Italian Journal of Food Safety

https://www.pagepressjournals.org/index.php/ijfs/index

eISSN 2239-7132

Please cite this article as:

D'Anza E, Di Paolo M, Alvino S, et al. **Assessment of environmental chemical contamination and histamine levels in the production of *Colatura di Alici di Cetara***. *Ital J Food Saf* doi:10.4081/ijfs.2024.12357

Submitted: 05-02-2024
Accepted: 03-06-2024

Assessment of environmental chemical contamination and histamine levels in the production of *Colatura di Alici di Cetara*

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Key words: fish sauce, food safety, heavy metals, fermented seasoning.

Contributions: VP, PG, conceptualization; MDP, ED, PG, methodology; MDP, ED, software; RM, PG, VP, validation; SA, AD, formal analysis; MDP, ED, PG investigation; VP, ED, resources; MDP, RM, PG data curation; MDP, ED, RM writing - original draft preparation; PG, RM writing - review and editing; SA, VP, AD, visualization; RM, PG, VP supervision, project administration. All authors have read and agreed to the published version of the manuscript.

Conflict of interest: the authors declare no potential conflict of interest.

Ethics approval and consent to participate: not applicable.

Funding: none.

Availability of data and materials: data and materials are available from the corresponding author upon request.

Conference presentation: this paper was presented at the XXXII National Conference of the Italian Association of Veterinary Food Hygienists (AIVI), September 13-15, 2023, Maierato, Italy.

Abstract
Colatura di alici di Cetara is a fish sauce obtained from the fermented seasoning of anchovies (*Engraulis encrasicolus L.*) in salt and produced in the province of Salerno (Campania, Italy). The anchovies used in its production could indicate the degree of the ecosystem's well-being through the study of heavy metals. Histamine represents one of the major hygienic and health concerns in fish products belonging to the *Engraulidae* family and those derived from them through enzymatic maturation. The current study aimed to: i) investigate the levels of heavy metals in anchovies; and ii) assess histamine content in two distinct production processes: the first following the Protected Designation of Origin (PDO) protocol, which involves anchovy's evisceration; and the second employing an experimental protocol using whole anchovies. The determined parameters were: i) heavy metals cadmium (Cd) and lead (Pb) using an atomic absorption spectrophotometer and mercury (Hg) using a direct mercury analyzer on the raw material (T0), coarse salt, and processed anchovies at T1; ii) polycyclic aromatic hydrocarbons (PAHs) using a high-performance liquid chromatography (HPLC) coupled to fluorescence detection at T1; and iii) histamine using an ultra HPLC-diode-array detection analysis at all stages throughout the process (T0 to T8). The results of the heavy metals analyzed show values below the permitted limits for Cd (0.013±0.006 mg/kg), Pb (not detected), and Hg (0.072±0.003 and 0.043±0.026 mg/kg). The PAHs were not detected. All histamine concentrations determined were below the maximum limit set by the European legislation for fish sauces. The highest values were found in the anchovies gutted at T2 (0.86±0.08), in the respective *colatura* (51.00±1.70) of the PDO procedure, and in the experimental procedure at T8 (7.00±0.60). No significant differences were found between the *colatura* obtained by both production processes. The study highlighted the importance of raw material selection and monitoring of the process for producing a product like *Colatura di Alici di Cetara*.

Introduction
Colatura di alici di Cetara is a fish sauce with a sharp taste, obtained from the fermented seasoning of anchovies (*Engraulis encrasicolus L.*) in salt. It is produced in the province of Salerno (Campania, Italy). The traditional processing of *Colatura di alici di Cetara* has been passed down through generations, earning it the protected designation of origin (PDO) status from the European Union in 2020 (European Commission, 2020 - Commission Implementing Regulation 2020/1529). *Colatura di alici di Cetara* is a culinary excellence of Southern Italy and represents a traditional condiment for various dishes, such as pasta, vegetables, and fish based meals (Di Nuzzo *et al*., 2017).
The anchovies (*Engraulis encrasicolus*) used to produce this fish sauce are pelagic fish caught in the Mediterranean Sea off the coasts of Amalfi and Positano. These small fish are among the most important pelagic fish resources in the Mediterranean Sea (Storelli *et al*., 2011). It is well known that most marine organisms, including fish, concentrate heavy metals at higher concentrations than those found in their environment (Sofoulaki *et al.,* 2018). Due to their position in marine food chains, short lifespan, early maturation, high fecundity, and plankton-based diet, anchovies reflect changes in the lower trophic levels and can indicate the degree of ecosystem wellbeing through the study of heavy metals (Bat *et al.,* 2014).
The anchovies used in its production are caught, gutted, beheaded by hand, and then carefully layered in wooden barrels called *terzigni*. These layers are separated by sea salt and left to season for 9 months. During this period, the synergistic interaction between halophilic microorganisms and enzymes facilitates the degradation of fish proteins and fats through various biochemical metabolic pathways, resulting in the flavor profile characteristic of fish sauce (Russo et *al*., 2020).
However, the evolution of volatile compounds during fermentation also results in the production of biogenic amines, including histamine. Histamine represents one of the major hygienic and health concerns in fish products belonging to the Engraulidae family and those derived from them through enzymatic maturation (Mercogliano *et al.*, 2008). Consuming food containing toxic levels of histamine can lead to food-borne chemical intoxication caused by histamine, thereby posing a health risk. European Regulation n. 1019/2013, which amended Regulation n. 2073/2005, set a maximum

tolerable limit of histamine at 400 mg/kg in fish sauce produced by fermentation of fishery products, like *Colatura di alici di Cetara*, to ensure consumer safety (European Commission, 2013). Moreover, sampling and compliance criteria were set for histamine levels (≤200 mg/kg, a maximum of c/n values observed are between 200 and 400 mg/kg or no values observed excess of the limit of 400 mg/kg) in fish species associated with a high amount of histidine, like anchovies (European Commission, 2005).

The careful handling and processing of fish from the moment of catch until consumption is critical for compliance with these maximum limits for histamine (Mohamed *et al.,* 2016). Effective storage, temperature control, and adherence to hygienic practices are essential in minimizing histamine production (Mohamed *et al*., 2016). Histamine is frequently produced when fish after the catch are exposed to temperatures, increasing enzymatic reactions and bacteria growth during both transportation and prolonged delay times before processing. This scenario permits the multiplication of histamine-forming bacteria and the release of histamine (Takahashi *et al.,* 2015). To preserve the quality of *Colatura di alici di Cetara*, the PDO outlines a specific production protocol, emphasizing a clear and precise timeline. For this reason, according to these guidelines, the duration from the moment the anchovy is caught to the final delivery at the processing plant must not exceed 12 hours. Overall, the current study aims to: i) investigate the levels of heavy metals cadmium (Cd), lead (Pb), and mercury (Hg) in anchovies and ii) assess histamine content in two distinct production processes: the first following the PDO protocol, which involves anchovy's evisceration, and the second employing an experimental protocol using whole anchovies.

Materials and Methods

Process description

Figure 1 reports the flow diagram to produce *Colatura di alici di Cetara*. On October 09, 2020, between midnight and 02:00 a.m., anchovies were caught in front of the Amalfi-Positano coast using a *lampara* fishing method. At the landing point in the port of Cetara, the size of anchovies and the presence/absence of *Anisakis* spp. were assessed. A representative sample was used to assess this. At 07:00 a.m., a batch of approximately 250 kg was delivered to a production plant following the PDO. The anchovies (9-12 cm) were allocated to produce two batches of fish sauce as follows: i) gutted anchovies of size 9-12 cm according to the PDO method (N1) and ii) whole anchovies of size 9-12 cm according to an experimental method (N2). For this production, anchovies were hand-headed, eviscerated (*scapezzate*), and put in alternating and ordered layers of salt and anchovies – in special oak containers called *terzigno* (one-third of a barrel).

Once the layers were completed, the containers were covered with a wooden disk called *tompagno*, on which weights (tradition says they are sea stones) were placed to keep the contents under pressure. The seasoning phase lasted 13 months (390 days) in *terzigni* that were never moved from the cooling room and were kept at a controlled temperature and humidity. The monitoring of the environments was carried out using an ESCORT mini-intelligent logger, Model MI-IN-D-2-L, Serial Number MI-BH-011-069 with temperature range between -40°C and +65°C, accuracy ±0.5°C and resolution 0.5°C.

Sampling and program of analysis

From October 2020 to November 2021, the sampling and analysis activities were carried out. Cd, Pb, Hg, and polycyclic aromatic hydrocarbons (PAHs), in the raw materials, were analyzed at time 0 (T0). In particular, the anchovies were taken to the zooprophylactic institute at the same time as they were processed in the factory; the salt used for processing was aliquoted and delivered with the anchovies. After an initial maturation period of one month, the anchovies were sampled from the *terzigni*. Each *terzigno* was opened and samples were taken from the different layers, from the surface to the bottom. Anchovies in the *terzigni*, N1 (muscle), and N2 (whole anchovies, muscle, and viscera) were tested monthly until February (T1 to T4), bimonthly until August (T5 to T7), and quarterly until November

(T8). The final products, *colatura,* extracted from N1 and N2 were analyzed at the end of the production period in November (T8).
Heavy metals (Cd, Pb, and Hg) were analyzed on the raw material (T0), coarse salt, and processed anchovies at T1 to determine their presence at the first steps of the process and also on the final product, *colatura di alici,* to evaluate a possible change in the concentration of the contaminants caused by processing. PAHs were analyzed at T0, T1, and in the final product. On the contrary, histamine content was evaluated throughout the whole process and in the final product, because it is correlated to the enzymatic fermentation of the *colatura*. To evaluate possible different contamination levels, the whole fish and their viscera and eviscerated bodies were tested. In particular, for N1, already gutted anchovies were sampled and analyzed, while for N2, whole anchovies, muscle, and viscera were analyzed separately, until product maceration.

Reagents and chemicals

All analytical grade reagents and solvents were from Carlo Erba Reagents S.r.l., Milan, Italy. High-performance liquid chromatography (HPLC)-grade water was in-house produced using a water purification system (Sartorius Arium® Pro, Sartorius Group, Göttingen, Germany). Ammonium di-hydrogen-phosphate $(NH_4)H_2PO_4$ and magnesium nitrate $Mg(NO_3)_2$ in 1% w/v water solutions were used as matrix modifiers (Perkin Elmer Waltham, Massachusetts, USA). Standard solutions of Pb, Cd and Hg were prepared by dilution of elemental standard solutions at 1000 mg L^{-1} (Merck, Darmstadt, Germany). Working-standard solutions for these elements were prepared daily by diluting stock solutions with water. Before use, all the glassware was washed with a solution of nitric acid (10% w/v) and then rinsed with HPLC-grade water.

Determination of histamine by ultra high-performance liquid chromatography with diode-array detection

Sample cleanup

Fish samples were homogenized by grinding; 5.00±0.01 g were weighed in a centrifuge tube, added with 30 mL of perchloric acid 0.4 M, then mixed by vortex. Further 20 mL of perchloric acid 0.4 M were added, and the sample was mixed then centrifuged at 4°C and 3500 rpm for 10 minutes. Then, 1.0 mL extract was drawn, added with 0.200 mL of sodium hydroxide 2 M, mixed by vortex; 0.300 mL of a saturated sodium hydrogen carbonate solution was added and mixed by vortex. Finally, a derivatization step was performed by adding 1.0 mL of dansyl-chloride, mixing, and incubating at 40°C for 45 minutes. To stop the reaction, 0.150 mL of ammonium hydroxide 30% w/v were added. The sample was mixed by vortex and then left in the dark for 1 hour. Then, 2.0 mL of acetonitrile was added, and after the mixing was centrifuged at 4°C and 3500 rpm for 10 minutes, the sample was analyzed by ultra high-performance liquid chromatography with diode-array detection. During each working session, precision and trueness quality controls were introduced, analyzing a sample spiked at 10 mg kg^{-1} and a sample spiked at 100 mg kg^{-1}, as well as reagent and process blanks.

Ultra high-performance liquid chromatography with diode-array detection analysis

Determination of histamine was performed using an ultra HPLC Dionex Ultimate 3000 system equipped with a quaternary pump, an autosampler, a diode array detector, and a thermostatic oven for column temperature control, managed by the software Chromeleon version 7 (Thermo Fisher Scientific, Milan, Italy). Chromatographic separation was performed on 1.8 µm particles, 50×4.6 mm Zorbax Eclipse Rapid Resolution XBD C18 stainless steel column (Agilent Technologies, Rome, Italy) at 25°C. Chromatography was run at a 1.0 mL min^{-1} flow rate, injecting 20 µL of sample, and applying isocratic elution by methanol/water 75/25 v/v. Detection was carried out at 254 nm to detect the derivatization product between histamine and dansyl-chloride. All samples were run in duplicate.
Histamine was determined by the external standard calibration curve method, calculating linear regression of the chromatographic peak areas *versus* standard solution concentrations. Interpolation of the linear regression curves was employed for quantification. The linearity of detector responses was assessed during each working session by the correlation coefficients (R^2) of standard calibration curves ($R^2 \geq 0.998$). The results are the mean of analyses in duplicate, corrected for mean recoveries of histamine. The test methods used are validated and

accredited according to the UNI EN ISO IEC 17025:2018 standard (UNI CEI, 2018), for official control of fish and derived products. The limit of quantification (LOQ) of the method is 0.20 mg/kg.

Determination of heavy metals by atomic absorption spectrophotometry

Sample preparation

Fish samples were collected at 4°C and homogenized by a mixer, then 0.75±0.01 g were weighted into a teflon reaction vessel, and 5.0 mL of 70% nitric acid, 2.5 mL of 30% hydrogen peroxide, and 2.5 mL of HPLC-grade water were added. After tight closure, the vessels were placed into a matrix-assisted microwave Milestone Ethos-One system (FKV S.r.l., Torre Boldone, Italy) and run using the following thermal program: 5 minutes at 800 W, from T=25°C to T=120°C, 2 minutes to 1000 W, T=120°C, 7 minutes at 900 W, T=120°C with T=190°C, 10 minutes at 700 W, T=190 °C. After acid digestion, the vessels were cooled to room temperature, and the samples were quantitatively recovered by filtration in 50-mL class A volumetric flasks and brought to a 50-mL final volume with HPLC-grade water. Likely, the samples of *colatura* were prepared by manual mixing, then 0.75±0.01 g were weighted and mineralized by matrix-assisted microwave as previously described.

Determination of lead and cadmium

Pb and Cd were determined by an atomic absorption spectrophotometer model PinAAcle 900T, equipped with a transverse heated graphite furnace; Zeeman effect correction for electrothermal atomization, and an autosampler were used (Perkin Elmer, Waltham, Massachusetts, US). The system was managed by WinLab software (PerkinElmer, Waltham, Massachusetts, US). Selective hollow cathode lamps (PerkinElmer, Waltham, Massachusetts, US) were used as line sources for Pb and Cd, respectively. The absorption wavelengths were 247.6 nm for Pb and 228.8 nm for Cd. Argon was used as the inert gas, and internal flow was set at 250 mL min^{-1}. The matrix modifiers $(NH_4)H_2PO_4$ and $Mg(NO_3)_2$ at 1% w/v were used during the determination of Pb and Cd. All samples were run in duplicate.

Determination of mercury (Hg)

Total Hg was determined by a direct mercury analyzer (DMA 80 Evo, Milestone, Sorisole, Italy). About 0.300 g of each sample were weighed in quartz sample vessels (DMA 8347, Milestone, Sorisole, Italy) introduced into the direct mercury analyzer system, where Hg is sublimed, adsorbed onto a gold amalgama, then thermally desorbed and determined by atomic absorption at 253.65 nm. All samples were run in duplicate.

Data quality assurance

Chemical blank determinations were made during each working session to check the purity of reagents or possible laboratory contamination and interferences in the whole analytical procedure. Data quality was assured through participation in a proficiency test with satisfactory results (z scores $\leq|2|$). Quantitative determination was carried out by interpolation of linear regression calibration curves of Pb, Cd, and Hg in standard working solutions at five concentration levels. The R^2 of standard calibration curves for all the elements were always higher than 0.99, showing an excellent linear relationship over the ranges of concentrations studied. Spiked samples were introduced during each work to verify the trueness of the analyses in terms of the recoveries of each element. The results are the mean of the analyses in duplicate, corrected for the mean recoveries of each element. The test methods used are validated and accredited according to the UNI EN ISO IEC 17025:2018 standard for official control of fish and derived products (UNI CEI, 2018). The LOQ are 0.010 mg/kg for Pb, 0.005 mg/kg for Cd, and 0.010 mg/kg for Hg.

Determination of polycyclic aromatic hydrocarbons by high-performance liquid chromatography coupled with fluorescence detection

The determination of PAHs in fish samples was performed by HPLC coupled with fluorescence detection (FLD) (HPLC-FLD). Samples were homogenized by a mixer, 2.00±0.01 g were treated with

2 M ethanolic KOH, then subjected to a liquid extraction with cyclohexane, and then the extract was cleaned up through a SPE C18 cartridge. Chromatographic analyses of PAHs were performed by HPLC-FLD by a system model alliance (Waters, Ireland). The LOQ for each PAHs was 0.2 µg/kg. The method allows to identify and quantify benzo[a]pyrene, benzo[a]anthracene, benzo[b]fluoranthene, and chrysene, selected as markers for the occurrence of the 16 EU priority PAHs in EU Regulations. The method was accredited according to the UNI EN ISO/IEC 17025:2018 international standard (UNI CEI, 2018); quality assurance controls were introduced during each working session to evaluate mean recoveries of PAHs from samples spiked at 1.0 µg/kg; quantitative analysis was carried out by external standard curve calibration.

Statistical data analysis

The data were statistically analyzed using IBM SPSS Statistics 29.0.1(171) software (IBM, Armonk, NY, USA). Data are presented as the mean ± standard deviation and were analyzed using one-way analysis of variance and nonparametric tests. The statistical significance of the comparison between the mean values was evaluated at $p<0.05$ and $p<0.01$.

Results and Discussion

Fermented fish sauces represent a highly nutritious food due to their nutritional composition and high digestibility, further enhanced during fermentation through the release of bioactive compounds such as peptides or amino acids (Han *et al*., 2023). However, compounds that could compromise food safety and pose health risks may be present in the raw material (anchovies). Fermented fish sauces may contain certain concentrations of heavy metals, including Cd, Hg, and Pb (Han *et al.,* 2023), deriving from both the muscle and viscera of fish. The results of the analysis of the raw materials (anchovies and salt) are shown in Table 1.

Based on the European Commission Regulation (EU) 2023/915, the maximum levels of Pb, Cd, and Hg are 0.30 mg/kg, 0.25 mg/kg, and 0.30 mg/kg, respectively (European Commission, 2023). The Pb level refers to the muscle meat of fish, and the Cd and Hg levels refer to *Engraulis* anchovies. Our analyses of the raw material (whole anchovies) showed no contamination by Pb and low levels of Hg and Cd, well below the maximum limits set by the European Commission Regulation (EU) 2023/915. Anchovies generally have lower Hg contamination than predatory fish (Costa *et al*., 2020), but they are a species susceptible to bioaccumulating Cd. In fact, the investigation of heavy metals at T1 in whole anchovies, muscle, and viscera showed a very low content of Hg only in the viscera, which therefore did not pose a concern for food safety.

The level of Cd and Pb was not detected, falling below the LOQ of the method, thereby excluding significant contamination in fish and later any potential cross-contamination during the production process. However, no specific Union maximum levels are set out in Annex I of European Commission Regulation (EU) 2023/915 for food that is dried, diluted, processed, or compound food (*i.e*., composed of more than one ingredient). For this reason, some aspects will be considered in the case of fish sauce. As indicated in Article 3 of European Commission Regulation (EU) 2023/915, changes in the concentration of the contaminant caused by processing or the relative proportions of the ingredients in the product should be considered. Therefore, it is important to note that fish sauce production includes salt as the second main ingredient, and analyzing this ingredient is crucial to determining potential heavy metal contamination such as Pb, Cd and arsenic (Heshmati *et al*., 2014). The oxidation rate of fatty acids in fish oil can be increased by heavy metal ions in the salt, which can lead to low-quality fish sauce (Han *et al*., 2023). This aspect highlights the importance of selecting raw materials for producing products such as *Colatura di Alici di Cetara.* The absence of heavy metals in the salt and in the final product (Table 1) proves that no risk derives from the salts used. Cd in the salt was not detected, avoiding possible cross-contamination of the final product. The presence of PAHs in fish is unusual; however, to exclude cross-contamination derived from the wood of the *terzigni* used for ripening, it was tested for PAHs possible presence in the anchovies at T0, T1, and in

the final product. No contamination by PAHs was observed in the raw materials or final product (Table 1).

Histamine, a biogenic amine, is primarily formed through the bacterial decarboxylation of free histidine in food (Burt *et al*., 1992). Fish preparations that require a long maturation period can be affected by histamine production during the production process (Shimoji *et al.,* 2019). Despite the significant concentration of free histidine in the muscle of fish belonging to the family *Engraulidae* (Burt *et al*., 1992), the highest histamine content determined in this study over the production stages was at 7.0 mg/kg for whole anchovies (Table 2). In the study by Pons-Sánchez-Cascado *et al.* (2005), the maturation period was half (26 weeks) that of the current study (56 weeks), and histamine values were lower (compared to batch 1) or similar (compared to batch 2) to those in our study, attributing the differences found between the batches to the different presence of microorganisms with amino acid decarboxylation capacity.

However, similar results about the histamine content in anchovies were reported by Mohamed *et al.* (2016). Production technology as well as the control of process parameters such as temperature are crucial aspects to monitor during the anchovy seasoning process and guarantee product quality. Citro *et al.* (2009) showed that high temperatures in the seasoning room during ripening increase the level of histamine. In this study, during the 13 months (390 days) of seasoning, *terzigni* were never moved from the cooling room and were kept at a controlled temperature and humidity. The average temperature and humidity stood at 12.8°C and 76%, respectively. This temperature and humidity management could have influenced the seasoning, keeping the levels of histamine production stable and low both in the production according to the PDO specifications (N1) and in the experimental one (N2).

Table 2 shows the histamine content (mg/kg) in anchovy samples at different stages of seasoning and in the *colatura*. In both experimental productions (N1 and N2), the histamine levels did not exceed the maximum limit established in Regulation EU n. 1019/2013 (European Commission, 2013). At the start of seasoning (T0), the histamine levels were undetectable in both experimental productions (N1 and N2). After one month of seasoning (T1), no detectable histamine content was observed in N1. Instead, at the same seasoning time (T1), N2 showed very low levels of histamine in the anchovies (2.37 mg/kg), in the muscle (2.30 mg/kg), and in the viscera (2.48 mg/kg). The histamine levels in the samples exhibited a reduction throughout the maturation process. At T2, the presence of residual histamine was detected in whole anchovies (0.56 mg/kg), muscle (0.50 mg/kg), and viscera (0.48 mg/kg), slowly decreasing compared to T1. All histamine concentrations determined were below the maximum limit for fish sauces. According to our results, Hernandez-Herrero *et al.* (2002) also noted a reduction in histamine content, which was statistically significant ($p<0.05$) only in the initial week of the ripening process, attributing the higher histamine content at T0 to the quality of the raw material. According to these researchers, histamine likely diffuses from the fish into the brine. At T3, T4, and T5, the histamine levels were undetectable in both experimental productions (N1 and N2). After 240 days of seasoning (T6), the anchovy's muscle of N1 showed a histamine level of 0.27 mg/kg. The same content of histamine (0.27 mg/kg) was detected in the anchovy's muscle in N2; instead, in the whole anchovies and viscera, the level of histamine was lower than LOQ and 0.42 mg/kg, respectively. After 13 months (T8) of seasoning, the anchovy meat was completely damaged; therefore, it was not possible to distinguish the muscles from the viscera. The analyses conducted on the material recovered in the *terzigni* N2 showed the highest histamine content. at 7.0 mg/kg in agreement with data reported by Mohamed *et al.* (2016). As regards the final products obtained (*colatura*), the dripping liquid extracted from the *terzigni* showed histamine concentrations at 51.0 mg/kg for N1 and 49.7 mg/kg for N2. In our study, the low level of histamine could be explained by the temperature and humidity control in the cooling room. In fact, controlling temperature and humidity helps reduce the enzymatic activity that causes histamine production (Takahashi *et al.,* 2015). Moreover, it is possible that the initial low histamine level in the product could be attributed to the use of fresh raw anchovies and the appropriate handling during ripening.

Conclusions

In the present study, chemical contaminants deriving from the raw material (anchovies), the salt, and the wooden containers (*terzigni*) were considered. The entire production process was managed, starting from the selection of fish, the control of the salt, and the monitoring of temperature and humidity to inhibit the enzymatic production of histamine, up to verifying no contamination by PAHs, which we hypothesized could derive from the wooden *terzigni*. The levels of contaminant residues found in these fish products do not show evidence of risk for consumers. The study highlighted the importance of raw material selection and monitoring of the process for producing a product like *Colatura di Alici di Cetara*. The management of temperature and humidity could influence the seasoning, maintaining stability and low levels of histamine production in both production processes analyzed. Controlling temperature and humidity may reduce the enzymatic activity that causes histamine production. During the process, under controlled conditions at low temperatures, the production of histamine is consistently low throughout the ripening process. This trial's information contributes to the understanding of this valuable product and can assist producers of *Colatura di Alici di Cetara*.

References

Bat L, Kaya Y, Oztekin HC, 2014. Heavy metal levels in the Black Sea anchovy (Engraulis encrasicolus) as biomonitor and potential risk of human health. TrJFAS 14:845-51.

Burt JR, Hardy R, 1992. Composition and deterioration of pelagic fish. In: Burt JR, Hardy R, Whittle KJ, eds. Pelagic fish: the resource and its explotation. Fishing New Books, Oxford, UK, pp 115-141.

Citro A, D'Antonio M, Bruno G, 2009. Differenti tecniche di estrazione e influenza della temperatura sulla produzione di istamina. Argomenti 1:73-6. [Article in Italian].

Costa F, Coelho JP, Baptista J, Martinho F, Pereira ME, Pardal MA, 2020. Mercury accumulation in fish species along the Portuguese coast: are there potential risks to human health?. Mar Pollut Bull 150:110740.

Di Nuzzo A, 2017. Approdi e rotte. Un esempio di trasmigrazioni mediterranee: il caso Cetara. Culture e Studi del Sociale-CuSSoc 2:171-87. [Article in Italian].

European Commission, 2005. Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. In: Official Journal, L 338/1, 22/12/2005.

European Commission, 2013. Commission Regulation (EU) No 1019/2013 of 23 October 2013 amending annex i to Regulation (EC) No 2073/2005 as regards histamine in fishery products. In: Official Journal, L 282/46, 24/10/2013.

European Commission, 2020. Commission Implementing Regulation (EU) 2020/1529 of 14 October 2020 entering a name in the register of protected designations of origin and protected geographical indications ('Colatura di alici di Cetara' (PDO)). In: Official Journal, L 349/3, 21/10/2020.

European Commission, 2023. Commission Regulation (EU) 2023/915 of 25 April 2023 on maximum levels for certain contaminants in food and repealing Regulation (EC) No 1881/2006. In: Official Journal, L 119/103, 5/5/2023.

Han J, Kong T, Wang Q, Jiang J, Zhou Q, Li B, Gu Q, 2023. Regulation of microbial metabolism on the formation of characteristic flavor and quality formation in the traditional fish sauce during fermentation: a review. Crit Rev Food Sci Nutr 63:7564-83.

Hernandez-Herrero MM, Roig-Sagues AX, Lopez-Sabater EI, Rodriguez-Jerez JJ, Mora-Ventura MT, 2006. Influence of raw fish quality on some physicochemical and microbial characteristics as related to ripening of salted anchovies (Engraulis encrasicholus L). J Food Sci 67:2631-40.

Heshmati A, Vahidinia A, Salehi I, 2014. Evaluation of heavy metals contamination of unrefined and refined table salt. Int J Res Stud Biosci 2:21-4.

Mercogliano R, D'Ambrosio R, De Felice A, Chirollo C, Citro A, Anastasio, 2008. "Colatura di alici "cetarese: evaluation of aminic profile. Ital J Food Saf 1:45-8.
Mohamed SB, Mendes R, Slama RB, Oliveira P, Silva HA, Bakhrouf A, 2016. Changes in bacterial counts and biogenic amines during the ripening of salted anchovy (Engraulis encrasicholus). J Food Nutr Res 5:318-25.
Pons-Sánchez-Cascado S, Veciana-Nogués MT, Bover-Cid S, Mariné-Font A, Vidal-Carou MC, 2005. Volatile and biogenic amines, microbiological counts, and bacterial amino acid decarboxylase activity throughout the salt-ripening process of anchovies (Engraulis encrasicholus). J Food Prot 68:1683-9.
Russo GL, Langellotti AL, Genovese A, Martello A, Sacchi R, 2020. Volatile compounds, physicochemical and sensory characteristics of Colatura di Alici, a traditional Italian fish sauce. J Sci Food Agric 100:3755-64.
Shimoji K, Bakke M, Hungerford JM, Mireles DeWitt CA, Köse S, 2019. Validation study of histamine test for the determination of histamine in selected fish products. J AOAC Int 102:164-80.
Sofoulaki K, Kalantzi I, Machias A, Mastoraki M, Chatzifotis S, Mylona K, Pergantis SA, Tsapakis, M, 2018. Metals and elements in sardine and anchovy: species specific differences and correlations with proximate composition and size. Sci Total Environ 645:329-38.
Storelli MM, Giachi L, Giungato D, Storelli A, 2011. Occurrence of heavy metals (Hg, Cd, and Pb) and polychlorinated biphenyls in salted anchovies. J Food Prot 74:796-800.
Takahashi H, Ogai M, Miya S, Kuda T, Kimura B, 2015. Effects of environmental factors on histamine production in the psychrophilic histamine-producing bacterium Photobacterium iliopiscarium. Food Control 52:39-42.
UNI CEI, 2018. UNI CEI EN ISO/IEC 17025:2018. General requirements for the competence of testing and calibration laboratories.

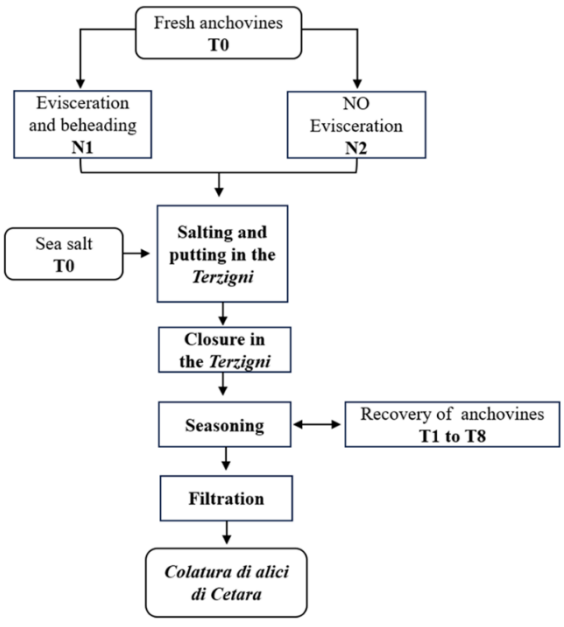

Figure 1. Flow diagram of three *Colatura di alici di Cetara* batches. N1, Protected Designation of Origin method; N2, experimental method.

Table 1. Heavy metals and polycyclic aromatic hydrocarbons in raw materials (achovines and salt) at T0 and T1.

Time	Items		Cd, mg/kg	Pb, mg/kg	Hg, mg/kg	PAHs, µg/kg
T0	Raw material	Whole anchovies	0.013±0.006	nd	0.072±0.003	nd
		Coarse salt	nd	nd	-	-
T1	N1	Muscle	nd	nd	0.068±0.001	nd
	N2	Whole anchovies	nd	nd	0.043±0.026	nd
		Muscle	nd	nd	0.043±0.017	nd
		Viscera	nd	nd	0.043±0.027	nd
	Final product[a]		nd	nd	nd	nd

Cd, cadmium; Pb, lead; Hg, mercury; PAHs, polycyclic aromatic hydrocarbons; N1, gutted anchovies of size 9-12cm following the Protected Designation of Origin method; N2, whole anchovies of size 9-12 cm following an experimental method; nd, not detected that is below the limit of quantification of the method; [a] the results in this row were the same for *colatura di alici* of N1 and N2. All data were presented as the least square mean ± standard deviation.

Table 2. Histamine content (mg/kg) in anchovy samples at different stages of seasoning and in the final product (*colatura*).

	Sample	T0	T1	T2	T3	T4	T5	T6	T7	T8	*Colatura*
N1	Muscle	nd^{A}	nd	0.86 ± 0.08^{B}	nd	nd	nd	0.27±0.06	nd	2.6 ± 0.3^{B}	51.0±1.7
N2	Whole anchovies	nd^{A}	2.37±0.18	0.56 ± 0.11^{B}	nd	nd	nd	nd	nd		
	Muscle	nd^{A}	2.30±0.26	0.50 ± 0.03^{B}	nd	nd	nd	0.27±0.11	1.10±0.6	7.0 ± 0.6^{B}	49.7±2.1
	Viscera	nd^{A}	2.48±0.29	0.48 ± 0.08^{B}	nd	nd	nd	0.42±0.09	nd		

N1, gutted anchovies of size 9-12 cm following the Protected Designation of Origin method; N2, whole anchovies of size 9-12 cm following an experimental method; nd, not detected that is below the limit of quantification of the method. All data were presented as the least square mean ± standard deviation. Different superscript uppercase letters indicate a significant difference at $p<0.01$; A, B, mean values in the same row with different letters presented significant differences.