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Microbiology of the food chain — Detection and quantification of histamine in fish and fishery products — HPLC method

Microbiologie de la chaîne alimentaire — Détection et quantification de l'histamine dans le poisson et les produits de la pêche — Méthode CLHP

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation on the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

This document was prepared by the European Committee for Standardization (CEN) Technical Committee CEN/TC 275, *Food analysis — Horizontal methods*, in collaboration with ISO Technical Committee TC 34, *Food products*, Subcommittee SC 9, *Microbiology*, in accordance with the agreement on technical cooperation between ISO and CEN (Vienna Agreement).

Introduction

Histamine is a causative agent of scombroid poisoning or histamine fish poisoning. Histamine can be present mainly in *Scombridae* (tuna, mackerel) and *Clupeidae* (herring, sardine), species which contain a high level of free histidine. Histamine is formed through the decarboxylation of histidine by microbiological histidine decarboxylase.

Histamine [2-(1H-imidazol-5-yl)ethanamine] is defined as a biologically active low molecular weight basic nitrogenous molecule. The consumption of food containing significant concentration of histamine can cause symptoms similar to those associated to food allergies.

This document was developed in response to the need to standardize a method for histamine detection and quantification in fish and fishery products, in particular for European Regulation 2073/2005[1] on microbiological criteria for foodstuffs.

Microbiology of the food chain — Detection and quantification of histamine in fish and fishery products — HPLC method

WARNING — Persons using this document should be familiar with normal laboratory practice. This document does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices.

1 Scope

This document specifies a high performance liquid chromatography (HPLC) method to analyse histamine in fish and fishery products (fish sauces, fish maturated by enzyme in brine, etc.) intended for human consumption.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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.

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

3 Terms and definitions

No terms and definitions are listed in this document.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at http://www.electropedia.org/

— ISO Online browsing platform: available at http://www.iso.org/obp

4 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. Precolumn derivatization is performed using dansyl chloride. The biogenic amines and the components in the solution are separated by HPLC with an appropriate column, using UV detection. Histamine mass concentration is calculated from the peak area ratio of histamine and internal standard with a calibration curve.

5 Reagents and materials

Use only reagents of recognized analytical grade and water complying with grade 1 of ISO 3696, unless otherwise specified. Solvents shall be of quality for HPLC analysis, unless otherwise specified.

5.1 Acetone, CH_3COCH_3.

5.2 Acetonitrile, CH_3CN.

5.3 Toluene, C_7H_8.

5.4 Water, HPLC grade.

5.5 Water, distilled or equivalent.

5.6 Nitrogen, gas.

5.7 Perchloric acid, c($HClO_4$) = 0,2 mol/l (recommended).

Dilute 19,5 ml of $HClO_4$ 65 % or 17,2 ml of $HClO_4$ 70 % to 1 000 ml of water (5.5). The solution is stable for six months if stored at room temperature (15°C to 25°C).

5.8 Saturated sodium carbonate solution, Na_2CO_3.

Dissolve 110 g of sodium carbonate in about 150 ml of water (5.5). The solution is in excess and stable for three months if stored at 5 °C ± 3°C.

5.9 Dansyl chloride solution, $\rho(C_{12}H_{12}ClNO_2S)$ = 7,5 mg/ml.

Dissolve 0,375 g of dansyl chloride in 50 ml of acetone (5.1). The solution is stable for three weeks if stored in the dark at a temperature lower than < −18 °C.

5.10 L-proline solution, $\rho(C_5H_9NO_2)$ = 100 mg/ml.

Dissolve 1 g of L-proline in 10 ml of water (5.5). The solution is stable for three weeks if stored at 5 °C ± 3°C.

5.11 Histamine stock solution, $\rho(C_5H_9N_3)$ = 12,5 mg/ml.

Dissolve 1,034 g of histamine dihydrochloride in 50 ml of water (5.5). The solution is stable for one year if stored at < −18 °C.

5.12 Internal standard (IS) 1,7-diaminoheptane stock solution, $\rho(C_7H_{18}N_2)$ = 6,4 mg/ml. (recommended).

Dissolve 0,320 g of 1,7-diaminoheptane in 50 ml of water (5.5). The solution is stable for three weeks if stored at 5 °C ± 3°C.

6 Apparatus

6.1 Grinder, e.g. mixer, blender.

6.2 Balances, (precisions 0,1 g and 0,001 g).

6.3 Crusher, (homogenizer) with metallic rods.

6.4 Centrifuge, refrigerated, capable of a centrifugal force of 8 000*g*.

6.5 Centrifuge tubes, (plastic) with closing caps.

6.6 Pipettes, ranges 20 µl to 200 µl and 100 µl to 1 000 µl.

6.7 Tubes, (temperature resistant glass) with closing caps.

6.8 Vortex.

6.9 Water bath, 60 °C ± 1 °C with dark cover or equivalent.

6.10 Refrigerator, capable of temperatures 5°C ± 3 °C.

6.11 Freezer, capable of temperatures < −18°C.

6.12 Nitrogen evaporator.

6.13 Needles, 20G 0,9 mm disposable.

6.14 Filters, 0,2 µm disposable [Polytetrafluoroethylene (PTFE)/Polypropylene (PP)].

6.15 Syringes, 2 ml disposable.

6.16 LC system, pump, refrigerated autosampler, column oven (25 °C ± 2°C), UV detector λ = 254 nm (UV).

6.17 LC column, Kromasil®[1)] C18 5 µm 100 Å (25 cm × 4,6 mm) or equivalent.

6.18 Glass autosampler vial, 2 ml with insert (200 µl) and cap.

7 Procedure

7.1 Sample preparation

Homogenize the sample (fish flesh) by grinding in a mixer (6.1). Transfer a test portion of 5 g ± 0,1 g of fish homogenate to a centrifuge tube (6.5).

7.2 Extraction

Add 10 ml of perchloric acid (5.7) and 100 µl of 1,7-diaminoheptane (5.12) to 5 g of fish in the centrifuge tube and mix (6.3). After complete homogenization, centrifuge (6.4) at 8 000*g* for 5 min at 4 °C.

7.3 Derivatization

Transfer 100 µl of the supernatant into a tube (6.7), add 300 µl of sodium carbonate solution (5.8) and 400 µl of Dansyl chloride solution (5.9).

Vortex (6.8) and incubate (6.9) for 5 min in the dark at 60 °C.

Cool the tube under cold tap water and add 100 µl of L-proline solution (5.10).

Vortex (6.8) and place the tube in the dark for 15 min.

NOTE Supernatant can be stored at < −18 °C for one week.

7.4 Purification

Add 500 µl of toluene (5.3) and vortex (6.8).

NOTE 1 Manipulation can be stopped at this step with storage at < −18 °C for one week maximum.

1) Kromasil® is the trademark of a product supplied by Sigma-Aldrich Co. LLC. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

Transfer as much as possible of the upper organic phase into a new tube (6.7) and dry it in the fume hood with a stream of nitrogen (6.12).

NOTE 2 The organic phase toluene contains the derivatized histamine and not the "non-organic" (aqueous) phase. The organic phase can easily be recovered by freezing the aqueous phase (< −18 °C for 30 min minimum). In addition, freezing can improve the quality of the upper organic phase.

Re-suspend the dry tube with 200 µl of acetonitrile (5.2)/water (5.4) (60/40 volume fraction) and vortex (6.8).

Filter the solution (6.13, 6.14 and 6.15) in a glass autosampler vial (6.18) and fill the autosampler (6.16).

7.5 LC condition

HPLC and UHPLC systems can be used. Indicative parameters depending upon the instrument and the column are given in Annex A.

7.6 Range of standard sample

Standard samples should be prepared by supplementation of volumes of histamine stock solution (5.11) to homogenate samples prepared according to 7.1 from a histamine free matrix.

Add the volumes as specified in Table 1 to the histamine free sample and proceed to the extraction step (7.2) and thus derivatization (7.3), purification (7.4) and LC (7.5).

To avoid matrix effect and bias, carry out a calibration line on the same matrix (histamine free) as the sample analysed.

Table 1 — Example for the preparation of standard samples

Level mg/kg	**Volume of the histamine stock solution (5.11)** µl
0	0
25	10
50	20
100	40
250	100
500	200

8 Calculation

8.1 Calibration line (curve)

Perform a calibration function by linear regression analysis, using histamine standard samples (7.6) and an internal standard with Formula (1):

$$f(C_{\mathrm{HS}}) = \frac{A_{\mathrm{HS}}}{A_{\mathrm{IS}}} \times C_{\mathrm{HS}} \qquad (1)$$

where

C_{HS} is the mass concentration of histamine in the standard sample (mg/kg);

A_{HS} is the area of the histamine standard peak;

A_{IS} is the area of the internal standard peak.

8.2 Histamine quantification

Calculate the concentration of histamine in the sample by using Formula (2) (regression equation):

$$C_H = \frac{\frac{A_H}{A_{IS}} \times \frac{5}{m}}{a} \tag{2}$$

where

C_H is the measured mass concentration of histamine in the sample (mg/kg);

A_H is the area of the histamine peak;

A_{IS} is the area of the internal standard peak;

a is the slope of the calibration line;

m is the mass of the sample taken.

The mass, m, usually corresponds to 5 g but if the sample concentration falls outside the range of the standard samples, conduct a new analysis with a smaller test portion in order to be in the linear range regarding representativity of the sample.

NOTE If the matrix is complex or difficult to obtain in free histamine condition (e.g. fishmeal, fish sauce, etc.), the spiking can be performed directly using the standard addition method.

9 Precision

9.1 Interlaboratory study

The results of the interlaboratory study to determine the precision of the method are summarized in Annex B. The values derived from the interlaboratory study may not be applicable to concentration ranges and food types other than those given in Annex B.

9.2 Repeatability limit

The absolute difference between two independent single test results or the ratio of the higher to the lower of the two test results on the normal scale, obtained using the same method on identical test material in the same laboratory by the same operator using the same apparatus within the shortest feasible time interval, will in not more than 5 % of cases exceed the repeatability limit r. The repeatability values for the collaborative test are given in Table 2.

9.3 Reproducibility limit

The absolute difference between two single test results obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases exceed the reproducibility limit R. The reproducibility values for the collaborative test are given in Table 2.

Table 2 — Mean values, repeatability (*r*) and reproducibility (*R*) limits for histamine in the collaborative test

	$\bar{x}$ mg/kg	*r* mg/kg	*R* mg/kg
Tuna	24,01	12,24	21,67
	93,10	15,93	34,02
	200,30	17,98	54,24
Herring	11,07	18,79	19,71
	26,87	3,86	10,98
	89,65	15,62	24,44
Mackerel	18,35	7,53	10,84
	70,11	7,45	25,17
	146,80	24,61	60,90

Annex A
(informative)

Recommendations for HPLC separation

A.1 LC conditions

HPLC and UHPLC systems can be used. The parameters given in this clause are examples and indicative because they depend upon the instrument and the column used. Follow the manufacturer's instructions.

Injection volume:	20 µl
Column:	C18 or equivalent
Column oven:	25 °C ± 2 °C
Tray temperature:	5 °C ± 2 °C
Mobile phase:	1 ml/min
Gradient:	acetonitrile/water

A.2 Order of sample injections

Inject samples in the following order:

— pure acetonitrile (check of interferences);

— calibration standard samples;

— samples;

— continuing calibration verification (CCV) samples (same concentration of one concentration calibration curve);

— pure acetonitrile (column washing).

A.3 Elution range

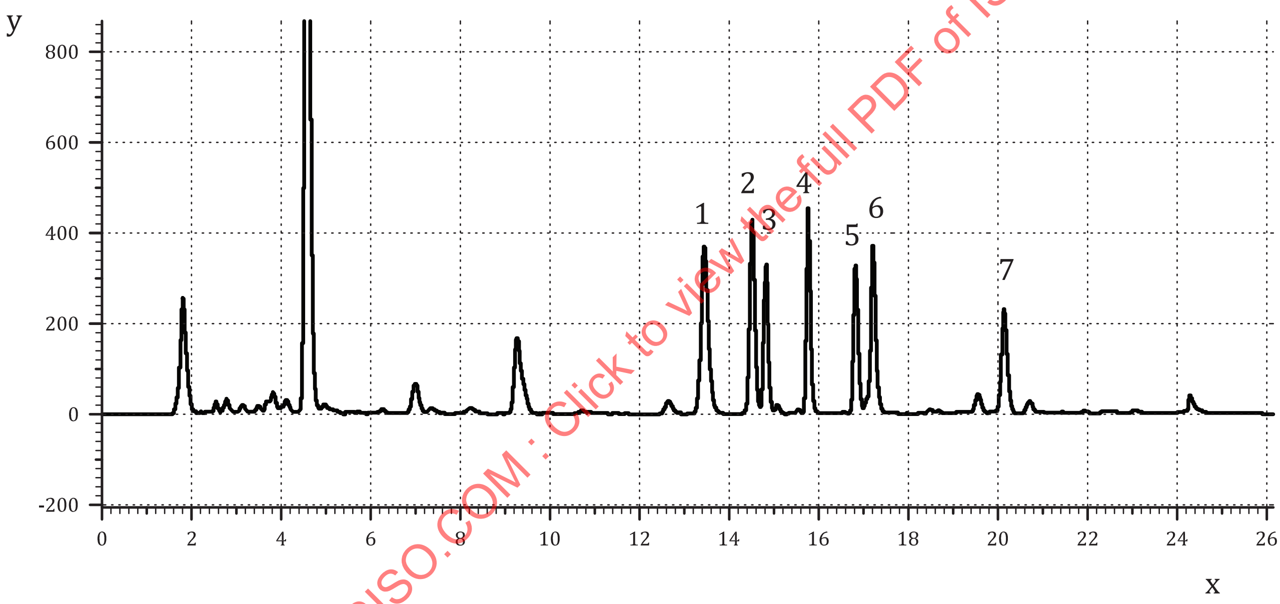
See Table A.1.

Table A.1 — Example of elution range for Kromasil®[2)] C18 5 µm 100 A (25 cm × 4,6 mm)

Time min	Water %	Acetonitrile %
0	40	60
11	25	75
11,1	5	95
20	5	95
20,1	40	60
30	40	60

A.4 Chromatogram

See Figure A.1.



Key

1 putrescine
2 cadaverine
3 histamine
4 internal standard
5 tyramine
6 spermidine
7 spermine
x minutes
y mAU

Figure A.1 — Example of chromatogram performed in tuna with biogenic amines (putrescine, cadaverine, histamine, tyramine, spermidine and spermine) at a level of 100 mg/kg and an internal standard (5.12)

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Annex B
(informative)

Performance characteristics of the method

B.1 Results of the interlaboratory study

An international interlaboratory study was organized by ANSES-Laboratory for Food Safety (Boulogne-sur-Mer, France) in the frame of the CEN Mandate M381. This study involved 9 laboratories from 7 countries and was conducted on tuna, herring and mackerel. Each species was tested at three levels of spiking. The study was organized in 2013 in accordance with ISO 5725-2:1994. Samples were sent in cans after sterilization. A calibration line was calculated on tuna free of histamine.[2],[3],[4],[5]

B.1.1 Interlaboratory study of tuna (*Thunnus albacares*)

The results are presented in Table B.1. No data were excluded.

Table B.1 — Validation data for tuna

Theoretical level	TUNA		
	25 mg/kg	100 mg/kg	220 mg/kg
Number of data	36	36	36
Mean value (mg/kg)	24,01	93,10	200,30
Recovery (%)	96,03	93,10	91,05
Repeatability standard deviation, s_r (mg/kg)	4,37	5,69	6,42
Coefficient of variation of repeatability, $C_{V,r}$ (%)	18,2	6,11	3,20
Repeatability limit (r = 2,8 x s_r) (mg/kg)	12,24	15,93	17,98
Repeatability limit (r = 2,8 x $C_{V,r}$) (%)	50,96	17,11	8,97
Reproducibility, S_R (mg/kg)	7,74	12,15	19,37
Coefficient of variation of reproducibility, $C_{V,R}$ (%)	32,23	13,05	9,67
Reproducibility limit (R = 2,8 x s_R) (mg/kg)	21,67	34,02	54,24
Reproducibility limit (R = 2,8 x $C_{V,R}$) (%)	90,26	36,54	27,07
HorRat value according to Horwitz	3,27	1,63	1,36

B.1.2 Interlaboratory study of herring (*Clupea harengus*)

The results are presented in Table B.2. No data were excluded.

To avoid matrix effect and bias above, carry out calibration line on the same matrix as the sample analysed.

Table B.2 — Validation data for herring

Theoretical level	HERRING		
	25 mg/kg	100 mg/kg	220 mg/kg
Number of data	36	36	36
Mean value (mg/kg)	11,07	26,87	89,65
Recovery (%)	44,29	26,87	40,75
Repeatability standard deviation, s_r (mg/kg)	6,71	1,38	5,58
Coefficient of variation of repeatability, $C_{V,r}$ (%)	61,61	5,14	6,22
Repeatability limit (r = 2,8 x s_r) (mg/kg)	18,79	3,86	15,62
Repeatability limit (r = 2,8 x $C_{V,r}$) (%)	169,72	14,38	17,43
Reproducibility, S_R (mg/kg)	7,04	3,92	8,73
Coefficient of variation of reproducibility, $C_{V,R}$ (%)	63,59	14,59	9,74
Reproducibility limit (R = 2,8 x s_R) (mg/kg)	19,71	10,98	24,44
Reproducibility limit (R = 2,8 x $C_{V,R}$) (%)	178,07	40,85	27,27
HorRat value according to Horwitz	6,45	1,82	1,37

B.1.3 Interlaboratory study of mackerel (*Scomber scombrus*)

The results are presented in Table B.3. No data were excluded.

To avoid matrix effect and bias above, carry out calibration line on the same matrix as the sample analysed.

Table B.3 — Validation data for mackerel

Theoretical level	MACKEREL		
	25 mg/kg	100 mg/kg	220 mg/kg
Number of data	36	36	36
Mean value (mg/kg)	18,35	70,11	146,80
Recovery (%)	73,39	70,11	66,73
Repeatability standard deviation, s_r (mg/kg)	2,69	2,66	8,79
Coefficient of variation of repeatability, $C_{V,r}$ (%)	14,66	3,79	5,99
Repeatability limit (r = 2,8 x s_r) (mg/kg)	7,53	7,45	24,61
Repeatability limit (r = 2,8 x $C_{V,r}$) (%)	41,05	10,62	16,77
Reproducibility, S_R (mg/kg)	3,87	8,99	21,75
Coefficient of variation of reproducibility, $C_{V,R}$ (%)	21,09	12,82	14,82
Reproducibility limit (R = 2,8 x s_R) (mg/kg)	10,84	25,17	60,90
Reproducibility limit (R = 2,8 x $C_{V,R}$) (%)	59,05	35,90	41,48
HorRat value according to Horwitz	2,14	1,60	2,09

B.2 Precision data

B.2.1 Study of performance at 400 mg/kg for different matrices

The study was performed by Anses (France). The results are presented in Table B.4.

Table B.4 — Determination of recovery for tuna, mackerel and fish sauce

	Tuna	Anchovy[a]	Fish sauce
Sample 1	396,32	414,28	423,19
Sample 2	387,52	365,17	397,62
Sample 3	401,88	421,81	402,08
Sample 4	381,78	378,73	398,44
Sample 5	422,09	434,21	394,99
Sample 6	401,70	425,71	403,28
Mean value (mg/kg)	398,55	406,65	403,27
Recovery (%)	99,64	101,66	100,82
SD	14,02	27,97	16,92

[a] Which have undergone enzyme maturation treatment in brine.

B.2.2 Intralaboratory performance study

The study was performed by Anses (France). A diagram of the accuracy profile is presented in Figure B.1.

Accuracy profile

Tolerance probability (beta)	80 %
Limits of acceptability	40 %

Levels	1	2	3	4	5
Theoretical level (mg/kg)	25,00	50,00	100,00	200,00	400,00
Mean value (mg/kg)	27,00	54,45	107,55	210,85	391,00
Precision standard deviation	4,67	5,44	8,95	17,14	41,89
Intermediate coefficient	1,02	1,05	1,07	1,08	1,08
Number of degrees of freedom	18,18	10,96	6,56	5,76	5,50
Low tolerance value (mg/kg)	20,59	46,60	93,77	183,90	324,59
High tolerance value (mg/kg)	33,40	62,29	121,33	237,79	457,40
Absolute bias (mg/kg)	2,00	4,45	7,55	10,850	−9,00
Recovery (%)	108,00	108,90	107,55	105,43	97,75
Low tolerance limit (%)	82,39	93,20	93,77	91,95	81,15
High tolerance limit (%)	133,61	124,60	121,33	118,90	114,35
Limit low acceptability (%)	60,00	60,00	60,00	60,00	60,00
Limit high acceptability (%)	140,00	140,00	140,00	140,00	140,00
Relative uncertainty (%)	38,51	23,02	19,33	18,60	22,77