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# The significance of cheese sampling in the determination of histamine concentration: Distribution pattern of histamine in ripened cheeses

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ABSTRACT

Cheeses are becoming a major safety and public health concern: cheeses available in supermarkets occasionally contain high histamine concentrations that can have negative effects on consumer health. In this study, we have attempted to assess the histamine distribution pattern in ripened cheeses, with the purpose of establishing a correct cheese sampling strategy for the quantification of histamine. To this aim, histamine was determined in four distinct areas of twelve long-ripened hard cheeses: the external and internal rind, along with the outer and inner core of the wedge. The concentrations measured were remarkably different: histamine accumulated in the central core, whereas the lowest amount was found in the peripheral rind. To explain this heterogenous distribution, histamine producers were determined in the four areas by identifying the *hdc* sequences obtained from cheese samples. Non-starter bacteria were identified as main histamine producers; however, these microbiota were homogeneously distributed throughout the wedge. Nevertheless, the analysis of psychochemical properties of the different areas revealed an observable trend: histamine tended to accumulate in the saltier, more humid, and less oxidized areas in a wedge. Overall, this study highlights the significance of a correct sampling strategy when histamine is quantified in cheese.

## 1. Introduction

Histamine is a biogenic amine synthesized from the precursor amino acid histidine through an oxidative decarboxylation reaction. It is frequently present in fermented products as a consequence of the metabolism of the microbiota that are present therein (Benkerroum, 2016). When it accumulates in food, histamine intoxication can cause severe systemic symptomatology including headaches, itching and rashes, vomiting, tachycardia, sweating, diarrhoea, respiratory distress, and hypotension (Comas-Baste, Sanchez-Perez, Veciana-Nogues, Latorre-Moratalla, & Vidal-Carou, 2020). Long-ripened cheese is the dairy product that tends to accumulate the most histamine: the content of the latter in cheese can reach values of up to 2,500 mg/kg (Madejska, Michalski, Pawul-Gruba, & Osek, 2018). Although the limit for histamine is not legally regulated in cheese, Regulation (EC) No 2073/2005 regarding microbiological criteria for foodstuffs establishes a limit of 200 mg/kg histamine in fish (Parliament, 2005). Nevertheless, 400 mg/kg of histamine has been proposed as a justifiable limit in ripened

*Abbreviations:* $a_w$, water activity; DSM, DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen; EFSA, European Food Safety Authority; HDC, histidine decarboxylase enzyme; *hdc*, histidine decarboxylase gene; LAB, lactic acid bacteria; NCIMB, National Collection of Industrial and Marine Bacteria; PCR, polymerase chain reaction; PDO, Protected Designation of Origin; TBARS, 2-Thiobarbituric acid reactive substances.

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cheese (Rauscher-Gabernig, Grossgut, Bauer, & Paulsen, 2009).

Cheese presents a strategically favourable microenvironment for the synthesis of histamine due to its composition and physical and chemical properties. Cheese is obtained from milk, by removing water and whey, and concentrating casein and fat. The first step consists in the clotting of milk to produce the curd by an enzymatic process; subsequently, the whey is removed by cutting the curd and stirring. Then, microbiota which are either naturally present in milk or artificially added produce lactic acid to render fresh cheese. Pressing the curd leads to form a rind, which limits oxygen and water transfer in the core of cheese. Finally, curing the cheese allows to obtain the final ripened product. Milk pasteurization, addition of selected starter cultures, or salting are optional steps in the cheesemaking process.

Microbiological and environmental factors can influence the cheesemaking process (Moniente, García-Gonzalo, Ontañón, Pagán, & Botello-Morte, 2021). On the one hand, microbiota involved in the synthesis of histamine comprise Gram-negative and Gram-positive bacteria, as well as yeasts. Lactic acid bacteria (LAB) are the main microorganisms responsible for histamine production in cheese (Barbieri, Montanari, Gardini, & Tabanelli, 2019): *Lentilactobacillus parabuchneri* stands out as the main histamine producer (Berthoud et al., 2017; Díaz et al., 2018; Diaz, Del Rio et al., 2016; Diaz et al., 2016; O'Sullivan et al., 2015; Wechsler et al., 2021; Wuthrich et al., 2017), although other LAB such as *Lactobacillus* sp., *Tetragenococcus halophilus*, and *Streptococcus thermophilus* have also been proposed as histamine-producing bacteria in cheese (Botello-Morte et al., 2022; Diaz, Ladero, Redruello, et al., 2016; Roig-Sangüés, Molina, & Hernández-Herrero, 2002; Rossi et al., 2011). Gram-negative bacteria can also contribute to the accumulation of histamine in cheese, usually as a result of spoilage or contamination in early steps of the cheesemaking process (Costa, Rodrigues, Frasao, & Conte-Junior, 2018). *Morganella morganii*, *Hafnia alvei*, and *Serratia* sp. are examples of Gram-negative histamine producers (Fernandez-Garcia, Tomillo, & Nunez, 2000; Roig-Sangüés et al., 2002). Finally, yeasts, including *Debaryomyces hansenii* and *Geotrichum candidum*, are likewise involved in histamine production in cheese (Gardini et al., 2006; Helinck, Perello, Deetae, de Revel, & Spinnler, 2013; Roig-Sangüés et al., 2002). Bacteria capable of producing histamine contain the *hdc* gene, codifying for the histidine decarboxylase (HDC) enzyme (Landete, de Las Rivas, Marcobal, & Muñoz, 2008). The *hdc* gene is mainly present in the bacterial chromosome, although certain bacteria, such as *L. hilgardii* and *T. halophilus,* feature the gene encoded in an extrachromosomal element: a mobile plasmid called pHDC (Lucas, Wolken, Claisse, Lolkema, & Lonvaud-Funel, 2005; Satomi, Furushita, Oikawa, Yoshikawa-Takahashi, & Yano, 2008; Wuthrich et al., 2017).

Environmental and physicochemical factors also determine histamine production in cheese (Moniente et al., 2021). As expected, microorganisms tend to produce more histamine when the precursor free histidine is available, which occurs as a result of proteolysis (Fernandez, del Rio, Linares, Martin, & Alvarez, 2006). The presence of other bacteria can determine histamine accumulation, either due to microbial competition and/or due to degradation of histamine (Coton et al., 2012). Further factors such as ripening temperature and time, as well as storage conditions, can also affect the formation of histamine in cheese. Histamine production is promoted by long ripening times combined with high ripening and storage temperatures (Linares et al., 2012), although some strains of *L. parabuchneri* can even synthesize histamine at 4–8 °C (Díaz et al., 2018). During the cheese ripening process, physical and chemical properties of the product evolve as the final cheese product matures. Those parameters tend to vary somewhat between the cheese core and the rind (Choi et al., 2020; Mayo, Rodriguez, Vazquez, & Florez, 2021). Whereas the surface is mainly aerobic and exposed to environmental microorganisms (Irlinger, Layec, Helinck, & Dugat-Bony, 2015), the core is more anaerobic, and microbiota in that area are mainly composed of LAB (Yeluri Jonnala, McSweeney, Sheehan, & Cotter, 2018). Physical and chemical properties of cheese, in turn, may exert an influence on the accumulation of histamine, thereby representing a key factor that needs to be carefully controlled in cheese (Linares et al., 2012). For instance, salt content above 5% (w/v) will generally tend to diminish histamine accumulation in cheese (Tabanelli, Torriani, Rossi, Rizzotti, & Gardini, 2012). Nevertheless, the halophilic bacterium *Tetragenococcus* sp. has been shown to produce histamine even at 20% (w/v) sodium chloride (Kimura, Konagaya, & Fujii, 2001; Satomi et al., 2008).

It is well established that neither microbiota nor physical and chemical properties, such as water activity ($a_w$) or salt content, are homogeneously distributed in cheese. Since histamine production in cheese is carried out by microbiota, which in turn are influenced by environmental factors, it would be expected that the distribution of histamine concentration in a cheese wedge would also be heterogeneous. This aspect is crucial when sampling for purposes of determining histamine concentration. The sampling area is a key point for a correct analysis of histamine concentration in cheese to obtain accurate, reliable values. Thus, a well-defined cheese sampling strategy could have an impact on results in terms of histamine content. Similarly, a correct cheese sampling strategy proved to be key in revealing the pattern of microbiota distribution in a wedge (Tilocca et al., 2020).

The objective of this study was to analyse the pattern of histamine distribution in long-ripened cheeses in order to establish an appropriate general sampling strategy for the determination of histamine concentration in cheese. We quantified histamine in different areas of cheese wedges, identified histamine-producing microbiota, and determined the cheeses' physical and chemical properties.

## 2. Material and methods

### 2.1. Cheese samples

For the purposes of this study, 12 long-ripened hard cheeses produced and commercialized in Spain were purchased. All of them were made from raw milk, and most of them could be considered "aged cheeses" (with ripening periods longer than 9 months), since it has been previously shown that these are key factors that play a role in histamine accumulation in cheese (Madejska et al., 2018). A recently published study shows that cheeses with higher histamine content are mostly made from raw sheep's milk (Botello-Morte et al., 2022). In that study, a commercialized Protected Designation of Origin (PDO) Idiazabal cheese (a hard raw sheep's milk cheese produced in the regions of the Basque Country and Navarra in Spain) displayed the highest histamine content, exceeding 500 mg/kg, which can be harmful or toxic to consumer health according to the European Food Safety Authority (EFSA, 2011). For these reasons, we selected two PDO Idiazabal cheeses (cheeses 1 and 2), along with a series of sheep's and mixed-milk cheeses in order to assess the pattern of histamine distribution within them. In total, half of the cheeses we selected (cheeses 1 to 6) were manufactured from sheep's milk, and the other half (cheeses 7 to 12) were made from milk blends. The main characteristics of the cheeses selected are summarized in Table 1.

The cheeses were received in the laboratory of the Centro Nacional de Tecnología y Seguridad Alimentaria (CNTA) and they were cut into nine wedges (Fig. 1A) in order to analyse a series of parameters in three different laboratories in triplicate. Each wedge was divided, in turn, into four areas to delimit the core versus the rind, and the inner area versus the outer area. The four areas thereby obtained are represented in Fig. 1B. Area 1 corresponded to the central rind of the wedge, Area 2 to the central core, Area 3 to the peripheral core, and Area 4 to the peripheral rind. Then, a set of samples of the different areas of 3 distinct wedges (twelve samples in total) were sent for the quantification of histamine concentration to the laboratory at BIOLAN Microbiosensores S.L. A second set of samples were sent to the laboratory of the Faculty of Veterinary in the University of Zaragoza, to characterize the histamine-producing microbiota present in the samples. Finally, a third set of samples were subjected to analyses of the physical and chemical

**Table 1**
Main characteristics of the cheeses used in this study: cheese variety, milk source and ripening time. The cheeses were made from raw milk and commercialized in Spain.

| Sample code | Cheese variety | Milk source | Ripening time (months) |
|---|---|---|---|
| **Cheese 1** | Idiazabal PDO | Sheep milk | ≥2.0 |
| **Cheese 2** | Idiazabal PDO | Sheep milk | ≥2.0 |
| **Cheese 3** | Aged sheep cheese | Sheep milk | 13.5 |
| **Cheese 4** | Aged sheep cheese | Sheep milk | 18.0 |
| **Cheese 5** | Aged sheep cheese | Sheep milk | 21.9 |
| **Cheese 6** | Aged sheep cheese | Sheep milk | 10.4 |
| **Cheese 7** | Aged cheese | Blended milk | 9.9 |
| **Cheese 8** | Aged cheese | Blended milk | 10.0 |
| **Cheese 9** | Aged hard cheese | Blended milk | 13.6 |
| **Cheese 10** | Aged cheese | Blended milk | 13.0 |
| **Cheese 11** | Aged cheese | Blended milk | 9.0 |
| **Cheese 12** | Aged cheese | Blended milk | 9.8 |

PDO: Protected Designation of Origin.

properties of the cheeses in the CNTA laboratory. Then, samples of 2–10 g (depending on the assay performed) of each of the four delimited areas were obtained in each laboratory and homogenized for purposes of analysis.

### 2.2. Histamine quantification

In recent years, the food industry has started to use biosensors for the quantification of histamine due to their low cost, speed, ease of use, and minimum amount of sample required (Moniente, Botello-Morte, García-Gonzalo, Pagan, & Ontanon, 2022). Samples were analysed for histamine content with the novel enzymatic BIO300 HIS biosensor (BIOLAN, Zamudio, Spain), according to an adapted protocol for histamine determination in cheese samples (Salleres et al., 2016). The test method consists in the extraction of histamine in aqueous solution and subsequent quantification by the biosensor after previous calibration. The enzymatic biosensor is a system that incorporates a biochemical sensing element (based on a specific oxidative enzyme) placed in close contact with or in close proximity to a transducer system that relates the concentration of an analyte to a measurable signal. The addition of the analyte causes sequential redox reactions that involve a release of electrons proportional to the concentration of the analyte. This type of biosensor thus combines the specificity and the selectiveness of enzyme–analyte reactions with highly sensitive electrochemical transduction.

For histamine extraction, a sample of 2 g of grated cheese was blended with 18 mL of distilled water and mixed by vortex. The internal calibration of the biosensor was 0–50.0 mg/kg, performed prior to the analysis of samples by adding, at two different times, 300 μL of a histamine standard of 25 mg/mL to the measuring cuvette, containing 10 mL of measuring buffer at pH 6.5. The range of quantification for the sample, taking into account the dilution factor performed for histamine extraction, was 100.0–500.0 mg/kg. To proceed with sample analysis, 300 μL of the extract was added to the measuring cuvette at the required moment. Once the measuring process had concluded, the device displayed the result of the analysis on the screen.

### 2.3. Total DNA extraction from cheese

DNA was isolated from cheese and quantified as described in Botello-Morte et al. (2022). Briefly, samples of 5 g of cheese were homogenized in sterile 2% (w/v) trisodium citrate (Panreac, Barcelona, Spain) and centrifuged for 10 min at 10,000 *g*. After removing fat layers and supernatants, cellular pellets were digested for 1 h at 37 °C in 20 mmol/L Trizma Hydrochloride pH 8 (Sigma Aldrich, St. Louis, MO, USA), 2 mmol/L EDTA (Panreac), 1.2% (v/v) Triton X-100 (Sigma Aldrich) with 20 mg/mL lysozyme (Sigma Aldrich), and 50 U/mL mutanolysin (Sigma Aldrich). Subsequently, 25 μL proteinase K and 200 μL buffer AL from a QIAGEN DNeasy Blood and Tissue kit (QIAGEN, Manchester, UK) were added, and incubated at 70 °C for 30 min. Samples were then subjected to a mechanical lysis by using a Precellys 24 homogenizer (Bertin Instruments, Montigny le Bretonneux, France) and PowerBead Tubes, Glass 0.1 mm (QIAGEN). Finally, supernatants were transferred to a column of the DNeasy Blood and Tissue kit (QIAGEN) and processed following the manufacturer's instructions.

### 2.4. PCR amplification of bacterial histidine decarboxylase (hdc) gene

PCR reactions were carried out in a T-100 thermal cycler (Bio-rad Laboratories, Madrid, Spain) with the Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, Massachussets, USA). The amplicons were visualized in a GelDoc EZ (Bio-rad Laboratories) documentation system, using Sybr-Safe DNA gel stain (Thermo Fisher Scientific). The *hdc* gene from Gram-positive bacteria was amplified by using the oligonucleotides JV17HC (AGACCATACACCATAACCTT) and HDC3 (GATGGTATTGTTTCKTATGA) according to Le Jeune, Lonvaud-Funel, ten Brink, Hofstra, and van der Vossen (1995) and Coton and Coton (2005), respectively. Genomic DNA from *L. parabuchneri* DSM 5987 was used as positive control in the PCR reaction. Oligonucleotides HIS2F (AAYTSNTTYGAYTTYGARAARGARGT)

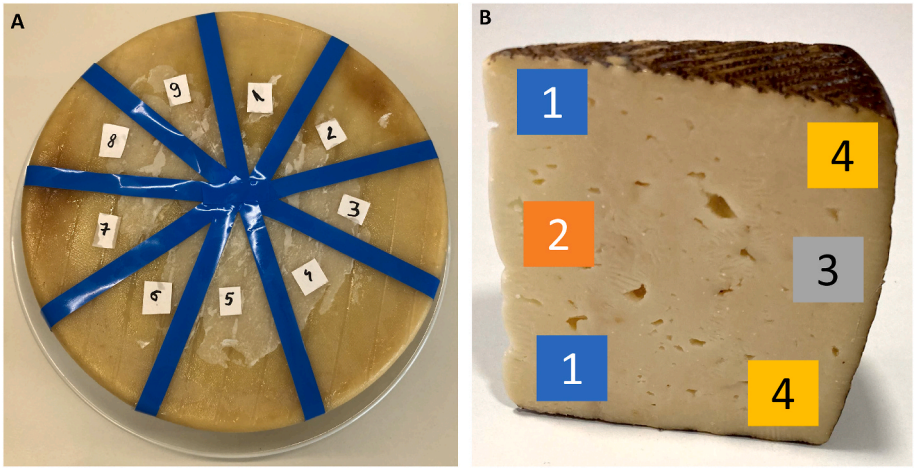


**Fig. 1.** Cheese sampling. A. The cheeses were cut into nine wedges. B. Each wedge was divided into four sampling areas. Area 1 (in blue) corresponded to the central rind of the wedge, Area 2 (in orange) to the central core, Area 3 (in grey) to the peripheral core, and Area 4 (in yellow) to the peripheral rind. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and HIS2R (TANGGNSANCCDATCATYTTRTGNCC), in accordance with de Las Rivas, Marcobal, and Munoz (2005), were used to assess the presence of the *hdc* gene amplified from Gram-negative bacteria, using total DNA of *M. morganii* subsp. *sibonii* NCIMB 865 as positive control. Since in certain histamine-producing bacteria the *hdc* gene can be codified in a mobile plasmid called pHDC, a sequence of the pHDC plasmid was amplified by using the pHDCF (CGCGGCAACAAAGGGTCC) and pHDCR (CGCTGATTCAGAATGACTTGAC) oligonucleotides from Botello-Morte et al. (2022).

### 2.5. DNA sequencing and BLAST analysis

Amplicons were purified with ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions, and sequenced by Macrogen Inc (Seoul, Korea). The analysis of nucleotide sequences was performed with the NCBI nucleotide database (BlastN: https://blast.ncbi.nlm.nih.gov/).

### 2.6. Analysis of physical and chemical properties of cheese

Several physical and chemical properties of the cheeses were analysed: humidity, salt content, and the oxidative state of the samples. Humidity was determined by drying the samples with a heating and drying oven (Memmert, Schwabach, Germany). Samples of 2 g of homogenized cheese measured in aluminium pans at 102 °C were used for this purpose. Salt content was determined by automatic potentiometric titration. Samples of 3 g of cheese were correctly homogenized, and then 0.1 mg of homogenate were subjected to salt content analysis, using a chloride-selective electrode and a 0.1 mol/L silver nitrate solution. The oxidative state of the four selected zones of the cheeses was determined by the 2-Thiobarbituric acid reactive substances (TBARS) assay (Pfalzgraf, Frigg, & Steinhart, 1995). For this purpose, samples of 4–10 mg of homogenized cheese were mixed with 35 mL of cold absolute ethanol (Sigma Aldrich). The mix was filtered, and distilled water was added up to the 50 mL mark. After that, 2 mL of 20% (v/v) trichloroacetic acid (Sigma Aldrich) and 1 mL of 0.67% (w/v) TBA were added to 2 mL of the extract previously obtained, and incubated for 15 min at 95 °C. Samples were then chilled for 5 min and centrifuged at 5000 *g* for 10 min. Finally, supernatants were subjected to spectrophotometric analysis at 440 nm and 532 nm wavelengths in a Jascov V-730 spectrophotometer (Jasco, Madrid, Spain). A blank sample was obtained using distilled water instead of the cheese sample. Calibration was obtained with different concentrations of malondialdehyde (Sigma Aldrich). Results were expressed as mg malonaldehyde/kg cheese. Water activity was measured in LabMASTER-$a_w$ equipment (Novasina, Lachen, Switzerland) at 25 °C. This equipment continuously measures water activity and temperature, based on a dimensionless scale of 0–1.0.

### 2.7. Statistical analysis

Statistical analysis was performed with one-way analysis of variance and Tukey's test with the XLSTAT software (version 2022.2.1; Addinsoft, Paris, France). The correlation among physical and chemical parameters in cheese areas was carried out with Excel (2019 version), establishing a *p*-value of 0.05 for significance.

## 3. Results and discussion

### 3.1. Pattern of histamine distribution in cheese

Histamine was extracted from the cheese samples and subsequently quantified with the novel enzymatic BIO300 HIS biosensor. Obtained data are summarized in Table 2. In general, histamine accumulation in the central areas (Areas 1 and 2) was higher than in peripheral zones (Areas 3 and 4), and the core generally exhibited more histamine than the rind (Area 2 versus Area 1, and Area 3 versus Area 4). This pattern recurred in most of the cheeses analysed. The differences observed indicated that the highest values of histamine were generally obtained in Area 2, whereas the lowest values appeared in Area 4, most of them lower than 100.0 mg/kg of histamine. Two of the twelve cheeses (Cheeses 8 and 12) did not exhibit histamine values higher than the biosensor's optimized limit of quantification (100.0 mg/kg) in any area of sampling. For that reason, cheeses 8 and 12 were excluded from further analysis in this study.

**Table 2**
Histamine concentration in the different areas of the cheeses. The mean histamine concentration value and the relative standard deviation of three independent measurements using the BIO300 HIS Biosensor are indicated.

| Sample code | Area 1 | Area 2 | Area 3 | Area 4 | Significance |
|---|---|---|---|---|---|
| **Cheese 1** | 149.5 ± 11.0 $^{a}$ | 178.3 ± 10.1 $^{a}$ | 145.5 ± 34.8 $^{a}$ | ≤100.0 $^{b}$ | ** |
| **Cheese 2** | ≤100.0 $^{b}$ | 221.5 ± 20.5 $^{a}$ | 115.0 ± 39.1 $^{b}$ | ≤100.0 $^{b}$ | *** |
| **Cheese 3** | 295.8 ± 20.0 $^{a}$ | 308.8 ± 15.8 $^{a}$ | 237.3 ± 22.1 $^{b}$ | 183.5 ± 10.2 $^{c}$ | *** |
| **Cheese 4** | 109.3 ± 6.6 $^{b}$ | 154.0 ± 5.0 $^{a}$ | ≤100.0 $^{c}$ | ≤100.0 $^{c}$ | *** |
| **Cheese 5** | 202.3 ± 17.3 $^{a}$ | 162.9 ± 29.6 $^{a}$ | ≤100.0 $^{b}$ | ≤100.0 $^{b}$ | ** |
| **Cheese 6** | 315.2 ± 28.2 $^{a}$ | 325.1 ± 30.1 $^{a}$ | 318.1 ± 19.2 $^{a}$ | 298.6 ± 15.9 $^{a}$ | n.s. |
| **Cheese 7** | 268.4 ± 54.7 $^{a}$ | 282.7 + 47.3 $^{a}$ | 160.7 + 44.8 $^{b}$ | ≤100.0 $^{b}$ | ** |
| **Cheese 8** | ≤100.0 $^{a}$ | ≤100.0 $^{a}$ | ≤100.0 $^{a}$ | ≤100.0 $^{a}$ | n.s. |
| **Cheese 9** | ≤100.0 $^{a}$ | 101.0 ± 3.5 $^{a}$ | ≤100.0 $^{a}$ | ≤100.0 $^{a}$ | n.s. |
| **Cheese 10** | ≥500.0 $^{a}$ | ≥500.0 $^{a}$ | 479.5 ± 21.9 $^{ab}$ | 450.0 ± 24.0 $^{b}$ | * |
| **Cheese 11** | 214.6 ± 28.6 $^{b}$ | 465.1 ± 34.9 $^{a}$ | 111.3 ± 19.2 $^{c}$ | ≤100.0 $^{c}$ | *** |
| **Cheese 12** | ≤100.0 $^{a}$ | ≤100.0 $^{a}$ | ≤100.0 $^{a}$ | ≤100.0 $^{a}$ | n.s. |

Area 1 corresponded to central rind of the wedge, Area 2 to the central core, Area 3 to the peripheral core, and Area 4 to the peripheral rind. The range of quantification optimized for the biosensor is 100.0–500.0 mg/kg: as a consequence, the histamine values lying outside of that range are mentioned as ≤ 100.0 mg/kg or ≥500.0 mg/kg n.s.: non-significant; *: *p*-value<0.05; **: *p*-value<0.01; ***: *p*-value<0.001. Lowercase letters indicate significant differences between Areas 1 to 4 in each cheese.

In order to highlight the heterogeneity of the distribution of histamine within a cheese wedge, we used box-and-whisker plots to reflect the trend observed in cheeses featuring significant differences among areas, regardless of whether the wedge belonged to a cheese with high or low histamine levels in general. This kind of plot allowed us to better differentiate the areas of the wedges that contained more histamine (with respect to the average value of histamine concentration in the cheese) from those that had less histamine. Fig. 2 shows the percentage of positive/negative deviation in each zone from the mean value of histamine concentration in the wedge, represented by 0% deviation. Our analyses of histamine content in cheese samples revealed that Areas 1 and 2 contained more histamine (17.8% and 54.4%, respectively) than the mean value of histamine content in the entire wedge, whereas the content of histamine in Areas 3 and 4 (−19.8% and −52.3%, respectively) was lower than the mean value of the wedge. Thus, histamine tended to have higher values in the central core (Area 2) of the wedge, which contained up to 115.4% more histamine than the mean value of the wedge's four areas. The peripheral rind (Area 4) displayed the lowest levels of histamine: its histamine content was always lower (up to −83.3%) than the mean value of the wedge's four areas. The central rind (Area 1) and the peripheral core (Area 3) of the wedge had mild histamine concentration values. In Area 1, histamine content tended to be higher than the mean value of the wedge: up to 73.3% higher, but it could also be lower, reaching −20.9%. Area 3 generally displayed values lower than the mean histamine content in the wedge: up to −50.3%

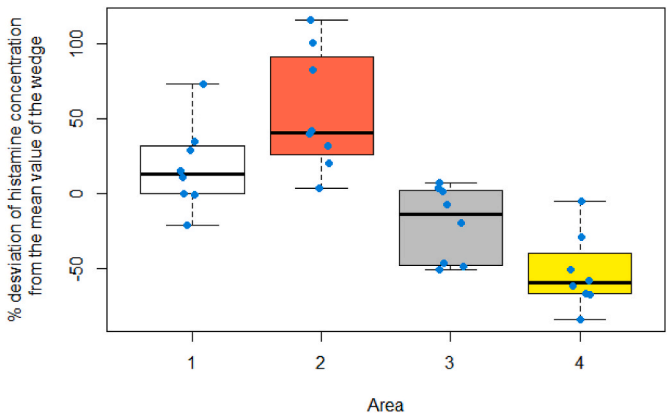


**Fig. 2.** The percentage of deviation of histamine concentration in each cheese area from the mean value of the entire wedge, represented in a box-and-whisker plot. Area 1 corresponds with the central rind of the wedge, Area 2 with the central core, Area 3 with the peripheral core, and Area 4 with the peripheral rind. Each blue point represents the deviation of histamine concentration from the mean value of the wedge. The median line of the box represents the medians of histamine concentration in each defined area of the wedge. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

lower, although it could likewise surpass 7.7% of the wedge's mean value (Fig. 2).

Previous studies performed on cheeses have suggested that biogenic amines might not be homogeneously distributed in cheese (Marijan et al., 2014; Novella-Rodríguez, Veciana-Nogués, Izquierdo-Pulido, & Vidal-Carou, 2003). In 1998, a team of authors suggested that biogenic amines could be more present in the central part of the wedge than close to the rind (Joosten & Stadhouders, 1987). Some years later, Novella-Rodríguez et al. (2003) and Marijan et al. (2014) confirmed this distribution in cheese for all the amines they tested, except for tyramine, which accumulates in the outer part of the cheese. However, Komprda et al. (2007) and Shehata and Rdwan (2017) found a higher content of biogenic amines in the cheese rinds. At any rate, the values of histamine quantified in all these studies were lower than 20 mg/kg in the two distribution zones; these values are thus not truly comparable with those of our study, where certain samples reached histamine concentrations higher than 500 mg/kg. Moreover, our study was performed in four accurately defined areas of each sample. However, it is worth noting that our proposal of a histamine distribution pattern could be more interesting when using ripened cheese with high histamine concentrations, even higher that the limit proposed by the authorities, since cheese sampling could thus represent a key point. It is possible that this pattern also occurs in cheeses with lower histamine amount, but in this case, it would be not as relevant.

Overall, histamine content was higher in the inner core of the wedge (Area 2), versus the other three areas in most cheeses (8 out of 12 cheeses). In 3 cheeses, the histamine values lay outside the range of quantification optimized for the biosensor: thus, in these cases, histamine content could not be accurately determined. Thus, the core of the wedge (Areas 1 and 2) generally exhibited a higher amount of histamine than the rind (Areas 3 and 4). This pattern of histamine distribution in four different areas highlights the importance of conducting a correct sampling procedure when determining histamine in cheese. Depending on the area of the wedge where the cheese sample is taken, the result in terms of histamine content can be extremely different. Taking different small-size samples from the four different areas of the entire wedge and homogenizing them can yield an intermediate value. However, to accurately determine the highest value of histamine present in the wedge, a sample from the cheese's inner core should be taken.

### 3.2. Molecular detection of the hdc gene and the pHDC plasmid

In order to search for a putative correlation between this pattern of distribution and histamine-producing microbiota in cheese, a molecular analysis of the presence of the *hdc* gene in the different areas of the wedge was performed. As evidenced in Botello-Morte et al. (2022), total DNA from cheese is the best starting material for this purpose. We therefore isolated total genomic DNA from each area in the cheese samples. Four cheeses were selected to perform molecular analysis: the two Idiazabal DPO cheeses (Cheeses 1 and 2), a further one made from sheep's milk (Cheese 3), and another one made from blended milk (Cheese 7). Bacterial *hdc* genes were PCR-amplified, using specific oligonucleotides previously described in the literature to amplify the gene from either Gram-positive or Gram-negative bacteria. Fig. 3 shows that *hdc* gene from Gram-positive bacteria was present in DNA isolated from all cheese samples, whereas the *hdc* gene from Gram-negative species was absent in all samples. Since the *hdc* genes of certain Gram-positive bacteria are occasionally located in the unstable pHDC plasmid, specific oligonucleotides that align within the plasmid sequence were also used in the PCR procedure to ascertain the presence of pHDC-containing bacteria. Certain areas, such as Area 1 in Cheese 3, also contained the pHDC plasmid, but not all of them did. Histamine producers with the *hdc* gene, belonging to the Gram-positive group, were present in all areas defined in cheese samples, and some of them contained this gene in an extrachromosomal, mobile plasmid.

### 3.3. Determination of histamine-producing microbiota in cheese

The *hdc* amplicons obtained by PCR were purified and subjected to Sanger sequencing to identify the histamine-producing microbiota. Two or more chromatographic peaks overlapped in each area, indicating that several *hdc* sequences, and, thus, histamine producers, were presumably present in the sample. The predominant species are listed in Table 3. The nucleotide sequences corresponded to *L. parabuchneri/buchneri* and the group *T. halophilus/T. muriaticus/Oenococcus oeni/Latilactobacillus sakei/L. hilgaldii,* whose *hdc* sequence is completely conserved and indistinguishable (Wuthrich et al., 2017). These histamine-producing bacteria are commonly found in cheese (Diaz et al., 2015; Diaz, Ladero, Redruello, et al., 2016; Møller, Ucok, & Rattray, 2020; O'Sullivan et al., 2015; Wechsler et al., 2021). We therefore presume that non-starter LAB were the main histamine producers in the ripened cheeses we analysed. The *hdc* gene of *L. parabuchneri/buchneri* is located in the chromosome, whereas the group *T. halophilus/T. muriaticus/O. oeni/L. sakei/L. hilgaldii* presumably contains the *hdc* genes in the pHDC plasmid (Lucas et al., 2005), which is in line with the results we obtained by PCR.

### 3.4. Analysis of physical and chemical properties of the different areas

The production of histamine is strongly influenced by microbiological and environmental factors present in food (Moniente et al., 2021). Since the same histamine producers were found in every cheese area subjected to analysis, the next step consisted in determining several of the cheeses' physical and chemical properties in order to elucidate their relationship with histamine accumulation in a particular pattern. Moisture (g/100 g cheese), $a_W$, salt content (g NaCl/100 g cheese), and oxidative state (measured by the TBARS assay as mg malonaldehyde/kg cheese) were determined in each area in order to correlate them with the amount of histamine in the samples. The values obtained for these measurements are summarized in Table 4. In order to globally represent the differences in each zone, the percentages of deviation of each parameter from the mean value of the wedge for each area are plotted in box-and-whisker plots in Fig. 4.

No relevant deviations of $a_W$ were found in any area with respect to the mean value of the entire wedge. Moisture and salt content were significantly higher in the cores (with deviations of 15.1% and 22.4% in Area 2, and 8.0% and 9.0% in Area 3, respectively) with respect to the

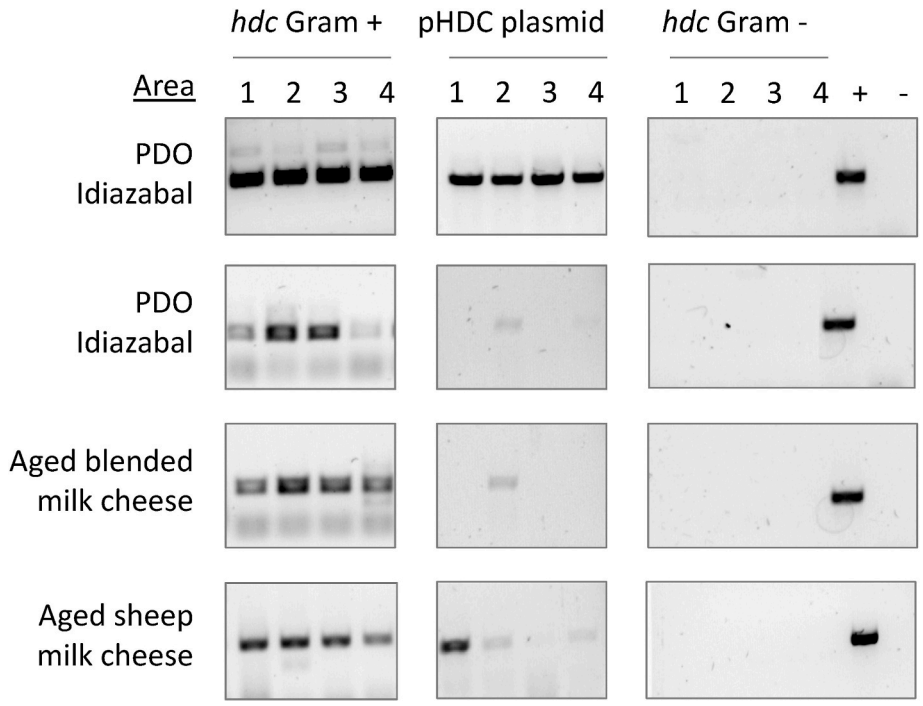


**Fig. 3.** PCR amplification of the *hdc* gene from Gram-positive and Gram-negative bacteria, as well as of the pHDC plasmid in the four areas of analysis established in the wedge of cheeses of several types of milk.

mean values in the entire wedge; whereas they were lower in the rind (moisture and salt content with deviations of −6.2% and −11.9% in Area 1, and -18.0% and −20.2% in Area 4, respectively). The same behavior was observed regarding histamine content distribution throughout the wedge, which was higher in the core (Areas 2 and 3) than in the outer parts.

Regarding oxidative state, despite the fact that non-significant differences have been found among areas, a slight tendency is observed: the core of the cheeses (Areas 1 and 4) was less oxidized (−29.6% and −40.3% in Areas 1 and 4, respectively) than the mean value of oxidation of the entire wedge, and also less oxidized than the outer portions. It is well known that lipid oxidation in cheese is associated with rancidity, flavor defects and oxidative deterioration, as well as loss of nutritional quality (Xiang et al., 2021). As a consequence of lipid oxidation, molecules such as aldehydes, ketones, alcohols, acids, and hydrocarbons are produced (Bergamo, Fedele, Balestrieri, Abrescia, & Ferrara, 1998). Additionally, the emergent trend of consumers towards low-fat dairy products results in an increase in the impact of protein oxidation in cheeses, which can also occur due to heat treatments applied during the cheesemaking process. Exposure to light during storage (frequently in packaging with transparent materials) can also lead to photo-oxidation of cheese (Kastrup Dalsgaard et al., 2010). The formation of sulphur compounds, such as dimethyl disulphide, and protein-bound carbonyls, is an indicator not only of the direct oxidation of aromatic and aliphatic amino acids, but also of secondary protein oxidation during glycation and lipoperoxidation (Rinaldi, Palocci, Di Giovanni, Iacurto, & Tripaldi, 2021). The influence of the glycation and the Maillard reaction during cheese manufacturing is worth highlighting (Fedele & Bergamo, 2001). Furfurals and advanced glycation end products are present in higher amounts in higher-fat and aged cheeses (Sharma, Kaur, Thind, Singh, & Raina, 2015) as well as in hard cheeses (Li et al., 2022). Since the cheeses used in this study were hard aged cheeses, it is possible that other TBARS products different than malonaldehyde could have been formed, leading to an overestimation of this product.

Despite the fact that the highest histamine content was found in the core of the cheeses (Areas 1 and 2), similarly to salt content and moisture, a positive, statistically significant correlation could not be established for any of these parameters (r −0.11, *p*-value 0.59 and r 0.33, *p*-value 0.082, respectively). Manca et al. (2020) were also unable to establish a relationship between histamine and water activity, moisture, or salt content in Fiore Sardo cheese. A significant correlation, negative in this case, between histamine content and oxidative state in the different areas of the cheese could not be determined either (r −0.37; *p*-value 0.056). However, a tendency to accumulate histamine in the wedge's saltier, more humid, and less oxidized areas was clearly notable. Other studies have indicated that high salt concentration could impair histamine production in food (Besas & Dizon, 2012; Møller, Castro-Mejía, Krych, & Rattray, 2021; Sumner, Roche, & Taylor, 1990; Tabanelli et al., 2012), probably due to the inhibitory effect of salt on microbial growth (Bansal & Mishra, 2020; Bisig, 2014). The same kind of microbial growth control can be exerted by $a_w$: the production of histamine can thus be inhibited when $a_w$ has been sufficiently reduced to impair the proliferation of histamine producers (FAO/WHO, 2013). However, it has also been reported that the halophilic bacterium *Tetragenococcus* sp. (obtained in this study as histamine producer, as shown in Table 3) has been able to grow (Unno et al., 2020), and even to produce histamine, at 20% (w/v) NaCl (Kimura et al., 2001; Satomi et al., 2008). *Staphylococcus epidermidis* and *S. capitis* also produced notable histamine amounts at 10% (w/v) NaCl (Hernandez-Herrero, Roig-Sagues, Rodriguez-Jerez, & Mora-Ventura, 1999). Furthermore, *Tetragenococcus* sp. has been proposed as a producer of a histamine under $O_2$-limiting conditions (Kimura et al., 2001). The latter bacterium could be associated with higher histamine content in the cheese core, where oxidative state is lower, as suggested by our data. Taken together, the analyses of the physical and chemical properties of the different areas in cheese samples revealed an observable trend: histamine tended to accumulate in the saltier, more humid, and less oxidized areas in a wedge. Further studies are nevertheless required to elucidate the microbiological/physicochemical causes of the characteristic distribution pattern of histamine accumulation in long-ripened cheeses with high histamine content.

**Table 3**
Summary of the main BLAST analyses of the amplicons of the *hdc* genes as obtained from the different areas of the cheeses.

| Cheese sample | | Main BLAST output | E-value | % identity | GenBank accession number |
|---|---|---|---|---|---|
| Cheese 1 | Area 1 | *L. parabuchneri* | 1E-151 | 99.67% | CP018796.1 |
| | | *T. halophilus* | 1E-137 | 95.24% | AB670117.1 |
| | Area 2 | *T. halophilus* | 5E-151 | 97.75% | AB670117.1 |
| | | *L. parabuchneri* | 2E-139 | 95.83% | CP018796.1 |
| | Area 3 | *T. halophilus* | 1E-152 | 99.67% | AB670117.1 |
| | | *L. parabuchneri* | 8E-149 | 98.37% | CP018796.1 |
| | Area 4 | *T. halophilus* | 1E-152 | 99.67% | AB670117.1 |
| | | *L. parabuchneri* | 6E-145 | 97.11% | CP018796.1 |
| Cheese 2 | Area 1 | *L. parabuchneri* | 1E-156 | 99.00% | CP018796.1 |
| | Area 2 | *L. parabuchneri* | 2E-150 | 99.01% | CP018796.1 |
| | Area 3 | *L. parabuchneri* | 4E-152 | 98.40% | CP018796.1 |
| | Area 4 | *T. halophilus* | 3E-74 | 86.13% | AB362339.1 |
| | | *L. parabuchneri* | 1E-72 | 89.43% | CP018796.1 |
| Cheese 3 | Area 1 | *T. halophilus* | 2E-135 | 95.75% | AB670117.1 |
| | | *L. parabuchneri* | 2E-125 | 93.79% | CP018796.1 |
| | Area 2 | *T. halophilus* | 3E-179 | 98.89% | AB670117.1 |
| | Area 3 | *L. parabuchneri* | 4E-173 | 99.13% | CP018796.1 |
| | Area 4 | *L. parabuchneri* | 9E-175 | 99.42% | CP018796.1 |
| Cheese 7 | Area 1 | *L. parabuchneri* | 3E-158 | 99.68% | CP018796.1 |
| | | *T. halophilus* | 4E-117 | 91.72% | AB670117.1 |
| | Area 2 | *L. parabuchneri* | 5E-151 | 98.00% | CP018796.1 |
| | Area 3 | *T. halophilus* | 2E-130 | 94.00% | AB670117.1 |
| | | *L. parabuchneri* | 8E-144 | 100.00% | CP018796.1 |
| | Area 4 | *T. halophilus* | 3E-115 | 92.86% | AB362339.1 |
| | | *L. parabuchneri* | 1E-148 | 97.76% | CP018796.1 |

## 4. Conclusions

This study highlights the relevance of choosing appropriate cheese sampling areas for the accurate quantification of histamine in long-ripened cheeses with an elevated histamine concentration. A clear pattern of histamine distribution was inferred, since it accumulated in the core, whereas the peripheral rind exhibited the lowest concentration. Extracting the sample from the inner and the outer parts of the wedge can allow researchers to represent an average value of histamine content, whereas sampling the inner core of the wedge will tend to yield the highest value. A causative relationship with the distribution of the histamine-producing microbiota could not be established. On the whole, nevertheless, histamine tended to accumulate in the saltier, more humid, and less oxidized areas of the cheese wedge.

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**Table 4**
Analyses of physical and chemical properties (water activity [$a_W$], moisture [g/100 g cheese], salt content [g NaCl/100 g], oxidative state [measured by the 2-thiobarbituric acid reactive substances [TBARS] test in mg malonaldehyde/kg]) of the selected cheeses in the defined areas (Area 1 corresponded to the central rind of the wedge, Area 2 to the central core, Area 3 to the peripheral core, and Area 4 to the peripheral rind of the wedge).

| Cheese sample | | $a_W$ | Moisture (g/100 g) | Salt content (g NaCl/100 g) | TBARS assay (mg malonaldehyde/kg) |
|---|---|---|---|---|---|
| Cheese 1 | Area 1 | 0.910 | 25.4 | 1.57 | 2.83 |
| | Area 2 | 0.917 | 29.6 | 1.88 | 2.32 |
| | Area 3 | 0.917 | 29.3 | 1.79 | 2.33 |
| | Area 4 | 0.903 | 22.2 | 1.47 | 3.28 |
| Cheese 2 | Area 1 | 0.929 | 26.9 | 1.30 | 2.60 |
| | Area 2 | 0.933 | 33.2 | 1.83 | 4.67 |
| | Area 3 | 0.930 | 29.8 | 1.60 | 1.75 |
| | Area 4 | 0.929 | 23.7 | 1.12 | 6.38 |
| Cheese 3 | Area 1 | 0.916 | 27.8 | 1.38 | 15.07 |
| | Area 2 | 0.910 | 30.4 | 1.48 | 1.94 |
| | Area 3 | 0.915 | 29.8 | 1.38 | 2.48 |
| | Area 4 | 0.919 | 25.7 | 1.16 | 5.04 |
| Cheese 4 | Area 1 | 0.915 | 26.0 | 1.39 | 5.87 |
| | Area 2 | 0.914 | 29.6 | 1.63 | 1.73 |
| | Area 3 | 0.917 | 28.6 | 1.55 | 1.79 |
| | Area 4 | 0.917 | 24.1 | 1.22 | 5.56 |
| Cheese 7 | Area 1 | 0.862 | 20.9 | 1.23 | 4.64 |
| | Area 2 | 0.840 | 28.1 | 2.36 | 2.84 |
| | Area 3 | 0.872 | 24.0 | 1.90 | 2.50 |
| | Area 4 | 0.850 | 13.8 | 1.15 | 11.09 |
| Cheese 9 | Area 1 | 0.870 | 20.5 | 1.62 | 14.12 |
| | Area 2 | 0.882 | 27.4 | 2.74 | 7.32 |
| | Area 3 | 0.876 | 25.9 | 2.37 | 5.99 |
| | Area 4 | 0.870 | 17.6 | 1.54 | 16.87 |
| Cheese 10 | Area 1 | 0.912 | 26.1 | 1.29 | 1.34 |
| | Area 2 | 0.916 | 31.2 | 1.80 | 0.93 |
| | Area 3 | 0.927 | 29.7 | 1.59 | 1.02 |
| | Area 4 | 0.903 | 24.3 | 1.18 | 0.97 |
| Average | Area 1 | $0.902^a$ | $24.8^a$ | $1.40^a$ | $6.64^a$ |
| | Area 2 | $0.902^a$ | $29.9^b$ | $1.96^b$ | $3.11^a$ |
| | Area 3 | $0.908^b$ | $28.2^b$ | $1.74^b$ | $2.55^a$ |
| | Area 4 | $0.902^a$ | $21.6^a$ | $1.26^a$ | $7.03^a$ |

Lowercase letters indicate significant differences in each cheese.

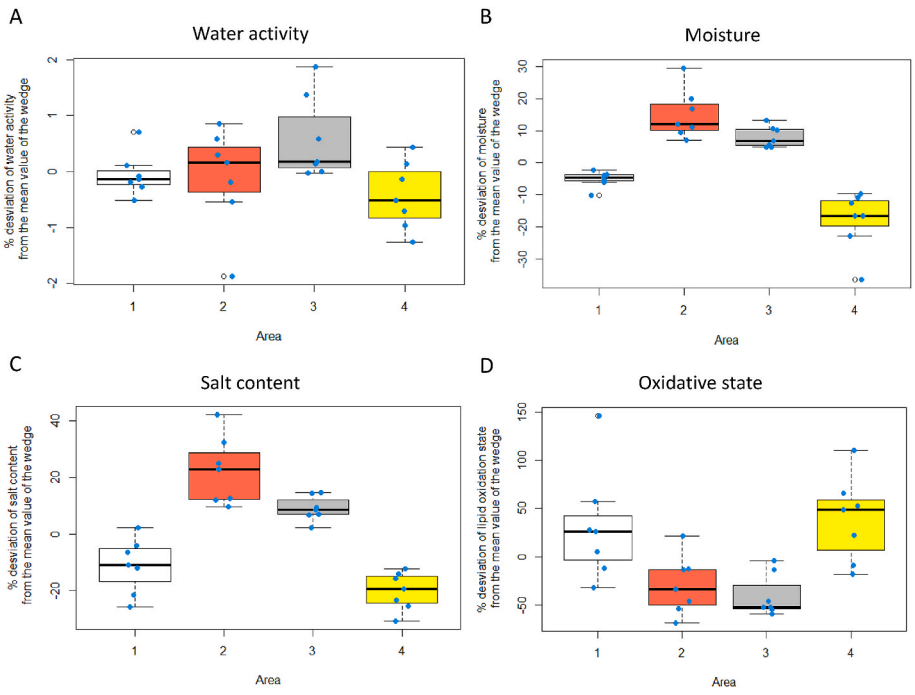


**Fig. 4.** The percentage of deviation of physical and chemical properties (water activity [A], moisture [B], salt content [C], and oxidative state [D]) measured in each cheese area from the mean value of the entire wedge, represented in a box-and-whisker plot. Area 1 corresponds with the central rind of the wedge, Area 2 with the central core, Area 3 with the peripheral core, and Area 4 with the peripheral rind. The median line of the box represents the medians of histamine concentration in each defined area of the wedge.

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## CRediT authorship contribution statement

**Marta Moniente:** Investigation, Writing – review & editing. **Diego García-Gonzalo:** Supervision, Writing – review & editing. **M. Goretti Llamas-Arriba:** Investigation, Writing – review & editing. **Jone Garate:** Investigation, Writing – review & editing. **Ignacio Ontañón:** Supervision, Writing – review & editing. **Arrate Jaureguibeitia:** Supervision, Project administration, Funding acquisition, Writing – review & editing. **Raquel Virto:** Supervision, Project administration, Funding acquisition, Writing – review & editing. **Rafael Pagán:** Supervision, Project administration, Funding acquisition, Writing – review & editing. **Laura Botello-Morte:** Investigation, Supervision, Writing – original draft, Writing – review & editing.

## Declaration of competing interest

None.

## Data availability

Data will be made available on request.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2022.114099.

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