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Equivalence testing of histamine methods - Final Report

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SANCO) and Joint Research Centre (JRC)*

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FINAL REPORT

ADMINISTRATIVE ARRANGEMENT

N °SANCO/2011/G4/JRC32515/SI2.611754

BETWEEN

DG Health and Consumers (DG SANCO)

AND

JOINT RESEARCH CENTRE (JRC)

Equivalence testing of the reference method for determination of histamine
mandated by Commission Regulation (EC) 2073/2005 to the Codex Alimentarius
standard AOAC 977.13

Joerg Stroka, Katrien Bouten, Carsten Mischke, Andreas Breidbach & Franz Ulberth

Contents

Executive Summary	3
1. Introduction.....	4
2. Objectives	6
3. Study protocol	6
4. Materials and Methods	8
4.1. HPLC method mandated by Regulation (EC) No 2073/2005.....	9
4.2. Fluorimeter method according to AOAC 977.13 (Codex Method).....	10
4.3. Statistical data evaluation	11
5. Results	11
5.1. Verification of the appropriate performance of the two methods.....	11
5.2. Conformity of the methods to standard method performance requirements.....	15
5.3. Method comparison	16
5.3.1. Hypothesis testing	16
5.3.2. Regression analysis.....	17
5.3.3. Reference materials and methods	19
6. Conclusion	21
7. References	22
8. List of Annexes.....	24

Executive Summary

Histamine fish poisoning is an allergy-like form of food poisoning that continues to be a major problem in seafood safety. The FAO/WHO Codex Alimentarius as well as EU legislation have therefore set maximum limits for histamine in fish and fish products. The analytical methods requested by Codex and by EU are different and concern has been raised that this could lead to disputes in the international trade of seafood.

This report describes the outcome of a study, commissioned by DG Health and Consumers and carried out by DG Joint Research Centre, that compared the performance of the method for determining histamine in fish as mandated by Regulation (EC) No 2073/2005 to the method mandated in Codex Alimentarius Standard 165-1989. The EU mandated method is based on HPLC separation of histamine and subsequent detection by a UV detector. It was published in the Journal of AOAC International, but has not been validated by a collaborative study. The Codex method is AOAC 977.13, which is based on the formation of a fluorescent derivative of histamine and subsequent measurement in a fluorimeter; it has been validated by collaborative trial.

The correct implementation of both methods by JRC was assessed by carrying out performance verification studies using various canned and fresh scromboid fish samples (tuna, macrel, and herring) taken from the Belgian market. Repeatability (RSD_r) and intermediate precision (RSD_{ip}) as well as recovery data were generated. Both methods conformed to specifications.

Various approaches were followed to test the equivalency of both methods, which were based on statistical hypothesis testing (t-test), regression analysis and benchmarking against established reference values.

All approaches indicated that the two methods are not fully equivalent. The EU mandated method has a tendency to overestimate, while the Codex method has a tendency to underestimate the histamine content in fish.

It was recognised that the EU mandated method was very accurate when applied to fresh tuna. A distinct matrix influence was noticed for all other fish species tested, leading to an overestimation of the histamine content.

It is therefore recommended to optimise the EU method so that matrix effects can be eliminated, or at least taken into account in an appropriate manner. In addition, a collaborative trial for the HPLC method to establish reproducibility data for the method should be organised. In line with current practice the collaborative study should also require to correct the reported data for recovery. Furthermore, as an ad-hoc measure the replacement of the HPLC method mentioned in Regulation (EC) No 2073/2005 by a ring-tested HPLC method, which are already available, could be considered.

1. Introduction

Histamine and other biogenic amines are generated in improperly stored raw fish by enzymatic conversion of free histidine and other amino acids. Decarboxylase producing Gram-negative enteric bacteria are primarily responsible for the formation of histamine in raw fish products. Improper storage conditions (time/temperature) are the main reason for bacterial growth. Consumption of such mishandled fish can lead to histamine fish poisoning, also termed scombroid poisoning. The symptoms are similar to those associated with sea food allergies. The term “scombroid” derives from the Scombridae family, such as tuna, albacore and mackerel, since these species have high levels of free histidine in their muscle tissues.

Regulation (EC) No 2073/2005 [1] limits the content of histamine in fishery products from fish species associated with a high amount of histidine to between 100-200 mg/kg, and in fishery products which have undergone enzyme maturation treatment in brine, manufactured from fish species associated with a high amount of histidine to between 200-400 mg/kg. The basis for decision making is a three-class attributes sampling plan ($n=9$, $c=2$, $m=100$ and $M=200$ mg/kg, or $m=200$ and $M=400$). Codex Alimentarius [2] limits histamine to 200 mg/kg for species of Clupeidae, Scombridae, Scombrosocidae, Pomatomidae and Coryphaenidae families, whereas the United States Food and Drug Administration has set a guidance level of 50 mg/kg histamine in the edible portion of fish [3].

As an accurate estimate of the histamine content forms the basis of acceptance or rejection of a lot, analytical methods have been specified by both EU legislation as well as Codex Alimentarius. EU legislation mandates the use of a method where a fish extract is derivatised and separated by high performance liquid chromatography (HPLC); quantification of the separated histamine derivative is done by using an ultra violet (UV) detector [4, 5]. Codex Alimentarius mandates the use of the AOAC 977.13 method [6], which is based on quantification of derivatised histamine by fluorescence measurements without prior HPLC separation.

Histamine in fish can be determined by various analytical methods and the ones applied frequently have been reviewed in 2007 by Onal [7]. Fluorimetry such as AOAC 977.13 is specific to histamine, and does not cover other eventually present biogenic amines. Chromatographic methods such as thin layer chromatography (TLC), gas chromatography (GC), capillary electrophoresis (CE) and HPLC offer as an advantage the simultaneous quantification of several biogenic amines next to histamine. These methods also have a broader dynamic range and have generally a lower detection limit compared to fluorimetry.

Commercially available immunochemistry based test kits for the determination of histamine are also available and the performance of several test kits were recently described by Köse [8].

An advantage of fluorimetry is its relative simplicity, freedom of intellectual property rights and the fact that the method only requires a fluorimeter as instrument.

Fluorimetry has been validated decades ago with success in a collaborative study for canned tuna and frozen Mahi Mahi. On the other hand chromatographic methods, especially HPLC, are more versatile, but require usually a higher level of maintenance and it appears that the methodology is rather susceptible to small changes in the method, having an impact on the precision. Three HPLC methods for histamine, which are based on HPLC using post-column derivatisation in combination with fluorimetric detection, have been validated in collaborative trial studies [9, 10, 11]. They are based on perchloric acid extraction of homogenized fish samples, HPLC separation of histamine on a reversed-phase column and fluorometrical quantification (excitation, 340 nm; emission, 455 nm) of histamine after post-column derivatisation with o-phthaldialdehyde (OPA). In one validation study [10] samples containing histamine at about 10-400 mg/kg were analysed. Repeatability relative standard deviations (RSD_r) varied from 2.1 to 5.6 %, and reproducibility relative standard deviations (RSD_R) ranged from 2.2 to 7.1 %. Averaged recoveries of histamine for this concentration range varied from 94 to 100 %. The German official method [11] was collaboratively validated for tuna, salmon and herring. At low histamine levels (12-18 mg/kg) RSD_r was 7 %, and RSD_R ranged from 6.8 to 17.2 %, while at high levels (372 mg/kg) the respective values were 3 % and 7 %.

The HPLC method mandated by Regulation (EC) No 2073/2005 is also based on extraction by perchloric acid, derivatisation using dansyl chloride, extraction of the derivative into toluene and HPLC separation with detection at 254 nm [4, 5]. The method has not been validated by collaborative study. The Nordic Committee on Food Analysis (NMKL) has recently published validation data for a similar method, although for the tuna sample included in the exercise precision was poor due to very low histamine levels [12]. The difference between the NMKL method and the EU mandated method is that the internal standard 1,3-diaminopropane (EU method) is replaced by 1,7-diaminoheptane (NMKL method).

Fluorimetric and HPLC methods have been subjected to a comparison study, commissioned by the UK Food Standards Agency (FSA) [13]. This comparison was based on data from two proficiency test (PT) rounds organised by the Food Analysis Performance Assessment Scheme (FAPAS®). Test materials included unprocessed and processed fish, and meat and cheese containing two different, known concentrations of histamine. Data reported by the participating laboratories were critically reviewed to assess the effect of methodological differences on the produced test results. The study came to the conclusion that fluorimetry as well as HPLC methods were suitable, under appropriate conditions, for the determination of histamine in fish. HPLC methods offered the advantage to be able to determine other biogenic amines as well. Among the HPLC methods tested those applying OPA post-column derivatisation had superior overall performance in all foodstuffs examined. The study further concluded that *"Variability between analytical procedures does not appear to be a significant problem when methods are under analytical control but it is apparent from the FAPAS data that some laboratories still have difficulties in this area. Analytical errors, lack of experience with the method used and incorrect selection of the appropriate method are all likely factors in this*

variability. From the work carried out, any of the methods studied, when properly validated, will give acceptable results for the determination of histamine".

The study also remarked that in the original publication [4] describing the HPLC method mandated by Regulation (EC) 2073/2005, many sample preparation steps were studied in order to optimise the procedure and small changes were observed to have large effects. Evidence of this variation was seen in the FSA study by measuring the absolute response of the internal standard used. In short, the signal magnitude (peak area) of the same amount of internal standard varied considerably between chromatographic runs. Care is therefore required to ensure that the internal standard is correctly used and quantified as any errors in this measurement will be directly reflected in the results obtained.

With mandate M/381 the European Commission requested the European Committee for Standardization (CEN) to provide standardized and validated reference methods within the framework of food hygiene legislation. Among the mandated items is also the validation of a method for the detection and quantification of histamine. A draft work instruction has been prepared by ISO/TC 34, Food products, Subcommittee SC 9, Microbiology in collaboration with CEN Technical Committee CEN/TC 275, Food analysis — Horizontal methods, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement). The draft [14] is based on the HPLC method [4, 5] mandated by Regulation (EC) 2073/2005, with the exception that the original internal standard 1,3-diaminopropane is replaced by 1,7-diaminoheptane. However, the method has not been submitted to validation by collaborative study yet.

2. Objectives

As the analytical method principle specified in Regulation (EC) No 2073/2005 for the quantification of histamine in fish deviates from AOAC 977.13 as mandated in Codex Alimentarius Standards, different results for the same samples of fish cannot be excluded. Therefore, DG SANCO.E2 has asked JRC-IRMM to study the equivalence of the two methods for the determination of histamine in fish.

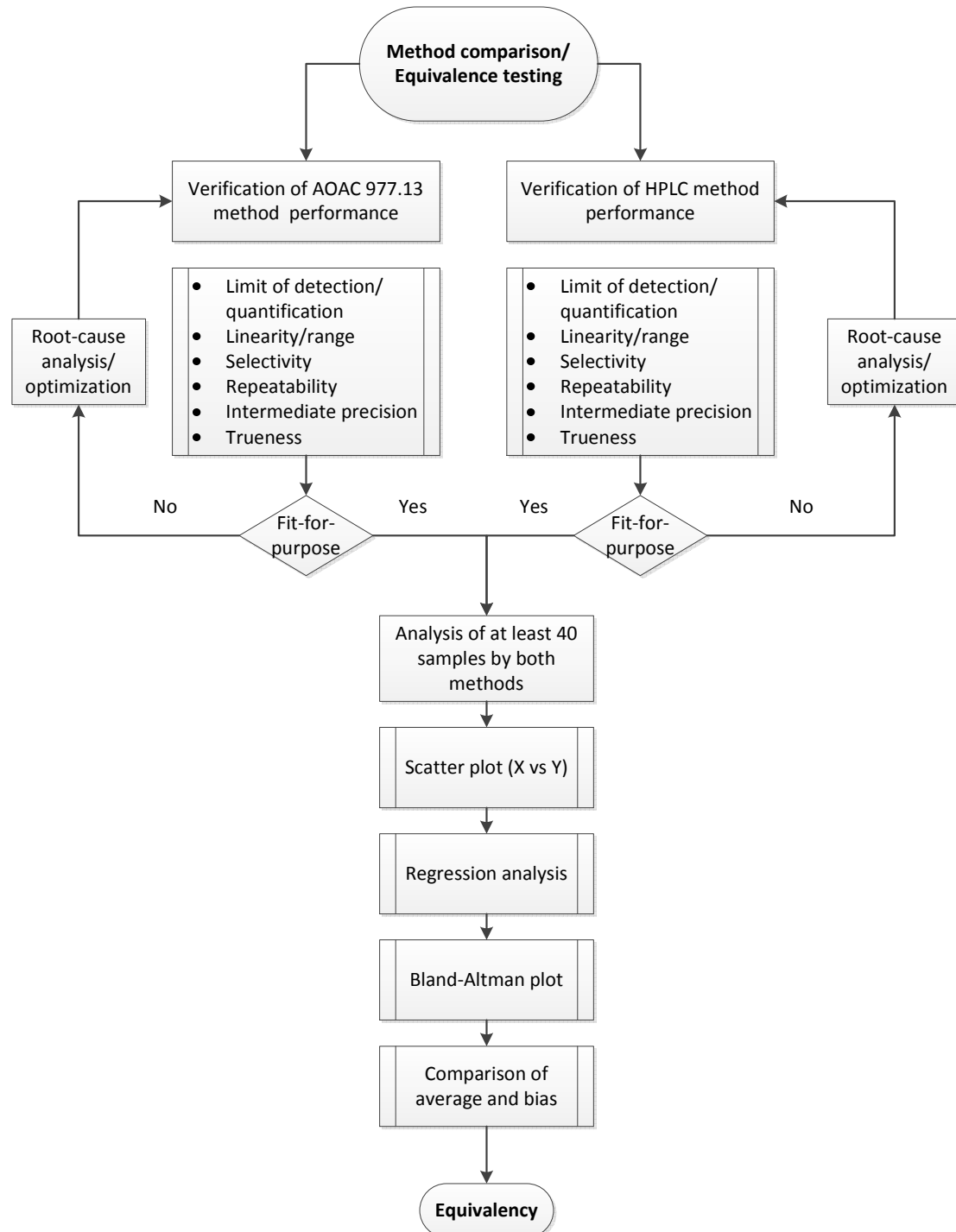
3. Study protocol

The goal of a method comparison experiment is to generate adequate data to evaluate the equivalency of the two methods over a range of concentrations [15]. Two independently validated methods are not inherently equivalent; validation typically determines the quality of a single analytical method, whereas equivalency demonstrates the sameness of the results produced by two analytical methods.

As the concept of equivalence of methods is not well covered in guidance documents issued by chemical societies such as the International Union of Pure and Applied Chemistry (IUPAC) or EURACHEM, relevant protocols related to the area of clinical chemistry and drug testing were used for developing the study protocol. In

particular, the Clinical and Laboratory Standards Institute (CLSI) EP9-2A Method Comparison and Bias Estimation Using Patient Samples; Approved Guideline [17] and the US Pharmacopeia, General Chapter <1010> Analytical Data: Interpretation and Treatment [15] texts were used.

The general outline of the method comparison study was as follows:



The correctness of the implementation of each method was verified at least at the decision levels of 100 and 200 mg histamine/kg using fresh and canned tuna, herring, and mackerel, and fresh cod (spiked samples). The following method performance characteristics were investigated:

- Limit of detection (LOD) and quantification (LOQ)
- Range and linearity
- Selectivity
- Repeatability
- Intermediate Precision
- Trueness (expressed as recovery)

Following the verification for each method, a direct method comparison (DMC) was carried out with the aim to test any observed difference in the methods for statistical significance. The design took into consideration a sufficiently large series of determinations carried out with both methods. Experiments were carried out on fish naturally incurred with histamine (either obtained contaminated or by controlled microbiological spoilage to induce histamine contamination) where possible.

A total of 104 duplicate analyses have been carried out for the DMC. This comparison included tuna, mackerel and herring all in fresh and canned state (including FAPAS® test samples). The majority of samples were initially found free of histamine. Therefore test samples were partially fortified with histamine and partially kept at room temperature for spoilage, resulting in samples naturally incurred with histamine.

This set of result allowed the generation of statistically meaningful data to test the equivalence of both methods in combination with the method performance obtained in the verification step.

Naturally incurred fish samples were in addition analysed with a reference method based on exact matching stable isotope dilution mass spectroscopy (IDMS) in order to test each method for trueness with an external reference. For the same purpose FAPAS® reference materials were used as well.

4. Materials and Methods

Fresh tuna, herring, mackerel and cod were purchased at local fish shops and canned tuna, herring, mackerel in a local supermarket.

4.1. HPLC method mandated by Regulation (EC) No 2073/2005

The work instruction as it was used in this study is described in the Annex. In short, five gram of the test material was extracted after addition of 100 μ L of internal standard (1,3-diaminopropane) with 10 mL 0.2 molar perchloric acid with a high speed blender (Ultraturrax).

The extract was centrifuged and 100 μ L of the supernatant was neutralized with 200 μ L of saturated sodium hydrogen carbonate solution. Four hundred μ L of the derivatisation agent (dansylchloride) were added. The reaction mixture was left for 10 min in the darkness at 60 °C for complete derivatisation of histamine. The mixture was cooled to room temperature and after the addition of 100 μ L of a L-proline solution it was left at room temperature to neutralize the surplus dansylchloride.

To this mixture 500 μ L of toluene was added, thoroughly shaken and put in the freezer to freeze out the aqueous phase. The toluene was completely transferred to a new vial, evaporated to dryness and reconstituted in 200 μ L acetonitrile for injection and determination by HPLC and detection at 254 nm.

The HPLC separation took place on a 15 cm, 4.6 mm i.d. 3.5 μ m particle size Agilent Zorbax SB-C18 column with a mobile phase gradient at a flow rate of 1.4 mL/min at 45 °C column temperature. The mobile phases were water (mobile phase A) and acetonitrile (mobile phase B). The gradient conditions are given in Table 1.

Time [min]	Mobile phase A [%]	Mobile phase B [%]
0.0	40	60
2.0	25	75
2.6	25	75
4.3	5	95
6.6	5	95
6.7	40	60
10	40	60

Table 1: Gradient flow of the HPLC mobile phase

The samples were injected in a sequence starting with neat mobile phase, and test samples in-between calibrants. Calibrants were injected at least every 10th test sample. Typical chromatograms for tuna samples containing histamine and other biogenic amines are shown in Figure 1.

Equivalence testing of histamine methods

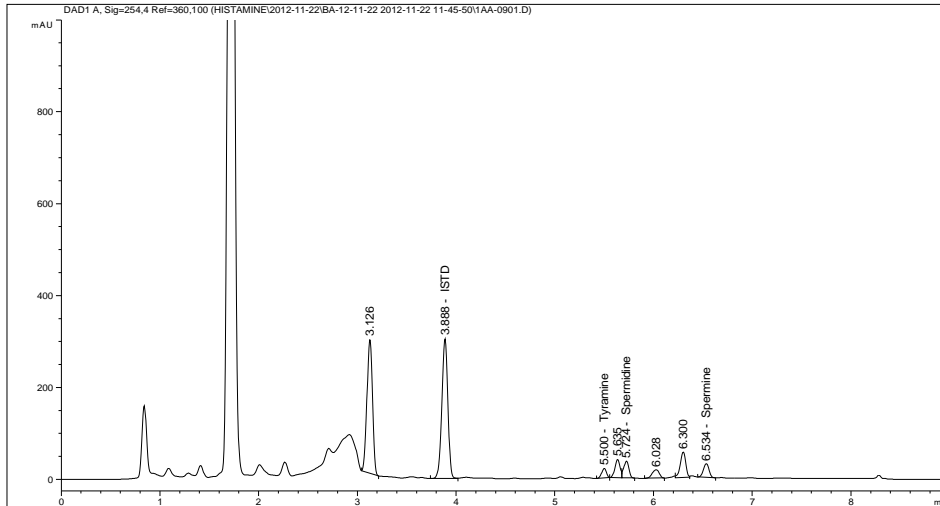


Figure 1a: Chromatogram of a raw tuna sample containing no histamine but a variety of biogenic amines and the internal standard (ISTD)

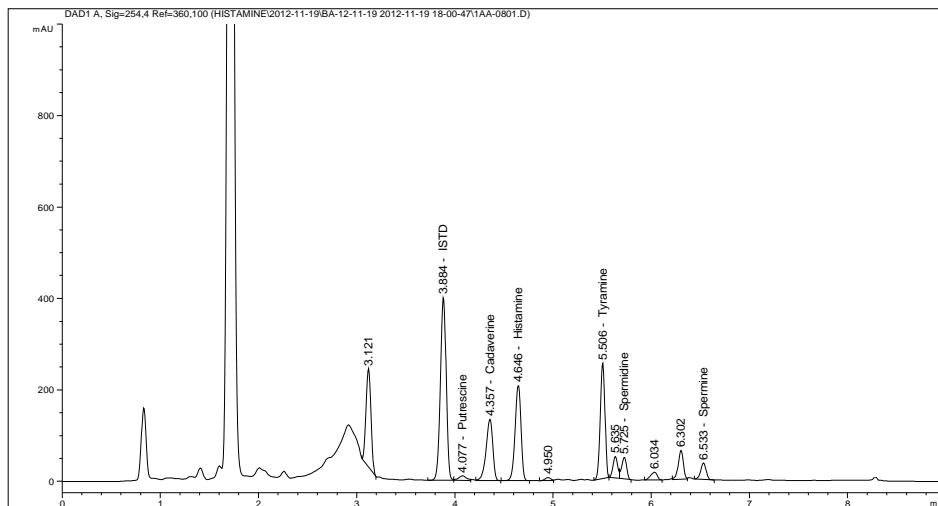


Figure 1b: Chromatogram of a spoiled canned tuna sample containing histamine (retention time 4.65 min) in the presence of a variety of other biogenic amines and the internal standard (ISTD)

4.2. Fluorimeter method according to AOAC 977.13 (Codex Method)

Histamine was determined following the protocol of the AOAC 977.13 method. The work instruction as used is listed in the Annex. In short the fish material was extracted with methanol, the filtered extract purified over an ion-exchange column and the obtained histamine determined in a fluorimeter after addition of the derivatisation reagent.

4.3. Statistical data evaluation

Data were evaluated using MS Excel 2010, SigmaPlot 12.0 and macros from the R Project for Statistical Computing (<http://www.r-project.org/>).

5. Results

5.1. Verification of the appropriate performance of the two methods

In the **matrix applicability** test the following fresh and canned fish species were involved: tuna, mackerel, herring and cod (only fresh).

The **concentration range** for the fluorimeter method was by the nature of the method design LOQ - 150 mg/kg; samples with a higher level of histamine had to be diluted prior measurement and the dilution factor taken into account. This extended the calibration range to 450 mg/kg. The concentration (operation) range for the HPLC method was LOQ to 400 mg/kg.

Both methods were found to be linear within their working ranges and no lack of fit was observed for linear regression. The lack of fit analyses have been performed using macros from the R Project for Statistical Computing.

Selectivity for the fluorimetric method was tested by determination of histamine in the presence of other biogenic amines (cadaverin, putrescine and tyramine) and no influence of these other biogenic amines was observed.

This observation is in line with the previously described findings of Rönnerberg and Håkanson [18] and Kuruma et al [19]. Histamine forms specific fluorescent adducts with OPA (Figure 2), while other amines will need an additional coupling reagent such as a thiol to form fluorescent derivatives. However both research groups claim slightly different adducts to be formed. One of the main reactions is proposed as (Figure 2):

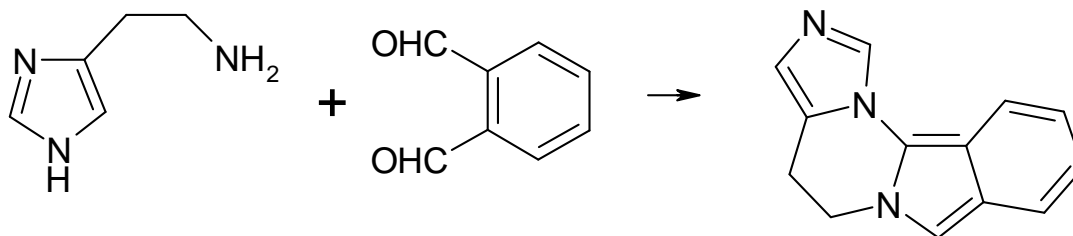


Figure 2: reaction of histamine and ortho-phthalaldehyde (OPA)

Biogenic amine selectivity for the HPLC method was demonstrated as the method resolves all biogenic amines to individual substances (signals) during the HPLC run. Representative chromatograms of are shown in Figure 1.

The precision parameters - **repeatability** (RSD_r) and **intermediate precision** (RSD_{ip}) - were tested for both methods with a variety of different fish types. Initially the validity of the fluorimeter method was tested by repetitive determination of histamine from independently prepared samples under repeatability conditions. The fluorimeter method gave a RSD_r value of 2 % analysing 10 replicates of a surplus FAPAS® canned tuna sample with an assigned value 153 mg/kg (satisfactory range 130-176 mg/kg). The mean level of the replicates was found to be 169 mg/kg and therefore in the satisfactory range.

For the generation of fish matrix specific precision parameter for both methods, different fish test portions were weighed independently and each test portion was subsequently analysed by both methods. This experiment was repeated on different days starting each day with a new analysis of a different portion of the sample material. Analysis of variance (ANOVA) was used to evaluate the data and estimate relative standard deviations for repeatability (RSD_r) and intermediate precision (RSD_{ip}). A summary is given in Tables 2 and 3.

Matrix	RSD_r [%]	RSD_{ip} [%]
Fresh tuna	6.3	6.3
Canned tuna	2.8	3.4
Fresh herring	5.1	8.9
Fresh mackerel	3.4	3.4
Fresh Cod	6.9	6.9

Table 2: Precision data for the fluorimeter method

The repeatability data (RSD_r) confirmed the initial finding of the experiment with the available FAPAS material, resulting in a RSD_r value of around 3-5 %. The intermediate precision (day-to-day variability) was found to be slightly higher ranging from 3.4 % for canned tuna to 8.9 % for fresh herring.

Matrix	RSD_r [%]	RSD_{Ri} [%]
Fresh tuna	3.6	14
Canned tuna	4.1	11
Fresh herring	3.9	12
Fresh mackerel	5.3	11
Fresh cod	5.2	14

Table 3: Precision data for the HPLC method

The resulting RSD_r for the HPLC method was of the same magnitude as the fluorimeter method, however with a somewhat higher day-to-day variability. This indicates that results generated within a sequence have a comparable precision for both methods, while the results vary slightly more when experiments are repeated on different days, compared to the fluorimeter method.

The **recovery rate** was determined for each method – fluorimetry and HPLC - by fortification of histamine free test material with histamine at levels ranging from 50 mg/kg to 400 mg/kg. This allowed monitoring if the recovery is a function of the spiked concentration. However no evidence that the methods recovery was dependent on the concentration (within the range of 50 – 400 mg/kg) could be found. The recoveries for the fluorimeter method were rather consistent with a relatively small variability between the different fish types tested (Table 4 and Figure 3).

Matrix	Recovery [%], (min-max)
Canned herring	85 (83-87)
Canned mackerel	86 (82-93)
Canned tuna	91 (85-97)
Fresh herring	82 (78-85)
Fresh mackerel	77 (70-85)
Fresh tuna	90 (86-94)

Table 4: Recovery rate for the fluorimetric method

Recoveries for the HPLC method were calculated on the basis of the internal standard (Table 5a) as described in the methods listed in Regulation (EC) No 2073/2005 [4, 5], and by external standard calibration (Table 5b) without making use of the internal standard.

Matrix	Recovery [%], (min-max)
Canned herring	140 (125-159)
Canned mackerel	126 (112-139)
Canned tuna	120 (101-166)
Fresh herring	113 (96-132)
Fresh mackerel	112 (100-129)
Fresh tuna	103 (93-120)

Table 5a: Recovery rate for the HPLC method and calibration with internal standard (1,3-diaminopropane)

Matrix	Recovery [%], (min-max)
Canned herring	103 (81-121)
Canned mackerel	109 (76-136)
Canned tuna	111 (86-143)
Fresh herring	99 (71-132)
Fresh mackerel	90 (75-108)
Fresh tuna	92 (82-106)

Table 5b: Recovery rate for the HPLC method and external calibration

All recovery rates from both method used and the different types of calibration for the HPLC method are graphically shown in Figure 3.

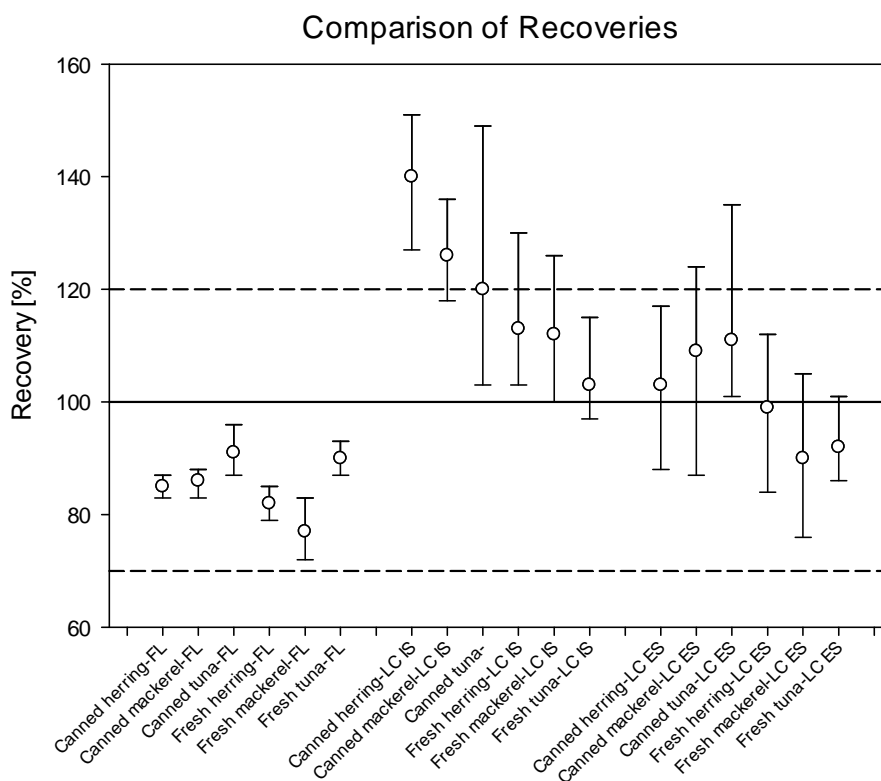


Figure 3: Summary of all recovery experiments:

FL refers to the recovery figures from the table above for the fluorimeter method, LC IS refers to the HPLC method using the internal standard and LC ES refers to the HPLC method calculating the results by external calibration only, not taking the internal standard into account. The upper and lower dashed horizontal lines refer to the recovery range considered by most analytical as acceptable recovery (120% and 70% recovery). The error bars reflect the upper (0.9) and lower (0.1) percentile of all recover results obtained for each scenario.

Limit of Detection (LOD) and **Limit of Quantification (LOQ)** were calculated on the basis of ISO 11843 part 2 based on data of recovery experiments for the fluorimeter method. For the HPLC method calculation of these parameters was done on the basis of the prediction interval of the calibration curve using macros from the R Project for Statistical Computing. The outcome of the LOD and LOQ experiments are summarised in Tables 6 and 7.

Matrix	LOD [mg/kg]	LOQ [mg/kg]
Tuna	5	21
Mackerel	7	19
Herring	4	44
cod fish	5	31

Table 6: LOD and LOQ of the fluorimetric method

Matrix	LOD [mg/kg]	LOQ [mg/kg]
Tuna	3.6	21
Mackerel	3.1	25
Herring	3.1	25
Cod fish	1	4

Table 7: LOD and LOQ of the HPLC method

Both methods showed comparable LODs and LOQs that demonstrated that both methods are fit for purpose with respect to monitoring samples for histamine at the levels of interest (100 mg/kg – 400 mg/kg histamine).

5.2. Conformity of the methods to standard method performance requirements

Matrix applicability was demonstrated by showing that the obtained performance parameters were satisfactory for all fish types tested for LOD/LOQ, selectivity, precision and recovery.

The **recovery rate** of both methods met method requirements for official control purposes such as those laid down in EU legislation for other contaminants. The results suggest, however, that the recovery rates for the fluorimeter method are systematically at the lower end of the accepted range (between 70 % and 100 %).

On the other hand the HPLC method showed a wider range of matrix specific recovery rates, ranging from just above 100 % to 140 %. This indicates that the proposed internal standard procedure compensates well for analytical factors related to the analysis of fresh tuna, but results do not allow the assumption that the use of an internal standard is suitable for other matrices such as canned herring (recovery 140 %). Contrary to this, recovery rates generated by means of an external calibration were nearly always in the range between 70 % and 120 %, independent of the fish species tested. Apparently, using an internal standard did not offer an improvement over the external standard calibration procedure, neither for recovery nor precision performance characteristics (Figure 3).

Both methods were also compared for **repeatability** and **intermediate precision**. Table 2 lists the precision data as obtained with the fluorimeter method, which are in good agreement with the validation data of AOAC 977.13 (RSD_r ranging from 2.4 to 6.5 % and RSD_R ranging from 5.4 to 11.0 %). As no collaborative study data are available for the HPLC method [4, 5] comparisons were made to the German official method, which is also based on HPLC [11]. RSD_r was 7 %, and RSD_R ranged from 6.8 to 17.2 % for low histamine levels (< 20 mg/kg), while at high levels (372 mg/kg) the respective values were 3 % and 7 %. The precision data of the HPLC method making use of an internal standard are in good agreement with those values (Table 3).

Both methods compare well for repeatability (RSD_r), thus results generated within a sequence and one day. The HPLC method however showed a larger dispersion of results if analytical sequences were run on different days (RSD_{ip}). This indicates that some factors, which could however not be identified by the JRC, have an influence on results between days.

5.3. Method comparison

5.3.1. Hypothesis testing

In a first attempt traditional **statistical hypothesis testing** was considered. The *t*-test is certainly the most widely applied method to compare two groups of results obtained in different ways. However, the *t*-test has several limitations and may not be the most appropriate technique when the objective is to show equivalence of two data sets. Although it is an appropriate test for proving that two data sets are different, problems arise when the two-sample *t*-test is used to show equivalence. Firstly, the traditional two-sample *t*-test can reward the analyst for having poor precision and/or a small number of observations, and secondly, it may lead the analyst to conclude that a statistically significant difference exists between the mean values when the magnitude of the difference is of no practical importance [20].

Nevertheless, a direct comparison of 95 different results across all fish matrices was attempted using traditional hypothesis testing techniques. The experiments were carried out at various levels ranging from LOQ to around 400 mg/kg. For statistical analysis the results were expressed as % recovery. This normalization (value observed/value expected expressed as % of the expected value) allows a comparison over different spiking levels. The expected value was calculated from spiking experiments.

To test whether data can be pooled irrespective of the fish species analysed, results obtained by one particular method were subjected to ANOVA. The analysis showed that recovery differed significantly between fish species. Therefore, the method comparison by *t*-test had to be carried out for each fish matrix separately. Figure 3 (comparison of recoveries) also support the ANOVA results; the ANOVA was not repeated for the HPLC method as a consequence.

The *t*-tests showed that there is a significant difference between the fluorimeter method and the HPLC method using an internal standard. As mentioned before, an external standard calibration procedure can be used for evaluating the results of the HPLC method, although this is not in compliance with the method protocol [4, 5]. Therefore, the results produced by the fluorimeter method and the HPLC method using external calibration were compared by using the *t*-test. In case of fresh tuna the test results did not differ significantly ($p=0.13$); for the all other fish species significant differences were observed.

However, these results have to be interpreted with caution, since the *t*-test is designed to prove that a difference between two mean values exists, and this feature renders the test not really appropriate for demonstrating equivalence.

5.3.2. Regression analysis

A better way of comparing the equivalence of methods is based on **graphical evaluation (scatter plotting) and regression analysis of the data** in accordance with CLSI EP9-2A [17]. For doing this, all data used for the *t*-test computation were scatter plotted (Figures 4a and 4b).

The plots clearly show the effect the internal standard or external standard calibration of the HPLC method has on the agreement with the data produced by the fluorimetric method.

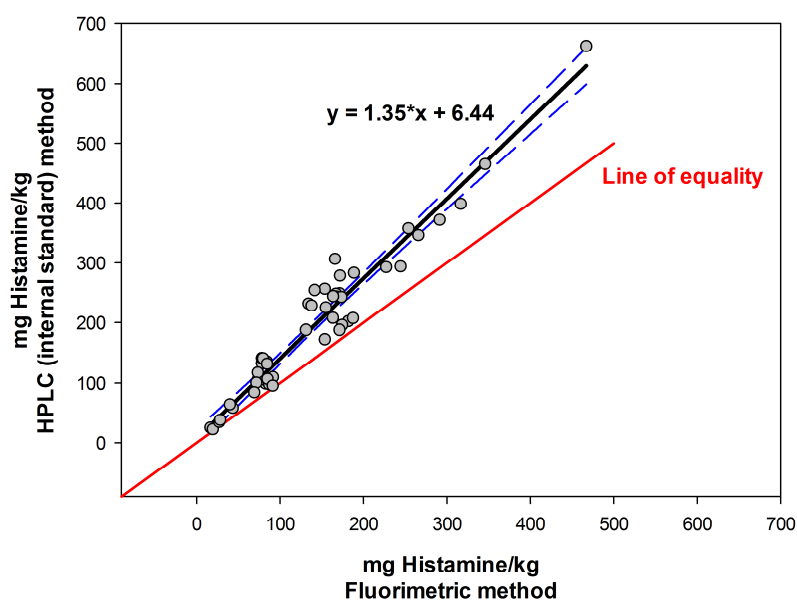


Figure 4a: Scatter plot of data obtained by the fluorimetric and the HPLC method using internal standard calibration

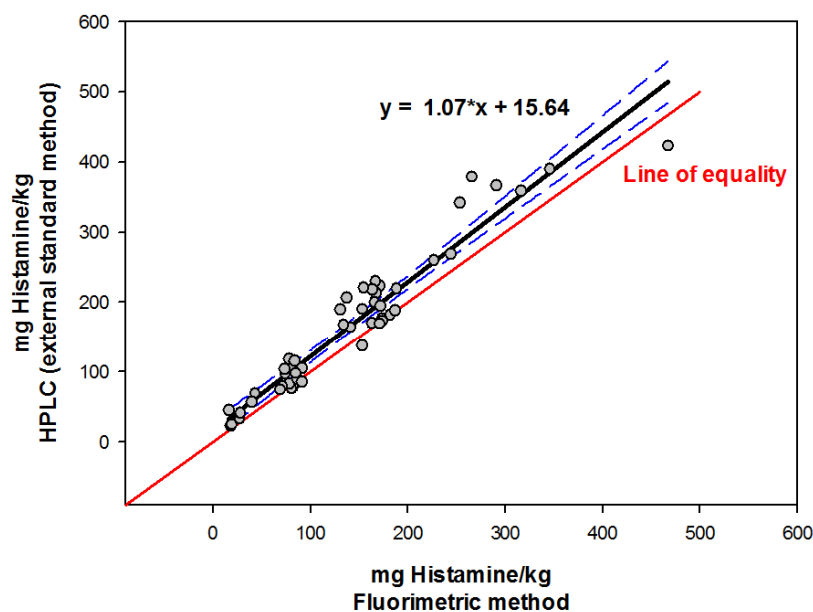


Figure 4b: Scatter plot of data obtained by the fluorimetric and the HPLC method using external standard calibration

In case the two methods would deliver exactly the same results, the slope of the regression line would be unity (line of equality). The deviation from unity can be interpreted as the proportional error and the intercept on the y-axis as constant error [8]. This means that on average the HPLC method applied as described in legislation (use of the internal standard) overestimates by 35 % the outcome of the reference method (Figure 4a), while evaluating the HPLC results without making use of the internal standard resulted in fairly good agreement between the results produced by the alternative and the reference method. In this case the HPLC method with external calibration led on average to an overestimation of 7 % (Figure 4b).

A basic assumption for linear regression analysis is that the X variable (values produced by fluorimetry) is known without error. This is rarely true, because every measurement has intrinsic error. However, if the range of the data is sufficiently wide, the effect of this error on the regression estimates can be considered negligibly small. To safeguard this assumption, slope and intercept were estimated by a technique that accommodates measurement errors in both variables (Deming regression) [22]. The slope and the intercept for the comparison with the HPLC method (internal standard) were 1.37 and 2.74, and 1.08 and 13.73 for the HPLC method (external standard). The Deming regression values were in very good agreement with the ones produced by linear regression (Figures 4a and 4b), underpinning the validity of the analysis.

5.3.3. Reference materials and methods

In a further step both methods were benchmarked against reference materials to see whether the values would fall into the confidence band of the reference values. For this exercise three FAPAS[®] test materials (canned tuna), for which reference values from proficiency tests were available, were used; in addition, exact matching stable isotope dilution mass spectroscopy (IDMS), which is a highly accurate analytical technique, was used to assign reference values to three different types of fish samples (canned herring, fresh mackerel and fresh tuna). The IDMS procedure followed an internal protocol that has been proven to generate best estimates of true values in a number of proficiency tests over the last years. The IDMS protocol is listed in the Annex.

The results obtained by IDMS are shown in Table 8, while those from FAPAS[®] samples are listed in Table 9.

Sample	IDMS [mg/kg]	HPLC IS [mg/kg]	HPLC ES [mg/kg]	fluorimetry [mg/kg]
Fresh tuna	83 ± 3	84	74	69
Canned herring	17 ± 2	24	23	18
Fresh mackerel	226 ± 16	283	219	189

Table 8: Comparison of results from IDMS with HPLC and fluorimetry

Sample	PT value [mg/kg] w/ acceptance range	HPLC IS [mg/kg]	HPLC ES [mg/kg]	fluorimetry [mg/kg]
FAPAS [®] T2760	342 (297-388)	385	362	304
FAPAS [®] T2772	153 (130-176)	239	167	138
FAPAS [®] T2775	253 (218-288)	294	264	236

Table 9: Comparison of FAPAS[®] test samples with average HPLC and fluorimetry results

For fresh tuna the HPLC method with internal standard calibration produced results in very close agreement to the reference values of the IDMS method (Table 8). This supports the observation that the HPLC internal standard method is very well suited for analysing fresh tuna.

As in previous experiments with spiked fish, the fluorimeter method recovery was 83 and 84 % of the reference values for the levels of 83 and 226 mg/kg. In contrast the HPLC internal standard method showed a recovery rate exceeding 120 % for fresh mackerel. The low level contamination (17 mg/kg) has to be considered with caution since the value is below the calculated LOQ for both method types (see Tables 6 and 7). However the obtained data indicate that all methods gave reasonable results at a level of 17 mg/kg.

Taking the consensus values from FAPAS® proficiency test materials as the reference, the fluorimeter method gave in all three instances consistent recoveries in the range of 89-93 %.

The HPLC external standard method resulted in fairly consistent recovery rates slightly above 100 % (104-109%) for canned tuna, while when using the internal standard it appears to add a bias and a wider dispersion of results (113-156%) which is in agreement with the previous findings from the spiking experiments.

As a result it appears that the HPLC internal standard method had been optimized for analysing fresh tuna. It must be noted however that the method has a bias for other fish species such as canned tuna, mackerel or herring, when compared with either FAPAS® consensus values or reference values from IDMS.

It can be anticipated that both methods come to much better agreement once the biases are properly compensated taking recovery figures into account. However, as mentioned before the fluorimeter method as published in the AOAC OMA does not consider recovery (bias) correction. At the time the study was conducted it was not considered to be critical, assuming that all laboratories use the same standardised method.

The comparison of both methods with external references (IDMS and FAPAS® values) supports the previous findings that the methods are not fully equivalent. In general, the fluorimetric method underestimates, whereas the HPLC method as described in the mandated protocol, overestimates the histamine content. This is also evident from blank fish samples (different species) spiked at the decision levels of 100 and 200 mg/kg (Figures 5 and 6).

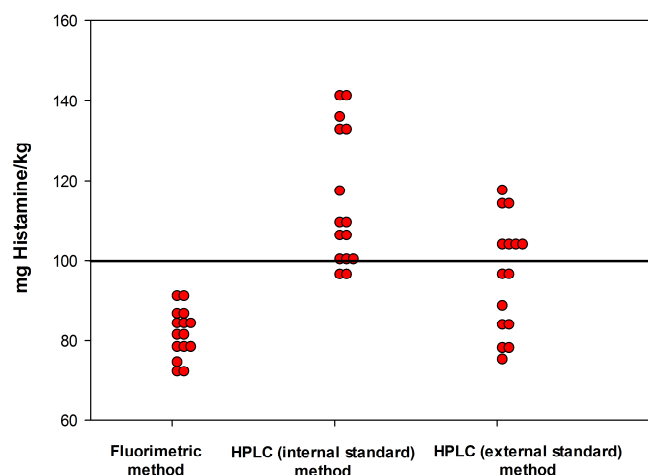


Figure 5: Performance of the fluorimetric and the HPLC method at a spiking level of 100 mg/kg in different fish species

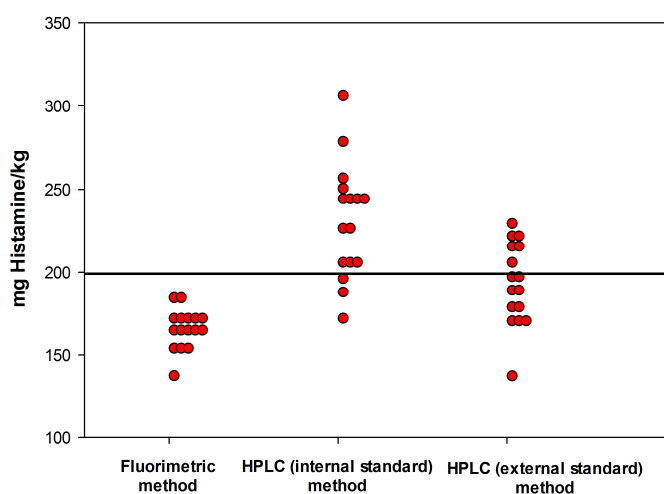


Figure 5: Performance of the fluorimetric and the HPLC method at a spiking level of 200 mg/kg in different fish species

The fact that both methods are not equivalent in a side-by-side comparison in one laboratory leads to the question whether results generated with these methods by a number of laboratories will differ significantly. This question must be asked as there are additional factors that were not subject of this study, which can however influence analytical results and lead to a further dispersion of results when generated in different laboratories. As a matter of fact these additional effects led to the larger acceptance range for FAPAS® consensus results as shown in Table 9, compared to the uncertainty figures obtained by IDMS. In essence, despite the finding that the fluorimeter method and the HPLC method are not fully equivalent, it is not certain that differences will become apparent when comparing results from a larger number of laboratories either using the fluorimeter method or the HPLC method. This was also the conclusion of the FSA study mentioned earlier [13].

6. Conclusion

A comparison of performance parameters generated for the fluorimeter method and the HPLC method with internal standard showed that both methods are selective to histamine, are linear over their working range (LOQ – 400 mg/kg) with a sufficiently low LOD and LOQ. The repeatability of both methods is practically the same, while intermediate precision is poorer for the HPLC method, indicating that the day-to-day variability is slightly higher for the HPLC method compared to fluorimetry.

A comparison of recovery rates showed that the fluorimeter method has a rather constant bias across different fish matrices with recovery rates between 77-90 %,

while recovery rates for the HPLC method are nominally higher, with a much higher dispersion of 103-140 % in dependence of the fish species analysed.

Statistical hypothesis testing (*t*-test) of a sufficient number of normalised data showed that the mean values produced with the two methods were significantly different. This does not necessarily mean that both methods cannot be considered to be equivalent.

Regression analysis indicated that the HPLC method, except for fresh tuna, overestimates, while the fluorimetric method underestimates the true histamine content.

The use of reference materials (consensus values of FAPAS® proficiency test materials and reference measurements done by exact matching stable isotope dilution mass spectroscopy) showed that the HPLC method is well suited for measuring histamine in fresh tuna, but it is positively biased for other fish species.

On the other hand the fluorimetric method showed a rather constant recovery rate of 80 % in all cases, leading to an underestimation which was, however, in all cases done on of FAPAS® reference material within the acceptance range.

The performance of the fluorimeter method has been proven for a larger number of laboratories in a previous collaborative trial. This collaborative trial did however not evaluate the bias (recovery rate) for the method, but it is within the generally accepted range of 70-120 % for analytical methods in other fields of food contaminants.

No collaborative study data is available for the HPLC method. It is therefore strongly recommended to organise a collaborative trial for the HPLC method to establish reproducibility data for the method. In line with current practice the collaborative study should also require to correct the reported data for recovery.

The data generated in this study further shows that matrix effects have to be addressed in an appropriate manner.

It is further recommended to consider the replacement of the HPLC method mentioned in Regulation (EC) No 2073/2005 by an already ring-tested HPLC method [10, 11].

7. References

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8. List of Annexes

ANNEX I	Work Instruction HPLC Method
ANNEX II	Work Instruction Fluorimeter Method
ANNEX III	Incurred Histamine in Fresh and Canned Fish by LC-IDMS
ANNEX IV	Collaboratively tested histamine methods

Annex I

Histamine determination by HPLC – UV

1. Scope of the method

This protocol describes the determination of histamine in fish (fresh tuna, mackerel, herring and canned tuna) in a working range up to 400 mg/kg with UV-HPLC.

2. Method principle

The test samples are extracted with perchloric acid (0.2M) and derivatised with dansylchloride. Surplus derivatisation agent is neutralized with proline. Histamine is separated on a reversed phase C18 column using a water/acetonitrile gradient. Histamine is identified by retention time as a baseline resolved peak. Quantification is carried out by UV absorption at 254 nm against an internal standard (1,3 dihydrochloride diaminopropane).

3. Reagents

All reagents are of analytical grade unless stated differently

- a) Water (ISO grade)
- b) Perchloric acid 65%
- c) Perchloric acid solution 0.2 M: either dilute 19.5 ml (65% HClO₄) or 17.2 ml (70% HClO₄) to 1 litre with water.
- d) Acetone
- e) Acetonitrile (HPLC grade)
- f) 1,3-diaminopropane dihydrochloride
- g) 1,3-diaminopropane solution (0.8 mg/ml): dissolve 40.0 mg in 50 ml water. The solution is stable for 3 weeks at 5±3°C.
- h) Sodium carbonate solution (Na₂CO₃): dissolve 110g (accuracy 0.1 g) until complete saturation in approximately 150ml Millipore® water.

- i) Dansylchloride
- j) Dansyl chloride solution (7.5 mg/ml): dissolve 375.0 mg dansylchloride in 50 ml of acetone. The solution can be stored in the dark at -18°C for 3 weeks.
- k) L-Proline
- l) L-proline solution (100 mg/ml): dissolve 1.000 g proline in 10 ml of water. The solution can be stored at 5±3°C for 3 weeks.
- m) Toluene

4. Equipment

- a) High speed blender (f.i. UltraTurrax)
- b) Analytical and laboratory balances (0.01g and 0.0001g resolution)
- c) Centrifuge tubes with screw caps
- d) Pipettes of various volumes: f.i. 20-200 µl and 100-1000 µl
- e) Refrigerated centrifuge
- f) Glass tubes of 10 ml
- g) Vortex mixer
- h) Water bath suitable for maintaining a temperature of 60°C (±1°C)
- i) Laboratory freezer capable of maintaining -18°C (±5°C)
- j) Evaporation block
- k) Syringes (2 ml)
- l) Syringe needles (20G, 0.9 mm)
- m) Luer lock filters 0.2 µm
- n) flask, vial, insert, cap

- o) HPLC system (injector, pump, UV-detector)
- p) Agilent ZORBAX SB-C18, 3.5 µm particle, 4.6 x 150mm

5. Method Protocol

Weigh exactly 5.0 g of test material into a centrifugation tube and cool in crushed ice until the test material reaches approx. 4°C.

Add 10 ml perchloric acid (3.c) previously cooled to 2°C, then add 100 µl of 1,3-diaminopropane solution (3.g) and turrax for 1 minute. Centrifuge with 12000 g at 4°C for 5 min.

Pipette 100 µl of the supernatant into a glass vial, add 300 µl of Na₂CO₃ solution (3.h) and 400 µl of dansylchloride solution (3.j) and close tube tight. Vortex and incubate for 5 min at 60°C in the dark. Cool tube to room temperature and add 100 µl proline solution (3.l). Vortex and place the tube in the dark for 15 min. Add 500 µl toluene (3.m), vortex and keep in the freezer at -18°C for at least 30 min.

Completely collect the non-frozen, organic phase in a new tube and evaporate the solvent under nitrogen flow at room temperature. Re-dissolve the evaporated residue in 200µl acetonitrile and vortex. Filter through inline syringe filter and inject 10 µL into the HPLC.

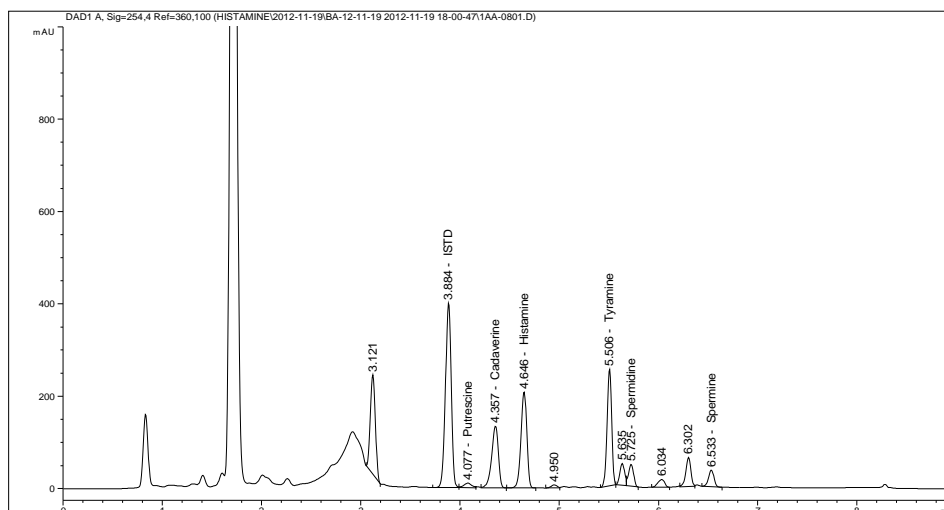
6. HPLC separation

Flow rate: 1.4 mL/min

Column temperature 45°C

Time, min	Acetonitrile %
0	60
2	75
2.6	75
4.3	95
6.60	95
6.61	60
10	60

Example chromatogram (naturally in incurred Tuna):



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Annex II

Determination of Histamine in Seafood with a Fluorescence spectrophotometer.

Rinse all plastic and glass containers with HCl [1 + 3] and H₂O before use

Apparatus

- (a) *Chromatographic tube.* -50 x 9 (id) mm polypropylene tube with 2 way valve.
- (b) *Photofluorometer.* Varian Cary Eclipse fluorescence spectrophotometer with excitation at 350nm and measuring emission at 444nm.
- (c) *Repipets.* -1 and 5 mL (Labindustries, 620 Hearst Ave, Berkeley, CA94710, or equivalent).

Reagents

- (a) *Ion exchange resin.* Bio-Rad AG 1-X8,50-100mesh (Bio-Rad Laboratories, 1414 Harbour, South Richmond, CA 94804)
Convert to -OH form by adding ca 15 mL 2N NaOH /g resin to beaker. Swirl mixture and let stand <30 min. Decant liquid and repeat with additional base. Thoroughly wash resin with H₂O, slurry into fluted paper (S&S No. 588, or equivalent), and wash again with H₂O. Prepare resin fresh weekly and store under H₂O. Place glass wool plug in base of tube, (a), and slurry in enough resin to form 8 cm bed. Maintain H₂O level above top of resin bed at all times. Do not regenerate resin in packed column; rather, use batch regeneration in beaker when necessary. Wash column with ca 10 mL H₂O before applying each extract.
- (b) *Phosphoric acid.* 3.57N. Dilute 121.8 mL 85% H₃PO₄ to 1L. For other concentration H₃PO₄, volume required for 1 L 3.57N acid = 17493/(density H₃PO₄ x % H₃PO₄). Standardize 5.00 mL by titration with 1.00N NaOH to phenolphthalein end point, and adjust concentration if necessary.
- (c) *o-Phthalicdicarboxaldehyde* (OPT solution. 0.1 %. Dissolve 100 mg OPT (Aldrich Chemical Co., Inc., No. P3, 940-0, or equivalent) in 100 mL distilled-in-glass methanol (Burdick & Jackson Laboratories, Inc., or equivalent). Store in amber bottle in refrigerator. Prepare fresh weekly.
- (d) *Histamine standard solutions.* Store in refrigerator.

- (1) Stock solution . 1mg/mL as free base. Accurately weigh ca 169.1 mg histamine.2HCl (98%,Aldrich Chemical Co., Inc., No.11,260-7,or equivalent) into 100 mL volumetric flask, and dissolve and dilute to volume with 0.1N HCl. Prepare fresh weekly.
- (2) Intermediate solution. 10µg/mL. Pipet 1 mL stock solution into 100 mL volumetric flask, and dilute to volume with 0.1N HCl. Prepare fresh weekly.
- (3) Working solutions. 0.5, 1.0, and 1.5 µg/5 mL. Pipet 1, 2, and 3 mL intermediate solution into separate 100 mL volumetric flasks, and dilute each to volume with 0.1N HCl. Prepare fresh daily.

Preparation of standard curve

Pipet duplicate 5 mL aliquots of each working standard solution into separate 50 mL glass or polypropylene Erlenmeyers.

Pipet in 10 mL 0.1 HCl to each flask and mix.

Pipet in 3 mL 1N NaOH and mix.

Within 5 min, pipet in 1 mL OPT solution and mix immediately.

After exactly 4 min, pipet in 3 mL 3.57N H₃PO₄ and mix immediately.

It is important to mix thoroughly after each addition and at least once during OPT reaction. (Run 6-10 OPT reactions simultaneously by adding reagents to Erlenmeyers in set order.)

Prepare blank by substituting 5 mL 0.1N HCl for histamine solution.

Within 1.5 h record fluorescence intensity (1) of working standard solutions with H₂O in reference cell, using excitation wavelength of 350nm and emission wavelength of 444nm.

Plot I (corrected for blank) against µg histamine/5 mL aliquot.

Determination

Transfer 5 g prepared sample to semimicro container of high-speed blender, add 20 ml methanol and blend ca 2 min.

Transfer to 50 mL glass stoppered volumetric flask.

Add another 20 ml methanol and blend on a high speed to rinse the blender, rinse the test tube and add rinsings to flask.

Heat in H₂O bath to 60°C and let stand 15 min at this temperature.

Cool to 25°C, dilute to volume with methanol, and filter through folded paper. Alcohol filtrate may be stored in refrigerator several weeks. (Light powdery precipitate separating on storage may be ignored)

Pass 4-5 mL H₂O through column, (a), and discard eluate.

Pipet 1 mL extract onto column and add 4-5 mL H₂O.

Immediately initiate column flow into 50 mL volumetric flask containing 5.00 mL

1.00N HCl. When liquid level is ca 2 mm above resin, add ca 5 mL H₂O and let elute.

Follow with H₂O in larger portions until ca 35 mL has eluted. Stop column flow, dilute to volume with H₂O, stopper, and mix. Refrigerate eluate.

Pipet 5 mL eluate into 50 mL Erlenmeyer, and pipet in 10 mL 0.1N HCl. Proceed as in 977.13C

Pipet in 3 mL 1N NaOH and mix.

Within 5 min, pipet in 1 mL OPT solution and mix immediately.

After exactly 4 min, pipet in 3 mL 3.57N H₃PO₄ and mix immediately.

It is important to mix thoroughly after each addition and at least once during OPT reaction. (Run 6-10 OPT reactions simultaneously by adding reagents to Erlenmeyers in set order.)

Prepare blank by substituting 5 mL 0.1N HCl for histamine solution.

Within 1.5 h record fluorescence intensity (1) of working standard solutions with H₂O in reference cell, using excitation wavelength of 350nm and emission wavelength of 444 nm.

Plot I (corrected for blank) against µg histamine/5 mL aliquot.

If sample contains > 15 mg histamine/ 100 g fish, pipet 1mL sample-OPT mixture into 10 mL beaker containing exactly 2 mL blank-OPT mixture, and mix thoroughly.

Read fluorescence of new solution. Dilute and mix aliquots with blank-OPT mixture as needed

to obtain measurable reading. This approximation indicates proper dilution of eluate required prior to second OPT reaction needed for reliable quantitation of sample.

Alternatively, use sensitivity range control of fluorometer (if instrument has one) to estimate dilution.

Use these approximations to prepare appropriate dilution of aliquot of eluate with 0.1N HCl, and proceed as in 977.13C, beginning "Pipet in 3 mL in NaOH ... "

Calculations

Calibration curve with 4 points: 0 µg/ 5ml, 0.5 µg/ 5ml, 1.0 µg/ 5ml and 1.5 µg/ 5ml

Determine intercept and slope.

Correct the signal for the exact weight of 5g

$(\text{Intensity} / \text{exact weight of fish sample}) \times 5 = \text{signal}$

Concentration of histamine in fish:

$(\text{signal} - \text{intercept}) / \text{slope} \times 100 = \text{concentration in mg/kg histamine}$

INSTRUMENT METHOD

CARY ECLIPSE – CONCENTRATION – method: HISTAMINE-201210.FMCM

Annex III**Incurred histamine in fresh and canned fish by LC-IDMS****Test materials:**

Five different test materials were investigated: canned herring, fresh herring, canned tuna (FAPAS PT material T2772), fresh tuna, and fresh mackerel. All test materials were minced and homogenized.

Reference solutions:

Solution Histamine A: 0.10046 g Histamine dihydrochloride (STD283, Sigma H7250 (Lot BCBG8381V) purity $\geq 99\%$) were dissolved in 2.01417 g 0.1 mol/L HCl for a solution of 0.0286844(74) g/g Histamine (free base; the numbers in parentheses are the numerical values of u_c referred to the corresponding last digits of the quoted result;).

Solution Histamine B: 0.11182 g Histamine A were diluted with 9.80705 g 0.2 mol/L HClO₄ for a solution of 0.32340 mg/g Histamine (free base).

Solution Histamine-d₄ A: 0.04333 g Histamine-d₄ dihydrochloride (STD237, CDN Isotopes D-2270 (Lot C262P13)) were dissolved in 0.86740 g 0.1 mol/L HCl for a solution of 0.029132(17) g/g Histamine-d₄ (free base).

Solution Histamine-d₄ B: 0.13613 g Histamine-d₄ B were diluted with 2.94660 g 0.2 mol/L HClO₄ for a solution of 1.28644(80) mg/g Histamine-d₄ (free base).

Sample preparation:

- 1 g of a test material into 15 mL PP screw-cap centrifuge tube
- Add 0.1 g histamine-d₄ B
- Add 4.9 mL 0.2 mol/L HClO₄
- High speed blend for 30 s
- Leave overnight in refrigerator (2-8 °C)
- Centrifuge 10 min at RCF of 12000 g and 5 °C
- Apply supernatant to SPE clean-up
- SPE clean-up:
 - Supelco Discovery SCX 500mg/ 3mL cartridges
 - Conditioned with 2 mL methanol and 2 mL water
 - Loaded with 2 mL supernatant
 - Washed with 2 mL water and 2 mL acetonitrile
 - Eluted with 2 x 1 mL water/diethyl amine (90/10,v/v)

- 10 µL of combined and mixed eluate to 990 µL acetonitrile/water (90/10,v/v), 20 mmol/L ammonium acetate
- After mixing this solution is injected into LC-MS

All weights were recorded with a precision of 5 decimal places. Each test material was prepared three times.

Calibration solutions:

- 0.05, 0.25, 1.0, and 6.0 g of histamine B into 15 mL PP screw-cap centrifuge tube
- Add 0.1 g histamine-d₄ B
- Mix and apply to SPE clean-up (as above)
- 10 µL of combined and mixed eluate to 990 µL acetonitrile/water (90/10,v/v), 20 mmol/L ammonium acetate
- After mixing this solution is injected into LC-MS

All weights were recorded with a precision of 5 decimal places.

Measurements:

All solutions were injected into a LC-MS consisting of a binary high-pressure gradient solvent delivery system (Shimadzu LC20-AD), an Accela auto liquid sampler, and a Thermo TSQ Quantum Ultra mass spectrometer.

Separation was afforded by an Ascentis express OH5 column (50 x 2.1 mm, 2.7 µm particle size) in HILIC mode with 20 mmol/L ammonium acetate as mobile phase A and acetonitrile/water (90/10,v/v) 20mmol/L ammonium acetate as mobile phase B in isocratic conditions of 75% B at 300 µL/min flow. Retention time under these conditions was 1.43±0.07 min. Effluent around the dead volume was diverted to waste.

MS settings were as follows: SRM in positive ESI mode with the following transitions for histamine: 112->68(21), 112->83(15), 112->95(15) and the following for histamine-d₄: 116->72(21), 116->85(15), 116->99(15) (always precursor->product(collison energy)); capillary temperature 320 °C, vaporizer temperature 250 °C, sheath gas press. 30, auxiliary gas press. 10, ion sweep gas press. 10 (all arbitrary units), spray voltage 3000 V, collision gas press. 1.5 mTorr, Q1 mass filter was set to a peak width of 0.2 (FWHM).

An initial measurement batch with all calibration solutions first, followed by all test solution, and finished by a repeat of the calibration solutions in reversed order was run to obtain an initial estimate of the contamination level. Based on these estimates test solutions were grouped with calibration solutions of similar ion ratios.

For the final measurements the canned herring injections were bracketed between two calibration level 1 injections since there was an exact match. Same

is true for the fresh tuna injections which were bracketed between two calibration level 2 injections. The canned tuna (FAPAS) and the fresh mackerel injections were bracketed between calibration level 2 and calibration level 3 injections. Finally the fresh herring injections were bracketed between calibration level 3 and level 4 injections. These set of injections were repeated three times each for each of the 15 test solutions.

Calculations:

The mass fractions of the canned herring and fresh tuna were calculated with the simplified model equation for exact-matching double IDMS:

$$w_{X,i} = w_Z \frac{m_{Y,i} m_{Z,i}}{m_{X,i} m_{Yc,i}} \bar{R}'_i \quad \text{Eq. 1}$$

where w_Z = mass fraction of analyte in reference material, $m_{X,i}$ = mass of test material in i -th SB, $m_{Y,i}$ = mass of spike added to i -th SB, $m_{Z,i}$ = mass of reference material in i -th CB, $m_{Yc,i}$ = mass of spike added to i -th CB, and \bar{R}'_i = mean of all measurements of $R'_{B,ij} / R'_{Bc,ij}$ for the i -th SB/CB pair with $R'_{B,ij}$ = observed isotope ratio of the j -th measurement of the i -th SB and $R'_{Bc,ij}$ = observed isotope ratio of the j -th measurement of the i -th CB.

The combined uncertainty of $w_{X,i}$ can then be expressed by Eq.2:

$$u_{c,i}(w_{X,i}) = w_{X,i} \sqrt{\left(\frac{u(w_Z)}{w_Z}\right)^2 + \left(\frac{u(m_{Y,i})}{m_{Y,i}}\right)^2 + \left(\frac{u(m_{X,i})}{m_{X,i}}\right)^2 + \left(\frac{u(m_{Z,i})}{m_{Z,i}}\right)^2 + \left(\frac{u(m_{Yc,i})}{m_{Yc,i}}\right)^2 + \left(\frac{u(\bar{R}'_i)}{\bar{R}'_i}\right)^2} \quad \text{(Eq. 2)}$$

where u denotes the standard uncertainty of the respective term of Eq. 2, e.g. $u(\bar{R}'_i)$ is the standard error of the mean of the three measured ratios $R'_{B,ij} / R'_{Bc,ij}$ in the i -th SB/CB pair.

Because for the canned tuna (FAPAS), the fresh mackerel, and the fresh herring no matching calibration solution existed a bracketing approach with a lower and a higher level was used. The following model equation applies to this scenario:

$$w_{X,i} = \frac{w_Z \left(\frac{m_{Z,high} - m_{Z,low}}{m_{Yc,high} - m_{Yc,low}} \right) \bar{R}'_i m_{Y,i}}{m_{X,i}} \quad \text{Eq.3}$$

where where w_Z = mass fraction of analyte in reference material, $m_{X,i}$ = mass of test material in i -th SB, $m_{Y,i}$ = mass of spike added to i -th SB, $m_{Z,high}$ = mass of

reference material in higher CB, $m_{Z,low}$ = mass of reference material in lower CB, $m_{Yc,high}$ = mass of spike added to high CB, $m_{Yc,low}$ = mass of spike added to low CB, and \bar{R}'_i = mean of all measurements of ion ration R'_B divided by the difference of $R'_{Bc,high}$ and $R'_{Bc,low}$.

The combined uncertainty of $w_{X,i}$ can then be expressed by Eq. 4

$$u_{c,i}(w_{X,i}) = w_{X,i} \sqrt{\left(\frac{u(w_Z)}{w_Z}\right)^2 + \left(\frac{u(m_{Y,i})}{m_{Y,i}}\right)^2 + \left(\frac{u(m_{X,i})}{m_{X,i}}\right)^2 + \left(\frac{u(\bar{R}'_i)}{\bar{R}'_i}\right)^2 + \left(\frac{u(\beta)}{\beta}\right)^2}$$

Eq.4

where u denotes the standard uncertainty of the respective term of Eq. 3, e.g. $u(\bar{R}'_i)$ is the standard error of the mean of the three measured ratios R'_B divided by the difference of $R'_{Bc,high}$ and $R'_{Bc,low}$. The term in parentheses in the numerator of Eq. 3 is denoted as β and its uncertainty $u(\beta)$ is calculated as follows:

$$u(\beta) = \sqrt{\left(\frac{m_{Z,high}}{m_{Yc,high}}\right)^2 \left(\left(\frac{u(m_{Z,high})}{m_{Z,high}}\right)^2 + \left(\frac{u(m_{Yc,high})}{m_{Yc,high}}\right)^2 \right) + \left(\frac{m_{Z,low}}{m_{Yc,low}}\right)^2 \left(\left(\frac{u(m_{Z,low})}{m_{Z,low}}\right)^2 + \left(\frac{u(m_{Yc,low})}{m_{Yc,low}}\right)^2 \right)}$$

The mass fraction w_T of a test material is then calculated by Eq. 5:

$$w_T = \bar{w}_X F_X \quad (\text{Eq. 5})$$

where \bar{w}_X = mean of all three $w_{X,i}$ of one test material and F_X = a factor of unity representing the mean of the relative combined uncertainties of $w_{X,i}$ of one test material. The combined uncertainty of w_T is then expressed by Eq. 5:

$$u_c(w_T) = w_T \sqrt{\left(\frac{u(\bar{w}_X)}{\bar{w}_X}\right)^2 + \left(\frac{u(F_X)}{F_X}\right)^2} \quad (5)$$

where $u(\bar{w}_X)$ = the standard error of the mean of \bar{w}_X and $u(F_X)$ = the mean of all $u_{c,i}(w_{X,i})/w_{X,i}$ per test material.

Annex IV

Collaboratively tested histamine methods

The full-text version of the two methods can be obtained from the respective standardisation bodies below:

[NMKL 196, 2013 -- Biogenic amines. HPLC determination in foods.](#)

[BVL L 10.00-5:1999-11 -- Untersuchung von Lebensmitteln - Bestimmung des Gehaltes an biogenen Aminen in Fischen und Fischerzeugnissen - Hochdruckflüssigkeitschromatographische Bestimmung; Referenzverfahren](#)

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Abstract

Histamine fish poisoning is an allergy-like form of food poisoning that continues to be a major problem in seafood safety. The FAO/WHO Codex Alimentarius as well as EU legislation have therefore set maximum limits for histamine in fish and fish products. The analytical methods requested by Codex and by EU are different and concern has been raised that this could lead to disputes in the international trade of seafood.

This report describes the outcome of a study, commissioned by DG Health and Consumers and carried out by DG Joint Research Centre, that compared the performance of the method for determining histamine in fish as mandated by Regulation (EC) No 2073/2005 to the method mandated in Codex Alimentarius Standard 165-1989. The EU mandated method is based on HPLC separation of histamine and subsequent detection by a UV detector. It was published in the Journal of AOAC International, but has not been validated by a collaborative study. The Codex method is AOAC 977.13, which is based on the formation of a fluorescent derivative of histamine and subsequent measurement in a fluorimeter; it has been validated by collaborative trial.

The correct implementation of both methods by JRC was assessed by carrying out performance verification studies using various canned and fresh scromboid fish samples (tuna, mackerel, and herring) taken from the Belgian market. Repeatability (RSDr) and intermediate precision (RSDip) as well as recovery data were generated. Both methods conformed to specifications.

Various approaches were followed to test the equivalency of both methods, which were based on statistical hypothesis testing (t-test), regression analysis and benchmarking against established reference values.

All approaches indicated that the two methods are not fully equivalent. The EU mandated method has a tendency to overestimate, while the Codex method has a tendency to underestimate the histamine content in fish.

It was recognised that the EU mandated method was very accurate when applied to fresh tuna. A distinct matrix influence was noticed for all other fish species tested, leading to an overestimation of the histamine content.

It is therefore recommended to optimise the EU method so that matrix effects can be eliminated, or at least taken into account in an appropriate manner. In addition, a collaborative trial for the HPLC method to establish reproducibility data for the method should be organised. In line with current practice the collaborative study should also require to correct the reported data for recovery. Furthermore, as an ad-hoc measure the replacement of the HPLC method mentioned in Regulation (EC) No 2073/2005 by a ring-tested HPLC method, which are already available, could be considered.

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