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High-throughput histamine analysis approach in an official control laboratory: Analytical methods and four years fish products results

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ABSTRACT

A highly practical two-tier approach involving a screening and a confirmatory method was set up to efficiently test histamine in fish products in the frame of official controls. After their validation, the routine management of the two procedures was simplified as far as possible ensuring a strict quality data control and maximizing the cost-effectiveness. Accordingly five hundred and ninety batches of fish products (3129 determinations) sampled from the Italian authorities were successfully analyzed over a four year period (2008–2012). Only a small percentage of analysed batches (4.9%) was judged non-compliant. The examination of irregular cases (fish species, processing technology and storage practices) suggests important considerations in order to minimise the histamine risk for consumers.

1. Introduction

Histamine is the vasoactive amine which causes the so called scombroid poisoning syndrome (Hungerford, 2010). Although scombroid poisoning is generally a mild illness characterized by rash, urticaria, nausea, vomiting, diarrhea, flushing, tingling and itching of the skin (Taylor, 1986), the type of symptoms may have different degree of severity depending on the amount of histamine assumed and on individual sensitivity. Scombroid poisoning may be a considerable risk for the consumers when fish belonging to the families of *Scombridae*, *Clupeidae*, *Engraulidae*, *Coryfenidae*, *Pomatomidae*, *Scombresosidae* are eaten, because their muscles are characterized by high amounts of histidine which is the precursor for the synthesis of histamine by histidine decarboxylase spoilage bacteria (Lehanea & Olley, 2000). The formation of histamine, other than the presence of spoilage bacteria and histidine rich muscle, depends on the storage condition of the fish and fish products and, once formed, there is no method of food preparation available, including boiling, which can degrade the toxin (Etkind, Wilson, Gallagher, & Cournoyer, 1987). Commission Regulation (EU) No. 2073/2005 (2005) and the subsequent amendment (Commission Regulation (EU) No. 1441/2007) defined the maximum levels of histamine in fresh and processed fish and gave the rules for a correct sampling. Sampling must always be in nine units per batch in consideration of the inhomogeneous distribution of the toxin (Tao, Sato, Yamaguchi, & Nakano, 2009). Therefore, to assess compliance of a commodity subjected to official control, it is necessary to perform nine independent determinations of histamine. The products will be judged compliant only if: (i) the mean concentration of the nine results below 100 mg/kg; (ii) no more than two results have a value between 100 and 200 mg/kg; (iii) no sample-unit has a level of histamine above 200 mg/kg. Fish products that have undergone enzyme ripening in brine (e.g. anchovies, sardines, sardinellas) have limits which are twice the above mentioned.

In 2007 a European Community inspection in Albania revealed the limited capability of this country to carry out the necessary checks to detect the histamine contents in fish and fishery products (Commission Decision (EU) No. 642/2007). In these cases measures must be taken to avoid serious risks to human health (Commission Decision (EU) No. 178/2002). Accordingly, the import and the marketing into the European Union of fish from Albania were authorised only if a systematic check of the compliance to European histamine limits was performed either by the exporter (Albania) or by the Member State. The batches of Albanian fish products belonging to the scombroid families that were arriving at the Italian Ancona Harbour lacking required analytical certificate were to be tested. Pursuant to Commission Decision (EU) No. 642/2007 (2007) border authorities asked the competent official laboratory (Istituto Zooprofilattico Sperimentale) for analytical support in order to quickly control all the imported lots which were lacking of the certificate. Consequently, there was an urgent need to

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implement a suitable laboratory strategy in order to efficiently process at once a large number of samples.

Considering both the matrix spoilage and the necessity to prevent scombroid poisoning episodes, speed turns out to be a fundamental requirement of histamine analysis: for this purpose a two-steps strategy involving an immunoenzymatic screening test and a confirmatory chromatographic procedure was set up. Both methods were developed, validated and managed according to European legislation, avoiding the investigation of performances no strictly required. The aim was to combine the necessary analytical reliability to suitable analysis time, dealing efficiently with histamine official testing in fish and fish products. Therefore over four years (2008–2012) of histamine analyses are summarised, comparing and discussing screening and confirmatory results.

2. Materials and methods

2.1. Reagents

All the chemical reagents and solvents were of analytical and chromatographic grade, respectively. Histamine dihydrochloride, acetone and dansyl chloride were supplied by Sigma–Aldrich (Steinhem, Germany). Trichloroacetic acid (TCA) and sodium bicarbonate were purchased by Carlo Erba Reagents (Rodano, Italy). Acetonitrile, diethylether and methanol HPLC grade from VWR International (Radnor, PA, USA).

2.2. Sample preparation

Upon arrival the samples were either immediately homogenized, weighted in a 50 mL polypropylene centrifuge tube and frozen or directly frozen until analysis (maximum 1 week time). Frozen fish was let to defrost at 4 °C and then prepared. In fresh fish, skin and bones were removed and the whole sample homogenized. Canned fish filets or pieces were paper dried to remove as much preserving liquid as possible (oil or water) and then homogenized. In salted products the salt was removed with the aid of a knife and of a paper cloth. Once removed the salt, the fish was cut to take off the bones. The fish filets were then homogenized.

2.3. Screening method

Screening analysis was performed using a competitive enzyme linked immunoassay (ELISA) RIDASCREEN® Histamine R1601 (R-Biopharm, Darmstadt, Germany). All the reagents used for the ELISA were included in the commercial kit. Sample preparation was carried out as described by the kit producer with minor changes. Briefly, one gram of the homogenized sample was weighted in a 50 mL centrifuge polypropylene tube, 9 mL of distilled water was added and stirred vigorously for 1 min prior to centrifuge separation at 18 g for 5 min at room temperature; 1 mL of the supernatant (taken cutting through the lipid layer) was mixed well with 9 mL of water. 100 μL for enzyme ripened product and 200 μL for non-enzyme ripened product were brought to the volume of 10 mL in a volumetric flask. 100 μL of the obtained solution were submitted to acylation reaction as described by R-biopharm and subsequently to the ELISA determination following the kit instructions. Only the standard zero (histamine: 0 ng/mL) and the standard at 15.0 ng/mL were analyzed instead of the whole calibration curve provided by the commercial kit (six points): the zero standard was used to verify the absence of laboratory contamination whereas the standard at 15 ng/mL to check the proper functioning of kit reagents. In each analytical batch both positive and negative quality control samples (QC_{pos} and QC_{neg}) were included (Galarini, Buratti, Fioroni, Contiero, & Lega, 2011). The negative quality control was a blank tuna or/and blank anchovies, depending on the type of samples analyzed in the daily batch. Accordingly, the positive quality control was the same blank tuna spiked at 100 mg/kg or/and the same blank anchovies spiked at 200 mg/kg. Unknown samples, standard solutions and quality controls were pipetted twice into the microplate wells.

A microtitre plate spectrophotometer (Multiskan FC, Thermoscientific, Waltham, MA, USA) was used to read the absorbance of the ELISA plate at 450 nm. The measured absorbances were inversely proportional to the histamine concentration. The results were expressed in percentages (B/B_0%) using the following Eq. (1):

$$\left(\frac{B}{B_0}\right)(\%) = 100 \times \frac{abs\ sample\ or\ \text{standard}}{abs\ zero\ \text{standard}} \quad (1)$$

where B is the absorbance of the sample or standard analyzed and B_0 is the absorbance of the standard zero (standard solution 0 ng/mL of histamine) provided by the ELISA kit.

2.4. Validation of the screening method

Since no EU specific rules are in force concerning the performance of analytical methods for histamine determination, in this work the validation study was carried out following the suggestions of Commission Decision (EC) No. 657/2002 (2002). Therefore the test was validated as a qualitative screening method studying the specificity, the detection capability and the ruggedness/applicability. The validation study was performed in intra-laboratory reproducibility conditions. Sixty blank samples, representative of the fish matrices collected for histamine controls (processed anchovies in different oils and aromatising agents, processed tuna belonging to different production brands, fresh anchovies, tuna and mackerel), were analyzed to assess specificity. The same sixty blank samples were divided into two sub-groups (30 samples each): one sub-group spiked with histamine at 100 mg/kg and treated as non-enzyme ripened products; the other at 200 mg/kg and treated as enzyme ripened products. These samples were then analyzed by ELISA.

The obtained data was tested for normality using the Shapiro–Wilks test. Mean and standard deviation of the signals (B/B_0) acquired from the two groups (blank and spiked samples) were calculated. The cut-off value was obtained as follows:

$$\left(\frac{B}{B_0}\right)_{\text{cut-off}} = \left(\frac{B}{B_0}\right)_{\text{mean,spiked}} + 2.39 \cdot \left(\frac{B}{B_0}\right)_{\text{SD,spiked}} \quad (2)$$

where $(B/B_0)_{mean,spiked}$ was the mean signal of the sixty spiked samples, $(B/B_0)_{SD,spiked}$ their standard deviation and 2.39 was the constant value corresponding to the one-tail t-Student ($p = 0.01$, $v = 59$).

The $(B/B_0)_{cut\text{-}off}$ was the key parameter to interpret the results of the screening assay: if B/B_0 of a certain sample was lower than $(B/B_0)_{cut\text{-}off}$, the sample was judged "suspect non compliant" and subsequently submitted to confirmatory analysis; vice versa if B/B_0 was higher than $(B/B_0)_{cut\text{-}off}$, the sample was judged "compliant".

Also the ruggedness (minor changes) was investigated applying the Youden approach. Seven operational variables (factors) were studied taking into account their possible influence on the test results. Two levels were set for each variable by multiplying its uncertainty by a factor 5 and then subtracting (lower level) and adding (upper level) this value to the nominal level of the variable (Vander Heyden, Nijhuis, Smeyers-Verbeke, Vandeginste, & Massart, 2013). The ruggedness experiment was repeated twice both in tuna spiked at 100 mg/kg and in anchovies at 200 mg/kg. The selected variables and their levels are reported in Table 1. The results