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EAST AFRICAN STANDARD

Frozen frog legs — Specification

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EAST AFRICAN COMMUNITY

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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

Frozen frog legs have attracted overseas buyers where frog meat is consumed. There is considerable scope for further development of export market of this commodity if the quality of the product is maintained continuously at a high level.

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

IS 2885:1975(2005), *Specification for Frozen Frog Legs*

CAC/RCP 52:2003(Rev. 4:2008), *Code of practice for fish and fishery products*

IS 4303-1:1975, *Code of hygienic conditions for fish industry — Part 1: Pre-processing stage*

IS 4303-2:1975, *Code of hygienic conditions for fish industry — Part 2: Canning stage*

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

USDA Foreign Agricultural Service website: http://www.mrldatabase.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 frog legs — Specification

1 Scope

This East African Standard prescribes the requirements and the methods of sampling and test for frozen frog legs.

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*

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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 B_1, and the total content of aflatoxin B_1, B_2, G_1 and G_2 in cereals, nuts and derived products — High performance liquid chromatographic method*

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

3.1 Definitions

For the purpose of this standard the following definitions shall apply.

3.1.1
count
number of pairs of frog legs per kilogram after thawing

3.1.2
frog legs
unseparated pair of hind legs of frogs of the species *Rana tigrina*, *R. hexadaetyla* and *R. catesbena* freed from skin, claws and saddle

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3.2 Product definition

Frog legs shall be prepared from the unseparated pair of hind legs of frogs of the species *Rana tigrina*, *R. hexadaetyla* and *R. catesbena* freed from skin, claws and saddle.

3.3 Presentation

There shall be three types of frozen frog legs, namely, (a) creamy white, (b) light pink, and (c) light blue.

3.4 Grades

Unless agreed otherwise between the purchaser and the vendor, the material shall be graded as follows:

Grade designation	Count per kg
Jumbo	4 - 8
Large	8 - 12
Medium	12 - 16
Small	16 - 24
Extra Small A	24 - 32
Extra Small B	32 - 36
Extra Small C	Above 40

The material with count, more than 80kg shall not, be processed.

3.5 Raw materials

The material shall be obtained from live healthy frogs. The legs shall be washed, trimmed, deskinned and cleaned properly. The raw material shall be clean, wholesome, complete whole pieces, generally free from blood clots and discolouration. The legs shall also be free from skin, any foreign matter before quick freezing. The muscles shall be complete and intact. They shall be free from any parasites.

4 Essential composition and quality factors

4.1 Preparation

The live frogs shall be washed in potable water to remove dirt and soil and later washed with water chlorinated to a level of 200 ppm chlorine. The frogs shall be paralyzed by dipping in 10 % sodium chloride solution for 10 minutes. The hind legs shall be severed from the trunk in such a way that the intestines are least disturbed. The severed legs shall be immersed in 3 % sodium chloride solution containing 200 ppm available chlorine for proper bleeding. The loosely hanging intestines, if any, shall be removed and washed in potable water. The legs shall then be dipped in 5 percent sodium chloride solution chlorinated to 500 ppm available chlorine for 15 minutes, and deskinned and the material dressed by removing the cloaca, the saddle, claws and hanging pieces of meat, etc. Each piece shall be washed three times using water chlorinated to a level of 200 ppm available chlorine and dipped in the same level of chlorine water for 10 minutes. The volume of water for dipping may be three times the volume of the frog legs. Finally, the legs shall be washed in 10 pprn chlorine water to remove excessive chlorine smell.

4.2 Freezing

The frog legs shall be either frozen in blocks or wrapped individually in polythene film or any other suitable moisture proof covering already dipped in 5 ppm chlorine water for 5 minutes. In case of delay in keeping the material for freezing, the material shall be temporarily kept in chill room in 20ppm chlorine water with suitable amount of ice. The material shall be quick frozen at or below -40℃: in the minimum possible t.ime. Broken or damaged legs shall not be used for freezing. The material frozen

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in trays without overwrap shall be uniformly glazed and filled in suitable containers. The frozen material shall be transferred immediately to the cold storage maintained at or below - 18℃.

4.3 Grading

The material shall be graded into different sizes on the basis of count (see 4.1). Grading may be done preferably before freezing but may also be done after freezing. The material used for a particular size of pack shall be of a reasonably uniform size.

4.4 Appearance

When thawed, frog legs shall have shining appearance and characteristic colour and odour of the species. They shall be soft but firm in texture with some degree of elasticity. The frog legs should be completely free from skin and any foreign matter, and reasonably free from dehydration, discolouration and blood clots. They shall not show any signs of deterioration and shall not have off odour.

5 Food additives

Only additives permitted in EAS 103 shall be used.

6 Hygienic requirements

6.1 The material shall be prepared under hygienic conditions, only in premises maintained in a thoroughly clean and hygienic manner complying with CAC/RCP 1, CAC/RCP 52 and duly approved by the competent authorities.

6.2 The microbiological count and heavy metal contamination of the frozen material shall not exceed the limits given in Table 1 and Table 2.

Table 1 — Microbiological limits

S/No.	Characteristic	Max. limit	Method of test
(i)	Total plate count per gram, max	500 000/g	ISO 4833
(ii)	Coliforms	100/g	ISO 4832
(iii)	*E. Coli*	10/g	ISO 7251
(iv)	*Salmonella*	Nil/g	ISO 6579
(v)	*Staphylococcus aureus*	100/g	ISO 6888
(ix)	*Faecal coliforms*	Nil/g	ISO 4832
(vii)	*Vibro parahemolyticus*	Nil	Annex C
(viii)	*Vibro cholerae*	Nil/g	ISO/TS 21872

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)	25 mg/100 g	Annex F
(iv)	Lead	0.5 ppm	Annex E
(v)	Cadmium	0.1 ppm	Annex E

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7 Packing and marking

7.1 Packing

The frozen material shall be packed in plywood or deal wood boxes or cardboard cartons. The containers shall be bound securely and stored at a temperature of -18℃. The mass of the thawed material shall be not less than that of the net mass declared on the package.

7.2 Marking

7.2.1 Bach container shall be marked with the following:

a) Name of the material with brand name, if any;

b) Name and address of the manufacturer (optional, for export purposes);

c) Size, grade or count;

d) Batch or code number;

e) Net mass of contents; and

f) Country of origin.

7.2.2 Each container may also be marked with a Certification Mark.

8 Sampling, examination and analyses

8.1 Sampling

(i) Sampling of lots for examination of the product shall be in accordance with CD/K/572:2010. Sampling of lots composed of blocks shall be in accordance with the sampling plan developed for quick frozen fish blocks (reference to be provided). The sample unit is the primary container or for individually quick frozen products is at least 1 kg portion of the sample unit.

(ii) Sampling of lots for examination of net weight shall be carried out in accordance with an appropriate sampling plan meeting the criteria established in CD/K/572:2010.

8.2 Sensory and physical examination

Samples taken for sensory and physical examination shall be assessed by persons trained in such examination and in accordance with CAC/GL 31.

8.3 Determination of net weight

8.3.1 Determination of net weight of products not covered by glaze

The net weight (exclusive of packaging material) of each sample unit representing a lot shall be determined in the frozen state.

8.3.2 Determination of net weight of products covered by glaze

As soon as the package is removed from frozen temperature storage, open immediately and place the contents under a gentle spray of cold water until all ice glaze that can be seen or felt is removed. Remove adhering water by the use of paper towel and weigh the product.

8.4 Procedure for thawing

The sample unit is thawed by enclosing it in a film-type bag and immersing in water at room temperature (not higher than 35℃). The complete th awing of the product is determined by gently

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squeezing the bag occasionally so as not to damage the texture of the squid until no hard core of ice crystals are left.

8.5 Cooking methods

The following procedures are based on heating the product to an internal temperature of 65 -70℃. The product must not be overcooked. Cooking times vary according to the size of the product and the temperatures used. The exact times and conditions of cooking for the products should be determined by prior experimentation.

Baking Procedure: Wrap the product in aluminum foil and place it evenly on a flat cookie sheet or shallow flat pan.

Steaming Procedure: Wrap the product in aluminum foil and place it on a wire rack suspended over boiling water in a covered container.

Boil-In-Bag Procedure: Place the product into a boilable film-type pouch and seal. Immerse the pouch into boiling water and cook.

Microwave Procedure: Enclose the product in a container suitable for microwave cooking. If plastic bags are used, check to ensure that no odour is imparted from the plastic bags. Cook according to equipment instructions.

8.6 Determination of histamine

Shall be determined in accordance with Annex B.

9 Definition of defects

The sample unit shall be considered defective when it exhibit any of the properties defined below.

9.1 Deep dehydration

Greater than 10% of the surface area of the sample unit or for pack sizes described below, exhibits excessive loss of moisture clearly shown as white or yellow abnormality on the surface, which masks the colour of the flesh and penetrates below the surface, and cannot be easily removed by scraping with a knife or other sharp instrument without unduly affecting the appearance of the product.

9.2 Foreign matter

The presence in the sample unit of any matter, which has not been derived from fish, does not pose a threat to human health, and is readily recognized without magnification or is present at a level determined by any method including magnification that indicates non-compliance with good manufacturing and sanitation practices.

9.3 Odour and flavour

A sample unit affected by persistent and distinct objectional odours or flavours indicative of decomposition, which may be characterized also by light pinkish to red colour.

9.4 Texture

Textural breakdown of the flesh, indicative of decomposition, characterized by muscle structure which is mushy or paste-like.

10 Lot acceptance

A lot shall be considered as meeting the requirements of this standard when:

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(i) the total number of defectives as classified according to Section 8 does not exceed the acceptance number (c) of the appropriate sampling plan in CD/K/572:2010;

(ii) the average net weight of all sample units is not less than the declared weight, provided there is no unreasonable shortage in any container;

(iii) the Food Additives, Hygiene and Labelling requirements of Sections 5, 6 and 7 are met.

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Annex A
(normative)

Sampling of frozen 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. Expose 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.

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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 % 0-phthalaldehydol (OPT reagent) — Phthalic dicarboxaldehyde (Aldrich, Milwaukee. WI), or o-phthaldialdehyde (Sigma, St. Louis. MO) C_6H_4 (CHO) L_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 to100 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.

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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 H_3PO_4 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. N 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 paraemolyticus* 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 3×10 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.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-parahaemolyticuls (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 ℃.

(b) V-parahaemolytiens produces an alkaline (red) slant and an acid (yellow) butt but no gas or H_2S 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 ℃. These cultures provide inocula for other tests as well as material for the gram stain and for microscopic examination.

(b) V-paraheamolyticus 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 ℃. A circular outgrowth from the line of stab

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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 H_2S 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_2S(-) GAS(-)
Hugh-Liefson glucose	+
Medium	-
Oxidase	+
Arginine dihydroloase	+
Lysine decarboxylase	+
Galatin	+
Halophilism (NaCl)	60 %, 8 %(+) 0 %, 10 %(-)
Growth at 42 ^{0}C	+
Voges-Proskaner	-
Indole	+
Cellulose	-
Sucrose	-
Maltose	+
Mannitol	+
Trehalose	+

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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) Drechset 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.

D1.3.3 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.

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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 t he 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.

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Annex E
(normative)

Determination of lead and cadmium using atomic absorption spectroscopy method

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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 lammonium 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 hydrochoric 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-a 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 homegenised sample (weighed to nearest 0.1 g) at 450-500 ℃ 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

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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 0.0.10, 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 zine 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 diethylcarbodioate 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.

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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

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

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

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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