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Short communication

Influence of storage temperature and freezing time on histamine level in the European anchovy *Engraulis encrasicholus* (L., 1758): A study by capillary electrophoresis

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Abstract

Histamine content in fish may increase by decarboxylation of free histidine to values that can be toxic, if storage conditions are not well controlled. We have studied the influence of storage temperature and time of freezing on histamine formation in the anchovy, *Engraulis encrasicholus* (L., 1758), for which little information is available. Analysis, carried out by capillary zone electrophoresis (CZE) without sample pre-treatment, was very simple, fast and reproducible. Results indicate that temperatures above 20 °C notably increase histamine production, whereas freezing can clearly prevent or slow down the process.

Keywords: Histamine; Anchovy; Capillary zone electrophoresis; Scombroid fish; Clupeid fish

1. Introduction

Histamine is a biogenic amine produced by decarboxylation of free histidine. Histamine is normally present at low levels in the human body and can be present in a variety of foods such as fish, cheese, meat, wine and fermented foods. Since histamine is involved as a primary mediator in many allergic reactions, the increase in its levels to values greater than 500–1000 mg/kg can be highly toxic giving symptoms that can be confused with alimentary allergies [1–3].

Histamine content in fish can rapidly increase during spoilage by bacterial histidine decarboxylases. Scombroid fish (Teleostea, Scombroidei), such as mackerel and tuna, or clupeid fish (Teleostea, Clupeoidei) such as sardines, anchovies and herring, are frequently involved in histamine toxicity. These fishes have relatively high free histidine levels in their muscles when alive [4]. Post-mortem proteolysis liberates additional histidine from muscle protein, and explains why histamine can reach high concentrations without the formation of organoleptic spoilage indicators.

Control of histamine levels in fish belonging to the Scombridae and Clupeidae families is regulated by European Union Directive No. 91/493.

The terms for testing indicate that nine independent samples from each batch should correspond to: (1) an average histamine concentration lower than 100 ppm (10 mg/100 g); (2) no more than two samples out of the nine can have a concentration between 100 and 200 ppm and (3) no sample may have a histamine content higher than 200 ppm.

The maximum level refers to fish products from the families Scombridae and Clupeidae that have not undergone enzymatic ripening treatment in brine. The maximum level doubles for fish products from the same family, which have undergone this treatment. Fresh fish contains negligible quantities of histamine, usually <0.1 mg/100 g [5].

At any time, exposure of certain fish to elevated temperatures after the catch and before consumption can cause formation of histamine from histidine by bacterial histidine decarboxylases, which are inevitably present [6]. Taylor cites a number of studies on the effect of storage temperature on histamine formation in various types of fish [7]. While all the studies agree

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that histamine formation is negligible in fish stored at 0 °C or below, data concerning storage conditions at higher temperatures are variable and do not allow the setting up of protocols that can avoid possible effects of storage conditions or transport stress on fish safety. In fact, it is not clear which are the lowest temperature limits for safe storage above 0 °C, and the optimal temperature for histamine formation. This is not surprising, given the variability of the nature of the microbial populations between fish species. In particular, information regarding histamine formation in anchovies is very limited [8].

The aim of this work was the evaluation of the effects of freezing or of temperatures above 0–4 °C on storage of anchovies with particular regard to histamine formation. Our study on histamine content of the extracts obtained with 5% trichloracetic acid (TCA) from anchovies was performed by capillary zone electrophoresis (CZE), a technique, which has been shown to be good for determining histamine levels in fish [9,10].

2. Experimental

2.1. Sample preparation

Six different batches of Anchovies – *Engraulis encrasicholus* (L., 1758) – were analysed. One of these – the BA batch – was captured directly by us in the Southern Adriatic sea during an experimental fishery project carried out by the Marine Biology Laboratory of Bari, Italy. The BA batch was stored on ice and transported to Potenza. The C1–C5 batches of anchovies were fished in the same sea (Southern Adriatic) by fishers and transported on ice to Potenza. The C1–C5 batches were purchased in different fish markets in Potenza the day after their capture. The samples were transported on ice to our laboratory and stored at 4 and −20 °C until use. Time zero for all samples was considered as the day of capture.

2.2. Capillary zone electrophoresis

Ten grams of gutted and filleted anchovies samples were blended with 50 ml of 5% TCA in a Waring Blendor for 3 min at room temperature. The homogenate was centrifuged at 4 °C for 6 min at 48,000 × *g*. Recovery of histamine was 87% with standard deviation of 4%.

Aliquots (2 ml) of supernatant extract were diluted with 10 mM sodium phosphate buffer at pH 2.5 (1:1), filtered through a Sartorius Minisart 0.2 μm pore size filter and then analysed using CZE.

Capillary zone electrophoresis separations were carried out with the BioFocus 2000 Capillary Electrophoresis System of Bio-Rad (Hercules, CA, USA), using the coated silica capillary BioCAP LPA of Bio-Rad (Cat. 148–3070); 50 μm I.D.; 47 cm total length; 40.5 cm effective length) at 20 °C. Samples were injected at nitrogen pressure of 1.362 atm × s and separation voltage of 15 kV. Detection was monitored at 210 nm. The run buffer was 100 mM sodium phosphate, pH 2.5 (Bio-Rad). Between analyses, the capillary was purged with deionised water for 2 min and with run buffer for 1 min.

The migration time of histamine was 4.9 ± 0.1 min. The ratio of the peak area to the migration time was used for quantification by the external standard method. A linear calibration curve was obtained using histamine hydrochloride (Fluka) as standard at 1.0, 2.5, 5.0, 15, 30, 60 and 100 ppm. Detection limit was 0.6 ppm. The minimum correlation coefficient of linearity was 0.999. Three different extracts were obtained from each anchovy sample, and each extract was analysed in duplicate.

3. Results

The histamine content of the extracts obtained with 5% TCA from gutted and filleted anchovies was determined by capillary zone electrophoresis. Fig. 1 shows a typical electropherogram of a TCA extract. The peak corresponding to the high resolution histamine signal was very well defined and without any interference. Addition of determined amounts of pure histamine as an internal standard increased the peak heights and clearly confirmed the identification of histamine.

The first approach to the study of histamine formation was to assess the maximum time allowed to store anchovies at 4 °C without detecting histamine. As shown in Fig. 2, the presence of histamine was observed in C5 starting from the second day, in C1–C4 batches from the third day and in the BA batch from the fourth day. After 5 days all samples showed levels of histamine higher than 100 ppm.

Since freezing may represent a common procedure for fish storage, the effect of freezing and thawing on histamine formation was also evaluated. C5 batch samples were stored at −20 °C for 3 h, 7 h, 24 h, 72 h, and 10 days. After freezing, samples were stored at 4 °C and analyzed using CZE after thawing (about 4 hours). The control sample was stored at 4 °C. As shown in Fig. 3, a significant decrease in histamine production rate was

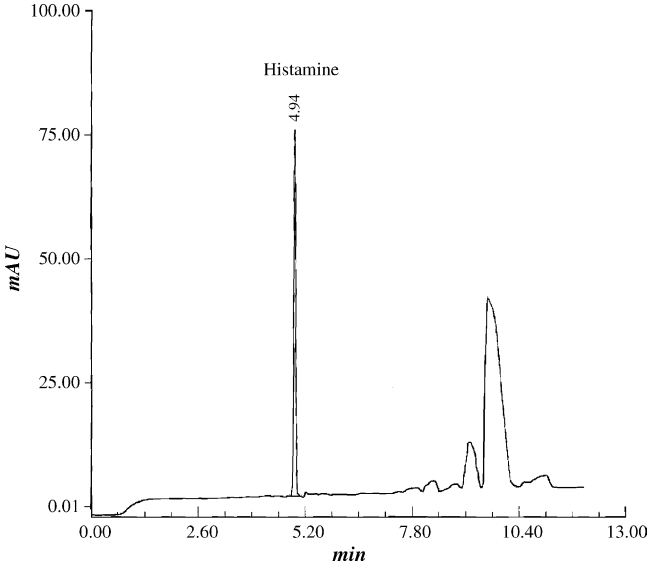

Fig. 1. Eletropherogram of anchovies extract for histamine analysis. Conditions: coated silica capillary (50 μm I.D.; 47 cm total length; 40.5 cm effective length), T = 20 °C, injection 1.362 atm × s, separation voltage of 15 kV, detection UV at 210 nm, run buffer: 100 mM sodium phosphate, pH 2.5 (Bio-Rad).

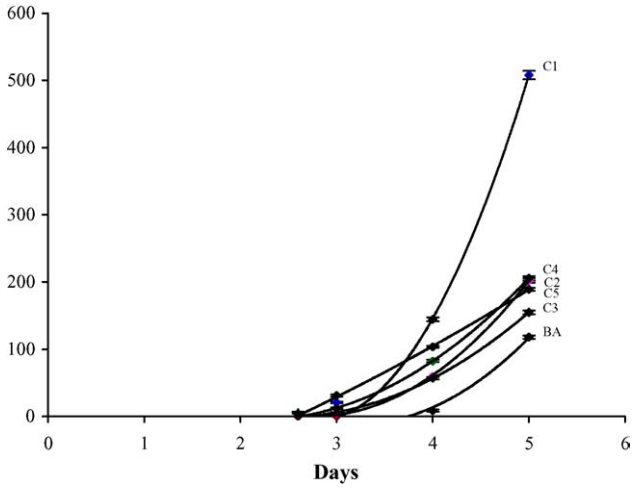

Fig. 2. Histamine formation against time in anchovies stored at 4 °C.

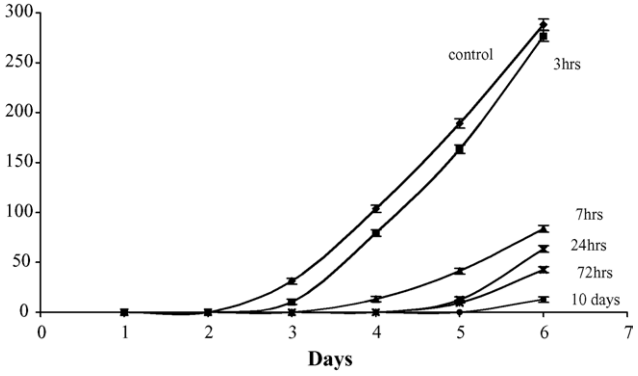

Fig. 3. Influence of freezing time on the histamine formation in anchovies. C5 batch samples were stored at −20 °C for 3 h, 7 h, 24 h, 72 h and 10 days. After these time periods samples were stored at 4 °C and analysed using CZE. The control was stored at 4 °C.

observed in the anchovies stored at freezing temperature for at least 7 h.

Finally, the influence of storage temperature on histamine formation was evaluated in one of our batches. Anchovy samples from the C1 batch were stored at 4, 10, 20 and 30 °C, respectively. Samples were analyzed in duplicate after 1, 3, 5, 8 and 24 h. Results only show the presence of histamine after 8 h in samples stored at 30 °C (Table 1). After 24 h the level of histamine in samples stored at 10 °C was about 10 ppm while in the samples stored at 20 and 30 °C it was higher than 700 ppm. Histamine was not detected in the samples stored at 4 °C.

Table 1
Influence of storage temperature on histamine (ppm) formation

Temperature (°C)	Time (h)				
	1	3	5	8	24
4	0	0	0	0	0
10	0	0	0	0	11.0 ± 2.8
20	0	0	0	0	749.9 ± 10.2
30	0	0	0	22.6 ± 2.9	1663.8 ± 19.3

Anchovies samples from C1 batch were stored at 4, 10, 20 and 30 °C, respectively.

4. Discussion

Formation of histamine in fish is related to the environmental conditions, histidine content, and the presence of bacterial histidine decarboxylase. High histamine levels can induce symptoms that are similar to allergic reactions and ingestion of 100–1000 mg at one time can induce so-called histamine poisoning.

While it seems to be well ascertained that formation of histamine increases with temperature during storage, there are very few studies concerning the formation of histamine in anchovies (*E. encrasicholus*) [8]. However, it is clear that the influence of storage conditions varies between different fish species.

On these grounds, the aims of this work were to assess the optimal storage conditions to avoid the increase of histamine in anchovies and to monitor the presence of toxic histamine levels in commercial products. There are several analytical methods available for the determination of histamine including high-performance liquid chromatography, ion exchange chromatography, thin layer chromatography, gas chromatography, mass spectrometry. Very often these methods are coupled to sophisticated techniques requiring derivatization and fluorimetric detection. The use of capillary zone electrophoresis for the determination of histamine levels in fish has been previously described [9,10] and its usefulness has been validated [11,12], also in comparison with HPLC. The study reported in the present paper represents the first example in which histamine has been determined in the anchovy by CZE.

The use of CZE for histamine analyses, as reported in this work, has several advantages on other techniques: it is simple (does not require preliminary modification of the molecules to be separated), rapid (a measurement is carried out in less than 15 min, including extraction by TCA), and very reliable. On this basis, CZE might be favored by the food industry where the analysis regards the assessment only of histamine levels. The use of other techniques may be more indicated to assess the presence of other biogenic amines. The analysis of histamine levels by CZE reported in this study differs in part both in the extraction procedure – based on TCA and the use of a Waring Blendor – as well as in the run conditions with a coated silica capillary.

Results shown in this paper clearly show the influence of different storage temperature values on histamine formation in anchovies, and the influence of freezing in stopping or slowing down histamine formation. In particular the results obtained show: (1) histamine only appears in anchovies after 2 days at 4 °C; (2) freezing time, when longer than 7 h, is important to reduce or stop the rate of histamine formation after thawing and storage at 4 °C and (3) histamine formation is greatly increased at storage temperatures higher than 4 °C. The differences observed in histamine formation between the BA batch and the C1–C5 batches might be due to different storage conditions on boat rather than to ground transport conditions. The BA anchovies

were putted on ice on board, this might be not the case for the other batches.

In conclusion, our procedure may be useful for determining the level of quality of the fish and for preventing its commercialization when contamination is present. This in turn will prevent food poisoning due to high histamine levels. Freezing can represent an alternative to the storage at 0–4 °C, when times longer than 3 days are foreseen before commercialization.

References

[1] J.E. Stratton, S.L. Taylor, Scombroid poisoning, in: D. Ward, C. Hackney, (Eds.), Microbiology of Marine Food Products, Van Nostrand Reinhold, New York, 1991, p. 331.
[2] J.D. Morrow, G.R. Margolies, J. Rowland, L.J. Roberts II, New Engl. J. Med. 324 (1991) 716.
[3] A. Halàsz, A. Baràth, L. Simon-Sarkadi, W. Holzapfel, Trends Food Sci. Technol. 5 (1994) 42.
[4] R.M. Love, The Chemical Biology of Fishes, Advances 1968–1977, vol. 2, Academic Press, London, New York, Toronto, Sydney, San Francisco, 1980, pp. 427.
[5] H.A. Frank, D.H. Yoshinaga, W.K. Nip, Mar. Fish Rev. 43 (1981) 9.
[6] L. Lehane, J. Olley, Int. J. Food Microbiol. 58 (2000) 1.
[7] S.L. Taylor, Crit. Rev. Toxicol. 17 (1986) 91.
[8] M.T. Veciana-Nogues, T. Hernandez-Jover, A. Marine-Font, M.C. Vidal-Carou, J. AOAC Int. 78 (1995) 1045.
[9] B. Mopper, C.J. Sciacchitano, J. AOOC Int. 77 (1994) 881.
[10] V.C. Trenerry, P.A. Marshall, K. Windahl, J. Capillary Electrophor. 5 (1998) 527.
[11] J. Lange, K. Thomas, C. Wittmann, J. Chromatogr. B 779 (2002) 229.
[12] A.L. Cinquina, F. Longo, A. Calì, L. De Santis, R. Baccelliere, R. Cozzani, J. Chromatogr. A 1032 (2004) 79.