosphate are unlikely to cause difficulties or precipitation in 0.5 M HCl.

E.4.1.1 Of the elements likely to be present in food digests only iron, copper and zinc could interfere in the determination using AAS. These elements are usually masked by forming their cyanide complexes at pH 8.5. In the AAS method given it has been found necessary to eliminate completely the interference from iron only by reducing with ascorbic acid any Fe (III) to Fe (II) prior to the extraction step. Three series of calibrations for 0.1 µg of lead were carried out by Snodin (1973).

- a) standard calibrations
- b) Calibrations in the presence of 50 µg each of iron (III), copper and zinc
- c) Calibrations in the presence of 100 µg each of iron (III), copper and zinc.

E.4.1.2 Lead is subject to slightly more interference than cadmium though the effect causes no more than a 15 per cent decrease in response even in calibration c). Moreover, since such excessive amounts of iron, copper and zinc as used in c) are likely to be present in foods, any interference by these metals should be within experimental error.

Experiments have indicated that the lead-DDCD complex is stable for several hours whilst that of cadmium is subject to slight decomposition (judged by a decrease of 10 to 20 per cent in the instrument response). For this reason cadmium is best determined first and lead determination completed within three hours of extraction as recommended by Brooks, Presley and Kaplan (1967). Nix and Goodwin (1970) showed that the sodium diethylcarboioate complexes of copper, iron, cobalt, nickel, chromium, lead, zinc, but not of manganese, were stable for at least 400 minutes.

NOTE In the paper referred to earlier by Roschnik, the use of xylene instead of MIBK is recommended and it is stated that some products such as juices and beverages need not be digested. The present author experienced some difficulty with the flame conditions but the method worked well using dinitrogen oxide/acetylene.

EU Directive

Histamine

Nine samples shall be taken from each batch and shall fulfil the following:

The mean value shall not exceed 100 ppm

Two samples may have value of more than 100 ppm but less than 200 ppm.

No sample may have a value exceeding 200 ppm.

Test to be done by HPLC.

Annex F (normative)

Determination of total volatile bases and trimethylamine

F.1 Principle of the method

This method is based on a semi-microdistillation procedure. Extracts or solutions are made alkaline with sodium hydroxide. The bases are steam distilled into standard acid and back titrated with standard alkali. Formaldehyde is added to the neutralized mixture and the acid released is equivalent to the volatile bases other than trimethylamine.

F.2 Procedure

Weigh 100 ± 0.5 of prepared sample into a homogenizer with 300 ml of 5 per cent m/v trichloroacetic acid. Run the homogenizer to obtain a uniform slurry, filter or centrifuge to obtain a clear extract. By pipette, transfer 5 ml of the extract to a semi-microdistillation apparatus. Add 5 ml 2M sodium hydroxide solution. Steam distil. Collect in 15 ml 0.01M standard hydrochloric acid. Add indicator solution (1 per cent rosolic acid in 10 per cent v/v ethanol). Titrate to a pale pink end point with 0.01M sodium hydroxide. Add 1 ml 16 % m/v neutralized formaldehyde for every 10 ml liquid in the tildrafim flask. Titrate the liberated acid with 0.01M sodium hydroxide.

F.3 Calculation

$$\text{Total base nitrogen} = \frac{14 (3 + W) \times V_1}{500} \text{ mg} / 100 \text{ g}$$

$$\text{Trimethylamine nitrogen} = \frac{14 (300 + W) \times V_2}{500} \text{ mg} / 100 \text{ g}$$

where;

V_1 is volume standard acid consumed in the first titration;

V_2 is volume standard acid released for the second titration;

W is water content of the sample mg/100 g.

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