



## Traceability

### Project 6.3 – Valid

# Methodology for histamine and biogenic amines analysis

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## Abstract

Histamine, putrescine, cadaverine, tyramine and agmatine are produced from the decarboxylation of histidine, ornithine, lysine tyrosine and arginine respectively. Histamine is associated of scombroid poisoning in conjunction with the ingestion of some fish species such as tuna, mackerel, sardine, herring, anchovy. The formation of histamine in fish products is directly correlated with the concentration of histidine in the tissue and the level of microorganisms present in the product, due to bacterial histidine decarboxylase action on histidine. The present review is an examination of representative methods used for fishery products rather than a broad review of the multitude of methods available for this goal. Routine analysis, semi-quantitative and quantitative methods for histamine and biogenic amines are briefly described, indicating the technical basis, the equipment and materials required, the strong and weak aspects of the methodology and the adequacy of the method in the distribution chain.

From the histamine and biogenic amines for fish quality assessment, two main conclusions can be withdrawn

1) With regard to quality **control methods for scombrotoxin**: To ensure the safety regarding histamine it is preferable to use a rapid method to do a screening, even if the method is semi-quantitative. Some convenient immuno-enzymatic kits are commercialised and other techniques such as colorimetric and TLC methods which require a small commitment of equipment and inexpensive reagents can be retained. However it is necessary to define the limits of the chosen method and to validate its reliability in comparison with an official method or a reference method; and in case of doubt regarding the results or in the event of dispute, it would be advisable to specify a reference method which may be used. Nowadays many rapid techniques are available and in the EU regulation it is specify ". Examinations must be carried out in accordance with reliable, scientifically recognized methods, such as high-performance liquid chromatography (HPLC).", but there are no reference method for histamine in Europe recognized by the member states. Histamine and biogenic amines are produced by enzymatic reaction, their level increases in the chain, even under chilling condition, so it is important to perform the analysis very quickly after the sampling or when it is possible, depending on the methods, to prepare the acidic extract that can be kept about one week at +4°C. The analysis result should be expressed clearly, i.e. histamine in mg/kg with the reference of the used method and details about the sampling (nature, date, place).

2) With regard to the **validation methodology**: for histamine numerous methods have been described, a few of them have been studied in interlaboratory trial, some are AOAC Official methods and in Europe there is a reference method since December 2005.

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# Histamine and biogenic amines as criteria for chemical quality assessment

Biogenic amines are chemically defined as aliphatic, alicyclic and heterocyclic organic bases of low molecular weight (Luten et al., 1992). Important biogenic amines in fish are histamine, putrescine, cadaverine, tyramine, agmatine, spermine and spermidine.

## 1. Nature and formation

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Histamine, putrescine, cadaverine, tyramine and agmatine are produced from the decarboxylation of histidine, ornithine, lysine tyrosine and arginine respectively.

Histamine is associated of scombroid poisoning in conjunction with the ingestion of some fish species belonging to *Scombroidae* (tuna, mackerel), *Clupeidae* (sardine, herring) or *Engraulidae* (anchovy) families, or other seafood such as mahi-mahi (*Coryphaena hippurus*), bluefish (*Pomatomus saltatrix*) which have naturally high quantities of histidine in their muscular tissue (Huss, 1995, Mariné-Font et al., 1995, Huss et al., 2000, Lehane and Olley, 2000, Becker et al., 2001). The formation of histamine in fish products is directly correlated with the concentration of histidine in the tissue and the level of microorganisms present in the product, due to bacterial histidine decarboxylase action on histidine. Biogenic amines have been identified in decomposed fish, and some index have been proposed to evaluate the degree of spoilage of fish (Mietz and Karmas, 1977, Staruszkiewicz, 1993, 2004, Sato et al., 1995, Tilve-Jar et al., 1996, Vallé et al., 1996, Rodriguez et al., 1997, Duflos et al., 1999, Jorgensen et al., 2000, Flick et al., 2001).

## 2. Regulation

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The Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs stated for histamine that :

"Nine samples must be taken from each batch. These must fulfil the following requirements :  
- the mean value must not exceed 100 ppm;  
- two samples may have a value of more than 100 ppm but less than 200 ppm;  
- no sample may have a value exceeding 200 ppm.

These limits apply only to fish species of the following families: Scombridae, Clupeidae, Engraulidae, Coryfenidae, Pomatomidae and Scombrosidae. However, fish belonging to these families which have undergone enzyme maturation treatment in brine may have higher histamine levels but not more than twice the above values. Examinations must be carried out in accordance with an HPLC analytical reference method, Male et al., 1996, Duflos et al., 1999.

The FDA guidelines 1998c for tuna, mahi mahi, and related fish advised histamine levels "500 ppm set based on toxicity, 50 ppm set as defect action level, because histamine is generally not uniformly distributed in a decomposed fish. Therefore, if 50 ppm is found in one section, there is the possibility that other units may exceed 500 ppm".

## 3. Methods of determination of histamine and biogenic amines

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A wide variety of procedure for the determination of histamine and biogenic amines have been published. Biological methods were the first methods of evaluation of scombroid toxine, they measured such parameters as amount of contraction of a histamine sensitive organ as guinea pig ileum and the first AOAC method of determination of histamine in food was a biological method (AOAC Official method 954.04), but these techniques have been supplanted by simpler and more convenient methods. For the most part the following presentation is an examination of representative

methods used for fishery products rather than a broad review of the multitude of methods available for this goal.

### 3.1. Routine analysis, semi-quantitative and quantitative methods for histamine

#### 3.1.1. Colorimetric methods

The earliest **colorimetric methods** such as the AOAC Official method 957.07 which required careful attention to procedural detail and were tedious, are not used today.

The new colorimetric assay proposed by Patange et al. (2004) appears to be simple; its limit of quantitation is 10 mg/kg. Most of the others colorimetric methods require prior purification by cation exchange chromatography, i.e. the reaction between purified histamine and copper which form a visible red complex (Bateman et al., 1994).

##### a) Technical basis

Principle: a saline extraction of histamine, followed by a centrifugation, a n-butanol extraction and an evaporation before the colorimetric reaction with *p*-phenyldiazonium sulfonate. The colour intensity pink-orange can be evaluated visually by comparison of a reference colour scale (Fig. 1) or by a spectrophotometer lecture at 496 nm.

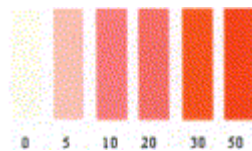


Fig.1: reference colour scale for histamine (concentration in µg/ml) according to Patange et al. (2004)

##### b) Equipment and materials required

A basic equipment for extraction (homogeniser, scale, filters and/or centrifuge), a refrigerate centrifuge, an ice production facility, a source of pure nitrogen, and for a quantitative measure a spectrophotometer

##### c) Strong and weak aspects of the methodology

###### *Advantages*

The method is rapid, approximately 45 min for a single assay including sample preparation. It is inexpensive, US\$ 1.0 of chemical and reagents per sample, and unskilled technicians can perform the assay if they are trained under scientific supervision.

###### *Disadvantages*

The method has not been tested in an interlaboratory trial.

##### d) Adequacy for each fish link (type of business) in the distribution chain

Quality control laboratories for screening test.

##### e) Bibliographic standards of reference

Patange et al. (2004)

#### 3.1.2. Thin layer chromatography (TLC) methods

Traditional TLC methods are old methods which are still used in control laboratories.

#### *a) Technical basis*

Principle:

- i) histamine extraction is made by methanol (Lin et al., 1977), by trichloroacetic acid (Taylor, 1983); or an aqueous fraction of the press juice from canned fish can be used (Schutz et al., 1976)
- ii) migration and separation is realized on an adequate stationary phase, Silica gel G in a chromatography chamber; the best solvent systems are [methanol : ammonia (20:1)] and [chloroform : methanol : ammonia (2:2:1)] (Lieber and Taylor, 1978a), these systems were originally published by Lin et al. (1976) and Brenner et al. (1969).
- iii) to reveal histamine the classical spray reagent is ninhydrin (Baranowsky, 1985); in a comparison study Lieber and Taylor (1978b) found specific reagents, fluorescamine and o-diacetylbenzene.
- iv) detection can be either quantitative by use of a densitometer, or semi-quantitative by comparing the colour intensity of the histamine spot in the sample to standards of histamine run at the same time on the chromatographic plate.

#### *b) Equipment and materials required*

A basic equipment for extraction (homogeniser, scale, filters and/or centrifuge), chromatographic plates and container, and a densitometer for a quantitative determination.

#### *c) Strong and weak aspects of the methodology*

Advantages

Rapid method, migration time about 2 hours with simultaneous analysis of 7/8 samples per plate at a time, and two plates can be simultaneously inserted in a migration container; the required material is not expensive, and unskilled technicians can perform the assay if they are trained. This method can be used as a screening test because it can separate the negative samples meanwhile the positive samples must be used for further analysis confirmation.

Storage of the reagents at ambient temperature

Disadvantages

Semi-quantitative results, lack of precision, detection limit near 50 mg/kg

Toxicity of some reagents (solvent)

#### *d) Adequacy for each fish link (type of business) in the distribution chain*

Quality control laboratories for screening test.

The classical TLC procedures are suitable for screening for histamine levels in excess of 50 mg/kg. However it is important to notice that recently, the development in the field of stationary phases for TLC and instruments for automated sample application and densitometric scanner has made it possible to obtain quantitative results similar to those from HPLC or GC (Lapa-Guimarães and Pickova, 2004). Now high-performance TLC (HPTLC) allows the simultaneous quantitative determination of biogenic amines (see 3.2 – Quantitative analysis).

#### *e) Bibliographic standards of reference*

Brenner et al. (1969), Schutz et al. (1976), Lin et al., (1976, 1977), Lieber and Taylor (1978a, 1978b), Taylor, (1983), Baranowsky (1985)

### 3.1.3. Enzymatic methods

#### *a) Technical basis*

The basis of this technique developed by Lerke et al. (1983) is that the enzyme diamine oxydase (DAO) catalyses the conversion of histamine to imidazole acetaldehyde with in parallel production of

hydrogen peroxide. Hydrogen peroxide is converted back to oxygen and water by the action of horse radish peroxidase (HRP), the second enzyme. Concurrent to this reaction is the oxidation of leuco-crystal violet (a colourless compound) to crystal violet, a purple compound. The intensity of the colouration is proportional to the amount of histamine, it can be evaluate visually (qualitative result) or by spectrometry (quantitative result). Some adaptations of the procedure have been described (Lopez-Sabater et al., 1993, Ben Gigirey et al., 1998), including one with a microplate reader (Etienne and Bregeon, 1992).

The limit of detection is 0.5 mg/kg and the limit of quantitation is 1.5 mg/kg (Lopez-Sabater et al., 1993).

NB: To simplify the analytical procedures, enzyme **biosensors** have been developped (Ohashi et al., 1994, Male et al., 1996, Bouvrette et al. 1997, Draisci et al., 1998a, Frebort et al., 2000, Lange and Wittmann, 2002) as well as a dipstick test, a solid phase assay based on the coupling of DAO to a peroxidase/dye system (Hall et al., 1995, 1999). The biosensors reported to provide simplicity and rapidity were realized in specialized laboratory but as they are not commercially available they can not easily be used.

#### *b) Equipment and materials required*

A basic equipment for extraction (homogeniser, scale, filters and/or centrifuge), water bath, pHmeter, enzymes and chemical reagents, tubes or microplates, and a spectrometer for a quantitative determination.

#### *c) Strong and weak aspects of the methodology*

##### Advantages

Rapid method with the simultaneous analysis of several samples, the incubation time of the enzymatic reaction varies from 20 min to 2 h. following the procedures.

Good correlation with AOAC fluorimetric method ( $r^2=0.829$  according to Ben-Girirey et al., 1998).

##### Disadvantages

DAO enzyme reacts with others amines such as agmatine, putrescine and cadaverine when they are present at high level (Etienne and Bregeon, 1992), Ben-Girirey et al. (1998) found that these methods tend to overestimate histamine at levels < 10 mg/kg

Storage of some reagents at  $-20^{\circ}\text{C}$  and at  $0-4^{\circ}\text{C}$

#### *d) Adequacy for each fish link (type of business) in the distribution chain*

Quality control laboratories for screening test.

#### *e) Bibliographic standards of reference*

Lerke et al. (1983), Etienne and Bregeon (1992, Lopez-Sabater et al., (1993), Ben Gigirey et al., (1998)

### 3.1.4. Immuno-Enzymatic methods

#### *a) Technical basis*

A enzyme-linked immunosorbent assay (ELISA) was developed for the measurement of histamine in food by Serrar et al. (1995). The test principle based on the competition principle is the following: an unknown amount of antigen present in the sample and a fixed amount of enzyme labelled antigen compete for the binding sites of the antibodies coated onto the wells. After incubation the wells are washed to stop the competition reaction. After the substrate reaction the intensity of the developed colour is inversely proportional to the amount of the antigen in the sample. Results can be determined directly using the standard curve. It was a very specific and sensitive method performed in a research laboratory.

Several ELISA procedures have been developed by companies which produce qualitative and/or quantitative analysis kits for the measure of histamine (see annex1 the list of the commercial kits). The analysis is realized in successive steps :

- 1) the extraction of histamine from the fish sample using water or an acidic solution followed by a filtration,
- 2) the addition of reagents to form an histamine conjugate (the nature of the reagents differs following the kits),
- 3) the competitive binding to the antibodies coated onto wells of microtiter or reaction tubes during a time of incubation which varies according the test, followed by a washing,
- 4) the enzymatic reaction to catalyse a colour change, the duration of the colorimetric reaction depends on the test and the intensity of the colour is inversely proportional to the concentration of histamine in the test solution,
- 5) the lecture of the colour intensity, it can be visual in quantitative tests by comparison with standards or more usually it is a spectrophotometric reading , in quantitative tests, with a calculation using a standard curve.

Five marketed test kits [ALERT® and Veratox® (Neogen), Histamarine (Immunotech), K1-HTM and K3-HTM test kit (IDR)] chosen to represent a variety of types of assays available in kit form have been tested by FDA (Rogers and Staruszkiewicz, 2000). The resultat was: "all the kits tested were acceptable for use as screening tests for histamine and were able to distinguish between products that contained less than 50 ppm and those that contained more than 50 ppm.". However the authors noticed a better accuracy of the ALERT® and Veratox® kits for low values, values near 50 ppm and very high values of histamine. They found that extraction of the test sample with water was easier, with fewer steps than extraction with dilute acid. The other considered points were the colour of the reaction, the difference in intensity in blue colour was visually easier to distinguish than the difference in intensity in the yellow colour, and the incubation times.

Histamarine (Immunotech) was approved by AOAC in September 1998. Another kit comparisons between two semi-quantitative rapid tests for the determination of histamine in fishery products were made by Pirazzoli and Incerti (1999), they found the Alert®ion exchange chromatography kit and the Alert®ELISA kit (Neogen) suitable for screening fish products.

#### *b) Equipment and materials required*

Using commercial kits no equipment are necessary except a spectrometer for a quantitative determination.

#### *c) Strong and weak aspects of the methodology*

##### Advantages

Rapid method with the simultaneous analysis of several samples, the incubation time of the immuno-enzymatic reactions varies from 15 min to 2 h15 following the procedures.

##### Disadvantages

Storage of some reagents at -20°C and at 0 - 4°C according to the test kit

#### *d) Adequacy for each fish link (type of business) in the distribution chain*

Immuno-enzymatic methods can be used in quality control laboratories and fish processing laboratories as a screening test for histamine. They are able to distinguish between products that contained less than 50 ppm and those that contained more than 50 ppm (Pirazzoli and Incerti, 1999, Rogers and Staruszkiewicz, 2000). Further confirmation of positive samples is needed with other techniques in more specialised laboratories (ex. HPLC).

#### *e) Bibliographic standards of reference*

Serrar et al. (1995), Pirazzoli and Incerti (1999), Rogers and Staruszkiewicz (2000), annex 1.



### 3.1.5. Flow injection analysis – FIA

#### a) *Technical basis*

The FIA is an analytical technique based on microfluidic manipulation of samples and reagents. Samples are injected into a carrier/reagent solution which transports the sample zone into a detector while desired (bio)chemical reactions take place. Detector response (absorbance, fluorescence, sensor...) yield a calibration curve quantifying the target analyte.

Flow injection analysis systems for the determination of histamine using a fluorimetric detection were developed to provide rapid screening of fish samples (Luten, 1981, Hungerford et al., 1990). The technique has been used in many laboratories with systems that were assembled from spare components. It does not require pre-treatment of the extract, however it does require careful selection of reagent concentrations and careful control of pumps to maintain specificity for the histamine derivative (Hungerford et al., 1990). Most recently commercial FIA systems have been tested for this screening (Hungerford et al., 2001), the method performance is excellent, with detection and quantitation limits near 0.8 and 2.4 mg/kg, and linearity to approximately 340 mg/kg.

Flow injection analysis line with biosensors based on the enzyme amine oxidase for the detection of histamine also have been proposed (Carsol and Mascini, 1999, Niculescu et al., 2000, Takagi and Shikata, 2004).

#### b). *Equipment and materials required*

A basic equipment for extraction (homogeniser, scale, filters and/or centrifuge), Auto-analyser assembled from spare components, pumps, tubing, detector or a commercial FIA system, an expensive equipment (auto-sampler, analyser and fluorescence detector interfaced to a computer)

#### c) *Strong and weak aspects of the methodology*

Advantages

This a rapid method, only one minute per extract and it gives quantitative results.

Disadvantages

It needs expensive equipment, sophisticated system and skilled technicians.

#### d) *Adequacy for each fish link (type of business) in the distribution chain*

It can be used for screening test in quality control laboratories.

#### e) *Bibliographic standards of reference*

Fluorimetric detection: Luten (1981), Hungerford et al.(1990, 2001)

***Conclusion of routine analysis: The above methods are relatively simple to use for routine screening of histamine in fish and fishery products, they allow to check a great quantity of samples. The colorimetric, TLC and enzymatic methods require in general a small commitment of equipment and inexpensive reagents, however they are qualitative or at best semi-quantitative. The immuno-enzymatic test kits giving quantitative results require few equipment, a photometer, and some reagents that are sold ready to use. Although the FIA method gives quantitative results very quickly, it requires sophisticated and expensive laboratory apparatus.***

## 3.2. Precise analysis – quantitative – for histamine and biogenic amines

Fluorimetric techniques have been developed for an accurate measurement of histamine and with the evolution of the techniques new procedures were tested. Now, the chromatographic techniques, such as gas chromatography (GC), high performance liquid chromatography (HPLC), and high performance thin layer chromatography (HPTLC), as well as capillary electrophoresis, offer one major advantage

over the previously discussed methods; they allow the simultaneous analysis of histamine and others biogenic amines in fish and fishery products.

### 3.2.1. Fluorimetric method for histamine

#### *a) Technical basis*

In this method, histamine is extracted with methanol, and purified on an anion exchanger column in order to remove interfering substances. The histamine is derived with o-phthalaldehyde and the fluorescence of the compound is measured spectrophotometrically.

#### *b) Equipment and materials required*

A basic equipment for extraction (homogeniser, scale, filters and/or centrifuge), chromatographic column, chronometer, fluorimeter

#### *c) Strong and weak aspects of the methodology*

##### *Advantages*

sensitive and reproducible method

It is a validated method which has been studied, standardized and tested in interlaboratory trial.

##### *Disadvantages*

Method complex and time consuming which needs skilled technicians. Although initially used, there is a tendency for being substituted by HPLC methods.

#### *d) Adequacy for each fish link (type of business) in the distribution chain*

not adequate for screening test quality in control laboratories (too tedious).

Now the AOAC fluorimetric method is used to compare the performance of new methods in studies

#### *e) Bibliographic standards of reference*

AOAC Official Method 977.13

### 3.2.2. Gas Chromatography (GC) methods

#### *a) Technical basis*

In the first procedures after the extraction of the amines, a step of complexation with a derivative, such as pentafluoropionic anhydride was considered necessary before the GC separation and the flame ionization detection (FID) (Staruszuszkiewicz and Bond, 1981, Rogers and Staruszuszkiewicz, 1997, Du et al., 2001).

More recently Hwang et al. (2003) described a new method of direct analysis of histamine or other biogenic amines by GC without derivatization using more performing GC column (CP-SIL 19CB) and alkaline methanol for the extraction. The detection limit for histamine by this method was about 5 mg/kg. This new procedure reduces the time for analysis, but also provides possibility to detect the compound itself and avoid errors derived from the reaction of derivative synthesis.

#### *b) Equipment and materials required*

A basic equipment for extraction (homogeniser, scale, filters and/or centrifuge)

A gas chromatograph with flame ionization detector

#### *c) Strong and weak aspects of the methodology*

##### *Advantages*

sensitive and reproducible method

rapid: one extract analysis requires about 10 min using the new protocol without derivatization, described by Hwang et al. (2003)

#### Disadvantages

This method needs an equipment and skilled technicians

#### *d) Adequacy for each fish link (type of business) in the distribution chain*

In Europe today it is not very used for screening test quality in control laboratories.

#### *e) Bibliographic standards of reference*

AOAC Official Method 996.07

Staruszkiewicz and Bond (1981), Rogers and Staruszkiewicz, 1997, Du et al. (2001), Hwang et al. (2003)

### 3.2.3. High Performance liquid chromatography (HPLC) methods

#### *a) Technical basis*

Over the years a number of different HPLC methods have been proposed. After the extraction an amines derivatization step is necessary, it can be done before or after the column separation, the principal derivatives used are o-phthaldialdehyde (OPA) and dansyl chloride, then the detection is done by fluorimetry. Several procedures using dansyl chloride for derivatization have been published (Mietz and Karmas, 1977, Rosier and van Peterghem, 1988, Malle et al. 1996, Valls et al., 1999). Methods with pre or post column derivatization with o-phthaldialdehyde were described (Walters, 1984, Gouygou et al., 1987, 1992, Ritchie and Mackie, 1989, Giorgio et al., 1993, Veciana-Nogues et al., 1995, Salazar et al., 2000).

In an EU project intituled "Biogenic amines in fishery products" (FAR-UP.1.46) some techniques have been studied and one HPLC method with a post-column OPA derivatization has been tested in an interlaboratory trial (Luten et al., 1992) the method is given in annex 2. A similar technique has been used in an interlaboratory exercise within eleven laboratories, the results indicated that the tested method was reliable and reproducible for determining histamine levels in various foods (Beljaars et al., 1998). In 1999, Germany adopted as reference method for the determination of biogenic amines in fish and fish products analysis of foods an HPLC method based on extraction of biogenic amines with perchloric acid, followed by direct HPLC analysis of the extract on a reverse phase column with on-line derivatization with o-phthaldialdehyde and fluorescence detection.

Other HPLC methods do not involve derivatization, they use an ionic chromatography followed by an electrochemical detection or a diode array detector ( Draisi et al., 1993, 1998b, asella et al., 2001, Cinquina et al., 2004a, 2004b).

#### *b) Equipment and materials required*

A basic equipment for extraction (homogeniser, scale, filters and/or centrifuge)

Liquid chromatography apparatus consisting in high pressure pumps, gradient system,

Detector and data acquisition system

For many procedures, a postcolumn reaction equipment (mixing chamber or capillary mixing T)

#### *c) Strong and weak aspects of the methodology*

##### Advantages

sensitive and reproducible method

Three similar procedures were standardized and tested in interlaboratory trial : Luten et al. (1992), Beljaars et al., (1998) and the German reference method.

##### Disadvantages

This method needs a sophisticated equipment and skilled technicians

*d) Adequacy for each fish link (type of business) in the distribution chain*

HPLC technique being sophisticated, the method can be used in specialized laboratories to check some samples after a first screening test.

*e) Bibliographic standards of reference*

The analytical reference method of the Commission Regulation (EC) No 2073/2005: Malle et al; (1996) and Duflos et al. (1999).

HPLC validated method by interlaboratory trial: German reference method (1999), Lutén et al. (1992), Beljaars et al.(1998).

Others references: Mietz and Karmas (1977), Rosier and van Peterghem (1988), Malle and Vallé (1996), Valls et al. (1999), Walters (1984, Gouygou et al. (1987, 1992), Ritchie and Mackie (1989), Giorgio et al.(1993), Veciana-Nogues et al. (1995), Salazar et al. (2000), Draisi et al.(1993, 1998b) Cinquina et al.(2004a, 2004b).

### 3.2.4. 3.2.4 - High performance thin layer chromatography (HPTLC) methods

*a) Technical basis*

The biogenic amines are extracted from fish with TCA solution, the extract are purified using solvents before a derivatisation of the amines with dansyl chloride, the separation of the dansylated amines is achieved on silica gel TLC plates, and the quantification is performed by UV densitometry. Some procedures have been described (Shalaby 1994, 1995, 1999, Shakila et al., 2001) and improved using less harmful solvent (Lapa-Guimarães and Pickova, 2004). Some results obtained by Shakila et al. (2001) are shown as example in Fig.2.

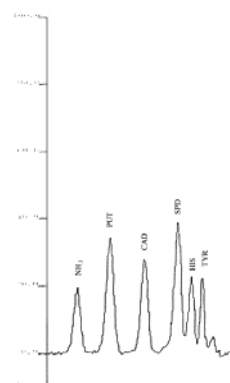
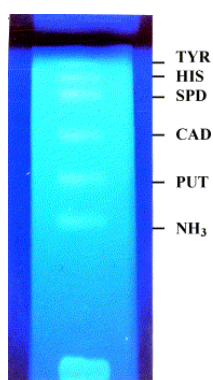


Fig. 2a: HPTLC separation of standard dansyl amines and ammonia on a pre-coated silica gel GF 254 plate

Fig. 2b: HPTLC densitometric scanning pattern of standard dansyl amines

According to Shakila et al., 2001

(NH<sub>3</sub> = Ammonia; PUT = Putrescine; Cad = Cadaverine; SPD = Spermidine; HIS = Histamine; TYR = Tyramine) -

*b) Equipment and materials required*

A basic equipment for extraction (homogeniser, scale, filters and/or centrifuge)  
Chromatographic plates and container,  
Densitometer and data acquisition system

*c) Strong and weak aspects of the methodology*

Advantages

it allows to fractionate 10–12 samples simultaneously on one plate at a time, and two plates can be simultaneously inserted in a migration container. The migration takes 30–40 min, and the time of scanning/calculation of one sample is 10 min. So as it allows simultaneous determinations can be considered rapid

It is not expensive and doesn't require sophisticated instrumentation.  
It is a sensitive and reproducible method

Disadvantages

It was not standardized neither tested in interlaboratory trial.  
Some reagents are toxic

*d) Adequacy for each fish link (type of business) in the distribution chain*

HPTLC can be used for screening test in quality control laboratories, it can replace old TLC methods, improving the quality of the results

*e) Bibliographic standards of reference*

Shablaby (1994, 1995, 1999), Shakila et al. (2001) Lapa-Guimarães and Pickova (2004)

### 3.2.5. Capillary Electrophoretic (CE ) methods

Capillary electrophoresis (CE) has developed enormously during the last decade

*a) Technical basis*

It is based on free-zone electrophoresis in buffer-filled capillaries which are used as the separation chambers. The detection is developed by ultraviolet or fluorescence detectors.

Protocol: The biogenic amines are extracted from fish with an acidic solution, the extract is purified before the injection under pressure in a capillary followed by a separation using a micro-current, at controlled temperature; in some procedures the amines are derivatized before the injection in CE. The detection is made using an UV detector or a diode array detector when the amines are directly separated or a fluorimeter when the amines are derivatized, i.e. with OPA

*b) Equipment and materials required*

A basic equipment for extraction (homogeniser, scale, filters and/or centrifuge)  
Capillary electrophoresis equipment

*c) Strong and weak aspects of the methodology*

Advantages

Disadvantages

This method needs a sophisticated equipment and skilled technicians  
Recent method which is no validated neither tested in interlaboratory exercises

*d) Adequacy for each fish link (type of business) in the distribution chain*

This new method could be used in specialized quality control laboratories for screening test.

*e) Bibliographic standards of reference*

Mopper and Sciacchitano, 1994, Gallardo et al., 1997, Shigeyuki et al., 1997, Mahendradatta and Schwedt, 1998, Du et al., 2001, 2002, Lange et al., 2002), Cinquina et al., 2004b.

## 4. Conclusion

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From the histamine and biogenic amines for fish quality assessment, two main conclusions can be withdrawn:

\* With regard to quality **control methods for scombrototoxin**

-To ensure the safety regarding histamine it is preferable to use a rapid method to do a screening, even if the method is semi-quantitative. Some convenient immuno-enzymatic kits are commercialised and other techniques such as colorimetric and TLC methods which require a small commitment of equipment and inexpensive reagents can be retained. However it is necessary to define the limits of the chosen method and to validate its reliability in comparison with an official method or a reference method; and in case of doubt regarding the results or in the event of dispute, it would be advisable to specify a reference method which may be used. Nowadays many rapid techniques are available and in the EU regulation it is specify ". Examinations must be carried out in accordance with reliable, scientifically recognized methods, such as high-performance liquid chromatography (HPLC).", but there are no reference method for histamine in Europe recognized by the member states.

- Histamine and biogenic amines are produced by enzymatic reaction, their level increases in the chain, even under chilling condition, so it is important to perform the analysis very quickly after the sampling or when it is possible, depending on the methods, to prepare the acidic extract that can be kept about one week at +4°C.

- The analysis result should be expressed clearly, i.e. histamine in mg/kg with the reference of the used method and details about the sampling (nature, date, place).

\* With regard to the **validation methodology**:

**For histamine** numerous methods have been described, a few of them have been studied in interlaboratory trial, some are AOAC Official methods and in Europe there is a reference method since December 2005.

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## Annex : Commercial kit tests for histamine

Test		Approx. Total Test Time	Supplier
ALERT® for Histamine [Sensitivity: 2.5 ppm]	Qualitative blue colour	35 min	Neogen Corporation Contact: Jennifer Baker 620 Leshler Pl., Lansing, MI 48912, USA Phone: 800/234-5333; 517/372-9004 E-mail: <a href="mailto:neogen-info@neogen.com">neogen-info@neogen.com</a> Web: <a href="http://www.neogen.com">www.neogen.com</a>
Veratox® for Histamine [Sensitivity: < 2.5 ppm, quantitative from 0 to 50 ppm]	Quantitative 650 nm	35 min	
Histamarine Test Kit <sup>1</sup> [Sensitivity: 0.5 ppm, quantitative from 1 to 500 ppm]	Quantitative 405-414 nm	1 h	Immunotech Contact: Alain Artus 130, av. Delattre de Tassigny, B.P. 177 13276 Marseille Cedex 9, FRANCE Phone: 33 491 17 27 46 E-mail: <a href="mailto:artus@immunotech.fr">artus@immunotech.fr</a> Web: <a href="http://www.immunotech.fr">www.immunotech.fr</a>
EIA for Histamine in Fish Extract, K1-HTM [Sensitivity: 2.5 ppm, quantitative 1-50 ppm]	Quantitative 405 nm	90 min	Immuno-Diagnostic Reagents Contact: Siong Wie P.O. Box 2659, Vista, CA 92085-2659, USA Phone: 858/350-9608 E-mail: <a href="mailto:idr@tiora.net">idr@tiora.net</a> Web: <a href="http://www.tiora.net/~idr/">www.tiora.net/~idr/</a>
EIA for Histamine in Raw and Canned fish, K3-HTM [Sensitivity: 5 ppm,]	Qualitative yellow colour	35 min	
HistaMeter [Sensitivity: 0-50 ppm,]	Qualitative	1 h	Biomedix Contact: Claver Bundack 1105 #F North Golden Springs Dr. Diamond Bar, CA 91765, USA Phone: 800/674-8648 #4282; 909/396-0244 E-mail: <a href="mailto:cb4biomedx@aol.com">cb4biomedx@aol.com</a>
HistaQuant [Sensitivity: 0-500 ppm,]	Quantitative	1-1/2 h	
Histamine in food ELISA [Sensitivity: 0-1000 ppm,]	Quantitative 450 nm	2h-15min	IBL - Immuno-Biological Laboratories Flughafenstr. 52 a D-22335 Hamburg, Germany E-mail: <a href="mailto:IBL@IBL-Hamburg.com">IBL@IBL-Hamburg.com</a> Web: <a href="http://www.IBL-Hamburg.com">www.IBL-Hamburg.com</a>

RIDASCREEN® Histamin R1602 [Sensitivity: 2.5 ppm;]	Quantitative 450 nm	2 h	R-Biopharm Landwehrstr. 64293 Darmstadt, Germany Phone: +49 6151 8102 - 0, Web: <a href="http://www.r-biopharm.com/foodandfeed/ridascreen_histamin.php">http://www.r-biopharm.com/foodandfeed/ridascreen_histamin.php</a>
RidaQuick Histamin (R1603-96 Wells) [Sensitivity 20 ppm;]	Quantitative	15 min	
Transia Tube Histamine [Semi- quantitative assay; from 25 to 200 ppm]	Semi- Quantitative 450 nm	1 h	Diffchamb AB Baika Barg` gata 7 42246 Hisings Baika Sweden Phone: 46-31-58-3270 E-mail: <a href="mailto:market.dept@diffchamb.se">market.dept@diffchamb.se</a> Web: <a href="http://www.diffchamb.com">www.diffchamb.com</a>
Transia Tube Histamine [Qualitative assay; threshold at 100 ppm]	Qualitative blue colour	1 h	

1AOAC Approved