CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO

ANALYSIS OF SULFITES IN CRUSTACEANS

by

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Training Placement Report presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Food Engineering

by

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Resumo

Devido à importância de manter os produtos com a maior segurança alimentar e melhor qualidade no ponto de venda, é necessário, por vezes, a utilização de conservantes. Estes devem ser devidamente selecionados e monitorizados, de modo a garantir o cumprimento dos limites legais definidos.

O projeto desenvolvido tem como principais objetivos avaliar tendências nos resultados obtidos, considerando diferentes metodologias de acondicionamento e preparação das amostras. Pretende-se avaliar como a concentração de sulfitos evolui ao longo do tempo, em camarões de aquacultura frescos e após congelação, com diferentes métodos de descongelação. Foi ainda realizada uma breve pesquisa para alternativa à utilização de sulfitos na conservação deste tipo de alimento.

Quanto à legislação de sulfitos, para camarões cozidos, o limite máximo de concentração de sulfitos residuais é de 50 mg de SO_2/ kg parte comestível. O valor máximo para as famílias *Penaeidae, Solinoceridae* e *Aristaeidae* até 80 unidades é de 135 mg de SO_2/ kg parte comestível. Para estas famílias, entre 80 e 120 unidades, o valor máximo é de 180 mg de SO_2/ kg parte comestível, e acima de 120 unidades o valor é de 270 mg de SO_2/ kg parte comestível (Regulamento (UE) n. 1129/2011).

Através da realização deste estudo foi possível visualizar que para o camarão fresco, ao longo da vida útil, a tendência da concentração residual de sulfitos é aumentar, tendo neste estudo existido um incremento em média de 50%. Em relação ao produto congelado, a tendência é de descida, estabilizando a concentração ao longo do tempo com uma média de 20%. Verificou-se ainda que a concentração de sulfitos não é dependente do tempo. Quanto às diferentes metodologias de análise utilizadas nas amostras congeladas, não houve diferenças significativas.

Palavras-chave: Crustáceos, Sulfitos, Conservação, Camarão, Segurança Alimentar

Abstract

Due to the importance of maintaining the products with the best food safety and quality at the point of sale, it is necessary to use preservatives that must be properly selected and monitored to guarantee compliance with the defined legal limits.

The developed project had as its principal objective to evaluate some tendencies in the results obtained, considering different storage and sample preparation methodologies. It is intended to evaluate how sulfite concentration evolves over time, in fresh aquaculture shrimp and after freezing, with different defrost methods. Also, brief research was done on some alternatives of sulfites for preserving this type of food.

Regarding sulphite legislation, for the cooked shrimps, concentrations of residual sulfites have a maximum of 50 mg of SO_2/ kg of edible part. The maximum level for *Penaeidae, Solinoceridae* and *Aristaeidae* family up to 80 units is 135 mg of SO_2/ kg of edible parts. For these families, between 80 to 120 units, the maximum level presented is 180 mg of SO_2/ kg of the edible part, and above 120 units, the level is 270 mg of SO_2/ kg of the edible part (Regulation (EU) n. 1129/2011).

Through the development of the study, it was possible to visualize that for fresh shrimps, over the lifetime, the tendency is to increase the residual sulfite concentration, and in this study, there was an increase with an average of 50%. Regarding frozen products, the tendency is to decrease, with an average of 20% and stabilise the residual sulfite concentration over time since sulfite concentration is not time-dependent. As for the different analysis methodologies used in the frozen samples, there were no significant differences.

Keywords: Crustaceans, Sulfites, Preservation, Shrimps, Food Safety

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List of abbreviations

FDA- Food and Drug Administration

IFA- Integrated farm assurance

PPO- Polyphenoloxidase

PV- *Penaeus Vannamei*

TMA-N- Trimethylamine Nitrogen

TVB-N- Total volatile basic nitrogen

1. Crustaceans

The crustaceans are a large class of marine animals. The number of different species inserted in this class is around 30 000. These animals, for their protection and muscle support, have an exoskeleton. This biological characteristic is composed of chitin, polysaccharide, and calcium. When crustaceans are in the growth phase, they need to regularly change their shell, a process called moulting (Dima *et al.*, 2017). The moulting cycle comprises four stages: pre-moult, moult, post-moult and inter-moult (Mähler *et al.*, 2020). This process is crucial not only for the growth of the animal but also for removing some parasites, damaged parts and infections (Lemos & Weissman, 2021).

Also, this class of animals is too perishable. Thus, there is a need to use good techniques, processes, and proper handling to ensure these animals do not have very fast spoilage (Nirmal *et al.*, 2015). This product is placed on a level of gourmet food, while their quality sometimes is not at this stage due to the onboard techniques (Huidobro *et al.*, 2002).

1.1. Melanosis

Melanosis is a process that occurs in crustaceans since they form a melanoid compound that has antibacterial and antifungal activities for their defence, and it is harmless for human consumption. This process occurs through the polymerisation of the quinones with proteins, amino acids or even auto-polymerized. This polymerisation forms an enzymatic browning that is undesirable and leads to the formation of black spots, being this a more limiting factor than microorganisms. The primary reaction in melanosis formation is the oxidation from phenols to quinones, which are present in the enzymatic complex Polyphenoloxidase (PPO) (Nirmal *et al.*, 2015) (Kimbuathong *et al.*, 2020).

Figure 1- Melanosis effect in raw shrimp (*in* Santos, 2017).

Figure 2- Melanosis effect in boiled shrimp (*in* Villa Camarão, 2023).

This PPO complex is crucial for some physiological functions of crustaceans, like sclerotization. This is a process of hardening the exoskeleton after the moulting process in the growth of the crustacean (Nirmal *et al.*, 2015). Also, melanosis has a higher presence when crustaceans are in the moulting season due to a more intense activity of the complex PPO. Therefore, there is a need to use higher concentrations of food preservatives (Gómez-Guillén *et al.*, 2005).

Since melanosis drastically affects the crustacean's market value, there was the need to use some melanosis inhibitors that bind with the quinones, preventing their polymerisation and forming noncolor compounds. Sulfites are the most utilised method to prevent or retard the formation of these black spots (Nirmal *et al.*, 2015). Also, through the study of Kimbuathong *et al.* (2020), it is possible to show that some concentrations of CO_2 and O_2 in modified atmosphere packaging can delay melanosis formation. Concentrations below 5% O2 and above 60% CO_2 can prevent melanosis in *Litopenaeus vannamei* (Kimbuathong *et al.*, 2020).

Storage, transport and exposure temperature are also an important factor. According to Regulation (EC) nº 853/2004, the correct way to store and commercialise crustaceans is at a temperature close to 0 ºC, while for more extended transportation, frozen storage is the best way to avoid the degradation of the product (Martinez-Alvarez *et al.*, 2020).

1.2. Food Safety

Since melanosis decreases the market value and acceptance of crustaceans by consumers, some inhibitors, like sulfites, prevent these blackspot formations (Nirmal *et al.*, 2015). These preservatives must be carefully added to the product to ensure that they will not cause damage to the consumer, and sulfites could be considered a hazard if not utilised correctly. The Hazard definition by *Codex Alimentarius* (FAO, WHO) is " A biological,

chemical or physical agent in, or condition of, food with the potential to cause an adverse health effect.". Also, according to *Codex Alimentarius* (FAO, WHO), food safety is the "assurance that food will not cause harm to the consumer when it is prepared and/or eaten according to its intended use.".

Thereby, not only are companies responsible for food safety, but consumers also present an essential role in this parameter, particularly concerning transport conditions after purchase and subsequent storage of the products. Companies must provide all the information they have on the product to the consumers through the label, such as if the product contains sulfites.

Regarding melanosis, if the consumer does not respect the storage indication at ambient temperature, the shelf-life of the shrimp is one day (Yu *et al.*, 2023). According to the Regulation (EU) n. 1169/2011, if the product contains more than 10 mg of SO_2/ kg of edible product, it is necessary to present on the label that it contains sulfites.

1.3. Principal Illnesses from Seafood for Human Consumption

According to Wallace *et al.* (1999), seafood is a huge source of outbreaks in the United States of America. At the same time, shellfish presented 64% of the seafood outbreaks, being the *Norwalk* virus and *scombrotoxin* the primary agents identified. Meanwhile, the Norwalk virus presented 42% of illnesses in the outbreaks, and scombrotoxin accounted for 19% (Wallace *et al.*, 1999).

Also, the prevalence of *Salmonella* and *Listeria* on shrimps was studied by Norhana *et al.* (2010), where it is possible to notice that the majority of shrimps that are recalled from the market is due to the presence of *Salmonella* or *Listeria* in the products. Therefore, through the investigation by Norhana *et al.* (2010), the most effective method to control the growth of *Listeria monocytogenes* is the modified atmosphere packaging (MAP) incorporating CO_2. Through this method, it is possible to extend the shelf-life of seafood due to a more present inhibition of microbial growth and lipid oxidation. According to the study developed by Kimbuathong *et al.* (2020), there is a need to use different compositions of CO_2, O_2 and N_2 for different species of shrimps. For *Litopenaeus vannamei,* studies showed that the best composition is 80% CO_2; 10% O_2; 10% N_2, while multiple combinations for *Penaeus duorarum* are possible, for instance: 80% CO_2; 5% O_2; 15% N_2 and 95% CO_2; 5% O_2; 0% N_2 and some others (Kimbuathong *et al.*, 2020). Furthermore, for cooked and peeled shrimps (MAP), the distribution might be realised at 2ºC with a shelf life of 20-21 days (Norhana *et al.*, 2010).

Vibrio parahaemolyticus is another gram-negative bacterium, like *Salmonella,* which is rising in outbreaks causing severe gastroenteritis. This bacteria lives in maritime environments

around the world. This bacteria concerns the consumer who eats raw shrimp and has lousy handling. Also, due to temperature fluctuations, the counts of *Vibrio parahaemolyticus* increase significantly (Letchuman *et al.*, 2015). A method utilised to control gram-negative bacteria is the High-Pressure Process (HPP) since they are sensitive to pressure due to the cell wall structure of these bacteria (Norhana *et al.*, 2010).

The study developed by Somorin et al. (2021) made it possible to identify the bacteria present in shrimps exported from Africa to European Union. *Salmonella* was the prevailing bacteria with 87.8% of the reports, followed by *Vibrio spp.* with 3.5%.

Not only microorganisms present hazards for consumers since food preservatives are added to shrimps to increase their shelf life, sodium metabisulfite being the most common. As referred by García-Gavín *et al.* (2012), "Even minimal (oral, parenteral, respiratory, or cutaneous) exposure in subjects with an intolerance may provoke various types of reaction, such as asthma, rhinoconjunctivitis, and even anaphylaxis leading to death.".

1.4. Shrimps

1.4.1. Aquaculture

Due to the fast growth of the world population, there was a need to accompany this with a larger volume of food production (Golden *et al.*, 2017).

Aquaculture is utilised for the farming of different marine species. Thereby, this method could be a solution for a cheap and easy way to get an excellent food source to the world, where diversity is one central aspect that differentiates it from other sectors (Golden *et al.*, 2017). Due to the increasing demand for fishery development and good quality, the aquaculture technique might be crucial since fishing areas reached their limit on ecosystem productivity (Fan & Li, 2019). There are different methods of shrimp aquaculture: extensive aquaculture, semi-intensive and intensive. Extensive aquaculture is a technique that utilises natural resources and conditions for collecting shrimps, and they are also fed by natural means. Intensive aquaculture, because of the need to use feeding and incubators for the growing tanks, a more considerable investment is necessary when compared with extensive aquaculture. On the other hand, it offers greater control (Dore *et al.*, 1991). In comparison, semi-intensive aquaculture depends on supplementing the natural feed (Kautsky *et al.*, 2000).

Figure 3- Example of Aquaculture Pools (*in* Panorama da Aquicultura, 2023).

Regarding shrimps, they can be farmed in seawater or freshwater, and due to their ease, intensive farming has changed their industry. Also, it is possible to induce moulting in shrimps through some events, which are: fast salinity reduction, lowering water levels, increasing temperature and addition of a nutrients mix (Lemos & Weissman, 2021). Several countries produce shrimp, Taiwan and Ecuador are the more prominent producers (Dore *et al.*, 1991). The farmed shrimps commercialised in Portugal come from Ecuador, India and Madagascar. According to Kautsky *et al.* (2000), Ecuador produces 60% of their shrimp from extensive aquaculture and 40% in semi-intensive aquaculture, while in India, 92% of shrimps come from extensive aquaculture and 8% from semi-intensive aquaculture.

Some dominant species are farmed with this method, such as *Penaeus vannamei,* known as whiteleg shrimp, and *Penaeus monodon* black tiger. Through this process, it is possible to reduce market fluctuations. Furthermore, the harvest could be carefully timed so that the product would be fresher when the shipment arrives (Dore *et al.*, 1991).

Certification Reference- GLOBALG.A.P.

Integrated farm assurance (IFA) is GLOBALG.A.P.'s principal standard, the only aquaculture certification recognised by the global food safety initiative (GLOBALG.A.P., 2023).

For producers, this standard has as its primary return better management of the farm, greater efficiency, and more outstanding care for environmental resources. It also has international advantages, such as accessing markets worldwide due to having an internationally recognised standard (GLOBALG.A.P., 2023).

This standard has six essential topics: animal health and welfare, food safety, environmental sustainability, production processes, workers' well-being, legal management and traceability (GLOBALG.A.P., 2023).

A label also guarantees the consumer responsible farming and transparency, allowing the consumer to trace the product from the beginning. There are some requirements to the possibility to achieve this label, such as the fish feed might be exclusively from manufacturers with GlobalG.A.P. certification, valid IFA certification, global risk assessment on social practice (GRASP) evaluation, chain custody certification, and GGN license (GLOBALG.A.P., 2023).

1.4.2. Wild Shrimp

Over the years, shrimp have become popular worldwide due to their taste and nutritional value. Thus, their prevalence in fishery trading commodities is increasing, and through the vast increase in shrimp commercialisation and overfishing, the available resources started to decline (Yu *et al.*, 2023).

The most typical fishing zones are in equatorial, intermediate ocean areas and subpolar (Arrasate-López *et al.*, 2012). The most utilised techniques as fishing methods are pole drag and bottom cage traps. The pole drag method uses a trap open in the front by the action of one or two rods and is towed with a boat to capture the shrimps. In comparison, cage traps are a method that directs the shrimps towards a trap that prevents escape (DGRM, 2023).

1.4.3. Most commercialised Species

Some dominant species are commercialised due to the availability and acceptance of consumers. One of the most common species is the *Parapenaeus longirostris*, also known as deepwater pink shrimp. In the 2000s, it was estimated that the fishing volume for *Parapenaeus longirostris* was higher than 1 000 tons (Mendes *et al.*, 2002).

Figure 4- *Parapenaeus longirostris* (*in* Shrimp Sea World Distribution, 2023).

Figure 5- *Penaeus monodon* (*in* Shrimp Sea World Distribution, 2023).

Another important specie commercialised worldwide is the *Penaeus monodon*, one of the biggest penaeids in the market (Cintra *et al.*, 2011). According to Tavares (2013), the shrimps from the *Penaeidae* family are the most produced and valuable commercialised species. Also, through farming, one of the most commercialised specie is the *Penaeus vannamei* (Dore *et al.*, 1991). According to Karunasagar & Ababouch (2012), the aquaculture production of *Penaeus vannamei* reached 1.8 million tonnes outside America in 2008, representing 80.7% of the worldwide aquaculture production.

Figure 6- *Penaeus vannamei* (*in* Prawn Sea World Distribution, 2023).

1.4.4. Processes Applied in Shrimps Preservation

As referred previously, shrimps are too perishable and have very fast spoilage. This could be due to a considerable composition of non-protein nitrogenous compounds quickly metabolised by microorganisms (Huidobro *et al.*, 2002).

The traditional method used on shrimps is to preserve them on flaked ice to decrease their corporal temperature and stress, followed by immersion in a solution of 1.25% sodium metabisulfite in water for 1 minute (Yu *et al.*, 2023) (Berardi *et al.*, 2022). According to Huidobro *et al.* (2002), preserving them in flaked ice shows some disadvantages, including injury and bruising of the flesh, and could leach some nutritional compounds. Through the study developed by Otwell & Marshall (1986), the best treatment for *Penaeus duorarum* is 2.5% of sodium bisulfite, while for *Parapenaeus longirostris* the best would be the immersion of the shrimp for 1 h in 50 g of SO_2/kg, with citric acid and chelating agents. Sodium metabisulfite is the method most utilised to prevent melanosis due to being one of the cheapest, easy to use, and most efficient (Surasani & Patange, 2012).

A substitution for this traditional method could be the utilisation of liquid ice. Liquid ice is a way to get a fast chilling of food products. With this technique, it is possible to get a lower temperature than would be possible with flaked ice. This process makes it possible to get a better quality due to the delay of enzymatic reactions and bacterial spoilage rate (Huidobro *et al.*, 2002).

Also, using liquid ice makes the addition of melanosis inhibitors possible, simplifying the process. From a microbiological and biochemical point of view, the liquid ice technique presents better results with fewer spoilage metabolites and fewer microorganisms. On the other hand, regarding sensorial aspects, the traditional method presents better results (Huidobro *et al.*, 2002).

The utilisation of a high-pressure process could be another substitute for flaked ice. This method presents some vantages, microorganisms and spoilage enzyme inactivation, with low effects on flavour and nutritional characteristics (Kaur *et al.*, 2013). According to Kaur *et al.*, 2013, the shrimps treated with higher pressures present a more rigid texture and a cooked appearance, being the most effective treatment at 435 MPa for 5 min.

1.4.5. Sulfites Legislation in European Union

The legislation on food products is crucial to maintain their safety since some compounds in different concentrations can harm consumers.

Regarding the levels of sulfites in shrimps, these suffer some alterations between the raw and cooked shrimp. For the cooked shrimp, concentrations of residual sulfites have a maximum of 50 mg of SO_2/ kg of edible part. However, legal limits are established depending on the calibre (number of units in 1 kg) and the family. The maximum level for *Penaeidae, Solinoceridae* and *Aristaeidae* family up to 80 units is 135 mg of SO_2/ kg of edible parts. For these families, between 80 to 120 units, the maximum level presented is 180 mg of SO_2/ kg of the edible part, and above 120 units, the level is 270 mg of SO_2/ kg of the edible part (Regulation (EU) n. 1129/2011).

Also, for consumer protection, the Regulation (EU) n. 1169/2011 establish that the products with more than 10 mg of SO_2/ kg of edible parts must be clear on the label that this preservative is present. Thereby, the consumer will be better informed and increase the product's food safety. Thus, some individuals develop allergies after ingesting sulphites (Armentia-Alvarez *et al.*, 1994).

1.4.6. Effect of Sodium metabisulfite in Melanosis

The utilisation of sulfites as a food preservative on shrimps is a methodology that serves to retard the enzymatic reactions of the complex PPO since they react with critical conditions of the enzymatic reactions, such as decreasing oxygen and lowering the pH (Andrade *et al.*, 2015). The sodium metabisulfite reacts with dissolved oxygen in water, forming sodium acid sulphate dissociating into sodium, sulphate ions, and hydrogen ions. The increase in hydrogen ions decreases pH (Albuquerque, 2005). According to Albuquerque (2005), each mg of sodium metabisulfite can consume 0.15 mg of dissolved oxygen.

As referred previously, the solution of 1.25% sodium metabisulfite in water for 1 minute is the traditional method used on shrimps to preserve them on flaked ice (Berardi *et al.*, 2022).

According to the research developed by Andrade *et al.* (2015), excessive concentrations of sulfites can cause collateral damage to the consumer. As the author said, " sulfite preservatives can cause nausea, abdominal pain, vomiting, skin reactions, as well choking and chemical pneumonitis in consumers or handlers".

Using the traditional immersion method in 1.25% sodium metabisulfite for 1 minute is ineffective for some species of shrimps. According to Mendes (2004), since this traditional method is sometimes ineffective, it is utilised 5 to 10% for 2 to 20 minutes in practice, even though the Food and Drug Administration of the United States had established 1.25% for 10 minutes as a limit. In the study realised by Gómez-Guillén *et al.* (2005), the immersion of pink shrimp in 12.5 g/ kg of sulfite for 2 hours was not capable of preventing melanosis for more

than three days. While in the treatment of *Penaeus duorarum,* with the same concentration and 1-minute immersion, the shrimps showed positive results after seven days (Gómez-Guillén *et al.*, 2005).

Therefore, it is necessary to adapt the treatments for the different species captured and the capture season. For instance, shrimps are more susceptible to melanosis in the moulting phase.

Regarding residual sulfites levels, according to Gómez-Guillén *et al.*, 2005, when shrimps are treated by immersion, the tendency after 4 days of storage is to decrease their levels due to the contact with ice, and this way, sulfites are dissolved and leave the muscle of shrimps. While shrimps treated with sulfite dust, the tendency after 4 days is to increase the residual sulfite levels since the contact with ice will help the dust penetrate the shell and bond with the muscle (Gómez-Guillén *et al.*, 2005).

1.5. Alternative Methodologies to Sulfites

1.5.1. Phenolic Compounds

Phenolic compounds, like vegetables and fruits, are present in food that is considered very healthy. These compounds are known for their health advantages due to their antioxidant activity. Their activity as metal chelators and bonding with free radicals could lead to an excellent alternative to the traditional use of sulfites (Nirmal *et al.*, 2015).

It is possible to know if some phenolic compounds will have a higher or lower activity as antioxidants through their reducing properties, like their capacity to donate electrons. High-performance liquid chromatography (HPLC) is the principal method for identifying and characterising different phenolic compounds (Nirmal *et al.*, 2015).

The characterisation of the mechanism that is the objective to inhibit must be considered. In this case, the PPO is responsible for the major degradation of crustaceans. This complex is a metalloprotein and depends on copper. Thus, to have a good inhibition of the PPO complex, a phenolic compound with a good copper chelating activity is necessary, like ferulic acid, catechin and mimosine (Nirmal *et al.*, 2015).

1.5.2. 4-hexylresorcinol

The 4-hexylresorcinol is a compound utilised to prevent melanosis in shrimps, being a potent inhibitor of the PPO complex. According to Otwell *et al.* (1992), this treatment does not negatively affect the organoleptic quality of shrimps, and the concentration of this compound is generally below 1 ppm on treated shrimp.

The concentration of 4-hexylresorcinol to achieve a comparable result with bisulfite, in terms of melanosis, is much lower. In the study developed by Otwell *et al.* (1992), the utilisation of 5 ppm (0.0005%) 4-hexylresorcinol was at the same level of melanosis inhibition, compared with the utilisation of 12 500 ppm (1.25%) bisulfite. The quality of the product, in sensory parameters, is not affected since the product is treated with low concentrations (Otwell *et al.*, 1992). Regarding the safety of this compound, the data shows that it acts as an irritant in very high concentrations, but in low concentrations, it does not present any reactions (Frankos *et al.*, 1991).

Through the research developed by Otwell *et al.* (1992), the immersion of the shrimps for 1 minute in a solution of 50 ppm 4-hexylresorcinol in seawater can prevent melanosis for 12 days. Also, regarding the study developed by Galvão *et al.* (2017), the shrimps treated with this compound presented a better result in the production of TVB-N, TMA-N and melanosis when compared with the sodium metabisulfite.

1.5.3. Chitosan

Due to the increase in sustainability among the companies, chitosan utilisation to prevent melanosis in crustaceans could be a way to achieve this objective since chitosan is biodegradable and obtained from waste of some industries, mainly crustacean companies. The utilisation of chitosan in some seafood products, such as salmon and oysters, has been studied as a protective film to extend their shelf life (Huang *et al.*, 2012).

According to the research developed by Huang *et al.* (2012), the shrimps treated with chitosan during cold storage have shown a significant difference in the increase of pH values, being this increase lower for the product with chitosan film. On the other hand, significant differences were not found between the pH values of the different treatments. This could be due to the thickness of the exoskeleton present on the shrimps that can work as a barrier to the chitosan. Furthermore, this treatment could be an excellent microbial enzyme inhibitor since the increase of pH values is lower when the shrimps are treated with chitosan (Huang *et al.*, 2012).

The total volatile basic nitrogen (TVB-N) analysis determines the freshness of seafood products. Since this factor will increase with enzymes and microbiological spoilage. The study developed by Huang *et al.* (2012) showed that the treatment with chitosan could make the passage of free amino acids to volatile basic nitrogen slower.

2. Work Objective

The main objective in the development of this study is the evaluation of the alterations in sulfite concentration that occurred on boiled shrimps from aquaculture over the lifetime of frozen and fresh storage products. Two suppliers were considered for the study, using different sample preparation methodologies as well as different analysis moments. It should be noted that the samples were collected from different suppliers with the sole purpose of carrying out the study. Throughout the study, these suppliers will be codified to maintain the confidentiality of the companies studied.

To reach this objective, the results of the different moments of analyses were analysed, trying to find some tendencies in each sample with the different preparations. Since residual sulfite concentrations are legislated, it is necessary to ensure that the level is not exceeded. Thereby, if the tendency is to increase sulfites during storage, the levels at the beginning of the shelf life should not be close to the limit to ensure that it is not exceeded during the product's lifetime.

3. Materials and Methods

The shrimps analysed from the two suppliers were treated with sodium metabisulfite (on average, 3% for 10 minutes). After capture, they were frozen and transported to the target suppliers of the study. At the supplier facilities, they were boiled for between 2 and 3 min at 90°C. Also, for this study's development, the residual sulfite concentration and pH on shrimps were analysed at two different moments.

The analyses were realised in two distinct phases (Phase I and Phase II) and at different moments: fresh products on the day of collection, 24 hours after the collection date, and fresh products at the end of the expiration date. On the collection date, some samples were frozen for after freezing analysis. Different defrost methods were considered: fully thawed with defrosting liquid; fully thawed without defrosting liquid; partially thawed with defrosting liquid, and partially thawed without defrosting liquid. The same frozen analyses were made 1 month after the collection date with the different defrost methods.

In phase I, the specie analysed was PV from Ecuador aquaculture. The samples from supplier X were collected for analysis on 30/11/2022 and from supplier Y on 20/12/2022. In phase II, the same specie with the same origin was analysed, and the samples from supplier X were collected on 02/05/2023 and from supplier Y on 09/05/2023. The samples analysed had different calibres, such as 30-50 and 60-80.

The method used to measure the pH values on shrimps was potentiometry, while Monier-Williams was utilised to analyse sulfite concentration (Figure 7). The analyses were carried out in an accredited external laboratory. This method makes it possible to measure the concentration of free sulfites and a percentage of bound sulfites in food.

In the first moment, the laboratory technician divides the samples into different bags to realise the different methodologies (Appendix Figure 8). The sample preparation is realised through the transfer of 50g of the food sample to a blender (Appendix Figure 9), adding 95mL of water and 5mL of ethanol, and continuing blending until the food is in small pieces (Appendix Figure 10) (Warner, 1992).

To measure this sulfite concentration, it is necessary to heat the food sample through reflux with 1 N HCl, so the sodium metabisulfite utilised in the treatment of shrimps could be converted to SO_2. Also, with a stream of nitrogen, the SO_2 goes through a water-cooled condenser, with a bubbler connected to a 3% H_2O_2 solution. When the SO_2 reach the solution of 3% H_2O_2, it is oxidised to H_2SO_4. After that, it is necessary to realise a titration with NaOH (Appendix Figure 11) (Warner, 1992).

After the realisation of the titration, it is possible to obtain the concentration of sulfites (µg SO_2/ g food) through the following equation (1) (Hillery *et al.*, 1989).

$$SO_2, ppm = \frac{(32{,}03 \times V_b \times N \times 1000)}{wt} \quad (1)$$

V_b- the volume of NaOH of normality N needed to obtain the endpoint (mL).
Wt- the weight of the sample portion inserted in the 1L flask (g).

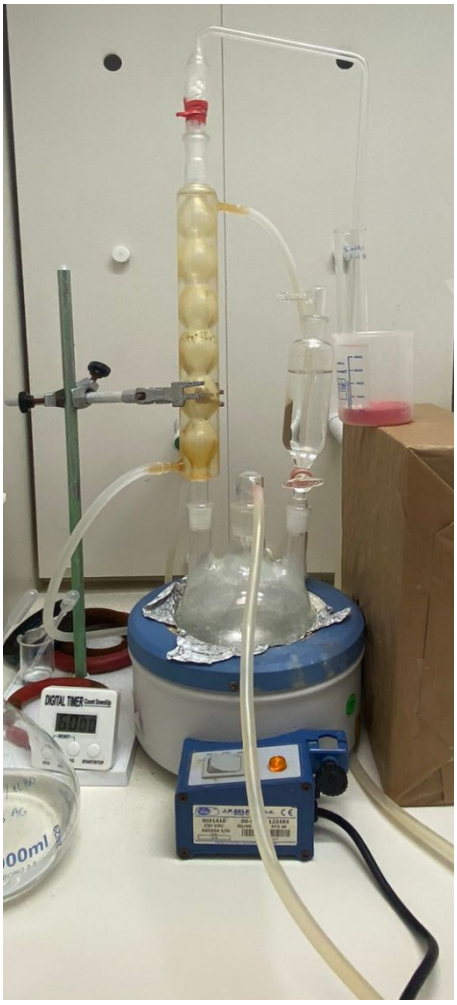

Figure 7- Equipment utilised in the Monier-Williams method.

The statistical analysis developed throughout the study, it was utilised the f-test to ensure the normality of the data and then the t-student tool. The confidence level utilised in the analyses was 95%. This statistical technique is used to analyse and compare the means of two independent samples. Assisting in making decisions based on statistical evidence (Ruxton, 2006).

4. Results

Different treatments were analysed to evaluate some tendencies in the residual sulfite concentration on boiled shrimps, as referred previously. Two phases of analysis were performed to achieve a more significant conclusion.

The first analysis phase is presented in Appendix Table 3 and Table 4, while phase II is present in Table 5 and Table 6. Where each table represents a different supplier. It should be noted that the company where the Training Placement Report took place does not consider the value of uncertainty associated with the result.

In phase I, it is possible to observe that supplier X (Appendix Table 3) presents 15% of results that are non-compliant with the legislation, based on the limit of 135mg of SO_2/ kg of edible product. The lowest result from this supplier is 68±14 mg of SO_2/ kg of edible product, with a pH value of 7.8±0.1, achieved on boiled shrimp with a calibre of 60-80 shrimps per kg and fully defrosted without the presence of the defrost liquid. While the biggest result was 154±31 mg of SO_2/ kg of edible product, with a pH value of 7.7±0.1, achieved on boiled shrimp with a calibre of 60-80 shrimps per kg, 24 hours after the collection. Also, it was possible to notice that the increase in the concentration of residual sulfites for fresh products was swift and notable. Calibre 30-50 per kg had a 93% increase in sulfites from the collection date to 24 h after and, for calibre 60-80 per kg, an increase of 100% (Appendix Table 7).

The values of pH from supplier X ranged between 7.1 and 7.8, being the highest value present on calibre 60-80 per kg fully thawed without the defrosting liquid, while the lowest pH value was in the analysis of fresh boiled shrimp in the collection date calibre 30-50 per kg.

The results from supplier Y in phase I, presented in Appendix Table 4, have 66% of the analyses above the legal limit (135mg of SO_2/ kg of edible product). These results are inserted in the category of boiled shrimp with a 30-50 calibre per kg from supplier Y and boiled shrimp with a 60-80 calibre per 600g. The most significant result obtained from supplier Y was 240±48 mg of SO_2/ kg of edible product, achieved in the analyse of boiled shrimp calibre 30-50 per kg frozen for a month and partially defrosted in the presence of the defrost liquid. The lowest result obtained from this supplier was 31±6 mg of SO_2/ kg of edible product in the analyse of the boiled shrimp with a calibre of 30-50 per 600g. The sample was frozen and then partially thawed without the defrost liquid. The analyses carried out in this supplier into the fresh product were after 24 h of the collection date for calibre 30-50 per kg and at the end of the expiration date for calibre 60-80 per 600g. The result obtained 24 h after the collection date was 35% higher than the result obtained on the collection date and, for calibre 60-80 per 600g measured at the end

of the expiration date, decreased by 9% when compared with the fresh product of calibre 60-80 per 600g measured on the day of collection (Appendix Table 7).

In supplier Y, the pH values ranged between 7.0 and 7.7, being the highest value present on three analyses: fresh boiled shrimp 24 h after the collection date with calibre 30-50 per kg, fully thawed without the presence of defrosting liquid and, frozen for 1 month fully thawed without the presence of defrosting liquid. The lowest pH level is present on the analysis 30-50 per 600g - Frozen (1 month)/ Partially defrosted/ with defrosting liquid, with a value of 7.0±0.09.

Table 1- Evolution of sulfites during frozen storage, Phase I.

Freezing Evolution (Phase I)							
	Calibre	**Fresh- Frozen**		**Fresh- 1 month Frozen**		**Frozen- 1 month Frozen**	
Supplier X	30-50/ kg	15%	Increase	10%	Increase	5%	Decrease
	60-80/ kg	29%	Decrease	31%	Decrease	3%	Decrease
Supplier Y	30-50/ kg	10%	Decrease	2%	Increase	13%	Increase
	60-80/ 600g	29%	Increase	33%	Increase	3%	Increase
	30-50/ 600g	21%	Decrease	17%	Decrease	5%	Increase

Also, regarding the frozen samples of phase I, it is possible to see the evolutions in both suppliers and the different methodologies in Table 1. Comparing the results obtained in fresh products with the frozen samples, analysed after 5 to 7 days, it is possible to notice that it increased for calibre 30-50 per kg from supplier X and 60-80 per 600g from supplier Y. The values decreased for the calibre 60-80 per kg from supplier X and for calibre 30-50 per kg and 30-50 per 600g from supplier Y. Thus, the result decreased for three of the five samples with an average of 20%. In contrast, the average increase was 22%. Concerning the frozen samples analysed 1 month after, only the calibre 30-50 per kg from supplier Y showed different results, increasing by 2% compared to the fresh products.

Regarding phase II results, it is possible to identify that supplier X (Appendix Table 5) presents 17% of non-compliant results. The higher value is presented in the analysis to calibre 30-50 per 600g, partially thawed without the defrosting liquid, with a value of 220±44 mg of SO_2/ kg of edible product, having a pH of 7.1±0.11. While the lowest value is present on the analysis to calibre 60-80 per 600g, partially thawed with the presence of the defrosting liquid, with a value of 51±10 mg of SO_2/ kg of edible product and a pH value of 7.8±0.12. Also, regarding the evolution of sulfites on fresh products (Appendix Table 8), it is possible to observe that the residual sulfite decreased on calibre 30-50 per kg after 24h and also on calibre

60-80 600g at the end of the expiration date. While for calibre 30-50 per 600g, at the end of the expiration date, the value was 18% higher.

Concerning the results from supplier Y (Appendix Table 6), 37% of non-compliant results exist. The highest value is found on the analysis to calibre 30-50 per 600g partially thawed with defrosting liquid, with a value of 322±64 mg of SO_2/ kg of edible product, having a pH value of 7.5±0.11. At the same time, the lowest value was 43±9 mg of SO_2/ kg of edible product, on the analysis of calibre 30-50 per kg partially tawed without defrosting liquid, with a pH value of 7.5±0.11. All analyses carried out on fresh products increased over time. The calibre 30-50 per kg after 24h had an increase of 24%, while at the end of the expiration date, the calibre 60-80 per 600g increased by 65% and 15% for 30-50 per 600g.

Table 2- Evolution of sulfites during frozen storage, Phase II.

Freezing Evolution (Phase II)							
	Calibre	**Fresh-Frozen**		**Fresh- 1 month Frozen**		**Frozen- 1 month Frozen**	
Supplier X	30-50/ kg	12%	Decrease	9%	Decrease	3%	Increase
	60-80/ 600g	2%	Increase	2%	Decrease	4%	Decrease
	30-50/ 600g	22%	Increase	23%	Increase	1%	Increase
Supplier Y	30-50/ kg	39%	Decrease	9%	Decrease	50%	Increase
	60-80/ 600g	18%	Decrease	16%	Decrease	3%	Increase
	30-50/ 600g	10%	Decrease	14%	Decrease	4%	Decrease

About frozen samples from phase II (Table 2), it is possible to observe that the sulfite concentration decreased in 4 of the 6 analyses with a short frozen storage period. The average decrease for these 4 analyses was 20%, while the average increase for the 2 samples that presented opposite results was 12%. The values did not oscillate too much when comparing the results of frozen samples with short-period storage and 1 month storage. Only on calibre 30-50 per kg from supplier Y did the average increase by 50%.

5. Discussion

This study was realised to understand possible tendencies of residual sulfite concentration levels on boiled shrimps over the lifetime in fresh and after-freezing products. Thus, different methodologies were used to understand possible interferences in the final result.

Concerning fresh samples, two moments of analysis were made for each calibre of the different suppliers. It is possible to observe from Appendix Table 7 and Table 8 that of the 10 different samples analysed, 7 increased throughout the time with an average of 50%. In comparison, the other three samples had a decrease of 14%. This increase could be due to the moisture loss in shrimps over time and the oxidation of sulfur compounds, leading to a higher sulfite concentration. However, this information is not present in the bibliography. Thus, developing a study on this perspective and purpose would complement the literature and allow more correct conclusions.

Regarding pH levels from fresh samples, the values significantly decreased from the collection day to the final expiration date (Appendix Table 11). This could be due to the significant increase in residual sulfites over time (Appendix Table 9) since sulfites react with critical conditions to preserve the food. As referred previously, sodium metabisulfite reacts with dissolved oxygen in water forming sodium acid sulphate, dissociating into sodium, sulphate ions, and hydrogen ions (Albuquerque, 2005). Regarding the pH range of the samples from both phases, the values were between 7.0 and 7.8. According to Mendes *et al.* (2002), the critical value of acceptability pH for shrimps is considered to be 7.8, suggesting that this parameter is a good freshness indicator. Thus, it was possible to observe that all the samples were fresh, and, with the utilisation of sodium metabisulfite, the pH value decreased over time, helping the product to maintain its freshness and reduce its degradation throughout time.

It should be noted that shrimps are living beings, and the more stressed the animal is in the harvest phase, the more sulfites will absorb (Oshimo *et al.*, 2021). This way, there may be some specimens in the different batches that are not representative of the total sample. Concerning the calibres studied, 30-50 and 60-80, there are no significant differences or consistency throughout the different analyses, making it possible to conclude that the calibre does not influence the sulfite concentration.

In reference to frozen samples (Table 1 and Table 2), as the bibliography predicted, most results showed a decrease in the sulfite concentration after a few days of frozen storage. For the 7 samples that decreased from both phases, the average decrease in sulfite concentration in frozen storage was 20%, close to the 17% decrease in Finne *et al.* (1986) study. According to

Finne *et al.* (1986), this decrease occurs due to the washing effect of the thaw drip. Concerning the different methodologies applied to frozen analyses (Partially/ Fully thawed, in the presence or without the presence of the defrosting liquid), the results did not present significant differences for both phases. It is possible to observe in Appendix Tables 12 and 13 that regarding phase I, the values are visually lower for the samples analysed without defrosting liquid and for the fully thawed samples. This could be due to the washing effect that occurs for a more extended period in fully thawed samples, and the sulfites that came out of the sample with the defrost were removed in this methodology. While for phase II (Appendix Tables 14 and 15), the opposite occurred. The analyses with defrosting liquid and partially thawed visually presented lower values. Nevertheless, the results are not significantly different.

Regarding frozen samples analysed 1 month after the collection day (Table 1, 2 and Appendix 16 and 17), it is possible to observe that the values are not significantly different and did not oscillate too much when compared with frozen samples for a short time. Excluding calibre 30-50 per kg from supplier Y values, the oscillation in sulfite concentration from a short storage period to a more considerable period is around 1% to 5%. Making it possible to conclude that the sulfite concentration decreases in frozen samples but stabilises over time. Thus, when the product is frozen, the sulfite concentration is not dependent on time. Also, the same result was achieved in the Rotllant *et al.* (2002) study, where sulfite concentrations were analysed in frozen samples for a short time and an extensive period. This could be explained through Finne *et al.* (1986) study that the crucial factor in the decrease in sulfite concentration in frozen samples is the washing effect, which does not depend on storage time.

In relation to both suppliers, it is possible to observe that supplier Y presents a more significant number of non-compliant results. In phase I, 66% of the analyses carried out to this supplier were above the legal limit, while supplier X presented 15%. In phase II, the same tendency continued with supplier Y having 37% of non-compliant results and supplier X having 17%. This could be explained by the fact that, sometimes, supplier Y performs a second application of sulfite when the sample of received shrimp presents a significant number of specimens with signs of melanosis. Although this second application was made by supplier Y, also supplier X presents some non-compliant results, which may be related to the practice of most operators, who use a concentration of sodium metabisulfite of 3% because, with the value of 1.25% that is established as a maximum by the FDA of the United States (Mendes, 2004), it is not possible to obtain a favourable result for the control of melanosis. Considering what was referred previously, it is essential that companies and competent authorities carry out studies to

assess the most appropriate concentration of metabisulfite, so that its use has the intended effect on melanosis without jeopardising consumer health.

6. Conclusion

In this chapter, the conclusions of the development study and some experiences acquired during the training placement are present.

Throughout the experimental activities mentioned previously, it was possible to observe the impact on the residual sulfite concentration through the varied methodologies applied. Verifying that frozen shrimps decrease residual sulfite concentration by around 20% and that the concentration is maintained over time on frozen samples. In contrast, in fresh samples, the residual sulfite concentration tends to increase throughout the lifetime of the shrimps. As mentioned before, from the 10 different fresh samples analysed, 7 increased throughout the time with an average of 50%.

It is possible to conclude that sodium metabisulfite is a good spoilage inhibitor for the product, reacting with critical conditions and allowing it to obtain a product with a better commercial appearance throughout its lifetime. Although several analyses have been conducted on frozen samples, this product is marketed as refrigerated. There are results with sulfite levels above the legislation limit, which may be due to the tendency to increase the sulfite concentration in fresh products over time, as evidenced in this study. In contact with the suppliers about alternative methodologies, it was possible to understand that this change would imply significant changes in the origin, mainly for Phenolic Compounds and 4-hexylresorcinol methodologies, requiring a careful evaluation by the companies.

Throughout the diverse parts of the training placement, it was possible to achieve greater know-how of the work market, get to know this sector better, some companies related to the thesis theme, and visit one of the most renowned laboratories at an international level.

In terms of conclusion, this training placement enabled to gain much knowledge throughout the entire distribution chain, a vast network in this area, and in practical terms, allowed to identify differences in the various methodologies analysed.

Future Work

This work was carried out based on two suppliers. Due to the internship time limitation, only two analysis phases were made. In order to obtain more robust results, more phases and analyses would be necessary, with a more significant number of suppliers and a greater variety of calibres.

Furthermore, more research could be done on some automation systems, like Flow Injection Analysis (FIA) and Sequential Injection Analysis (SIA), to analyse residual sulfite concentration. This way, these systems could be implemented in factories or on product reception platforms of retail companies, allowing a quick analysis of residual sulfites in the product and rejecting products that do not comply with the legislated value.

Also, due to one of the significant shrimp farmed in aquaculture being the *Penaeus vannamei*, more detailed research about the optimum level of sodium metabisulfite to apply could be done in this specimen.

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Appendix

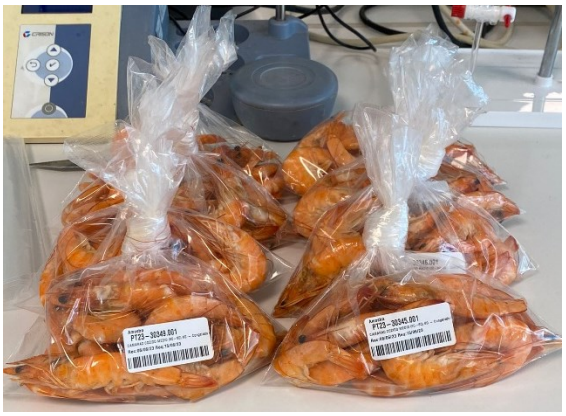

Figure 8- Division of samples for different methodologies.

Figure 9- Sample weighing.

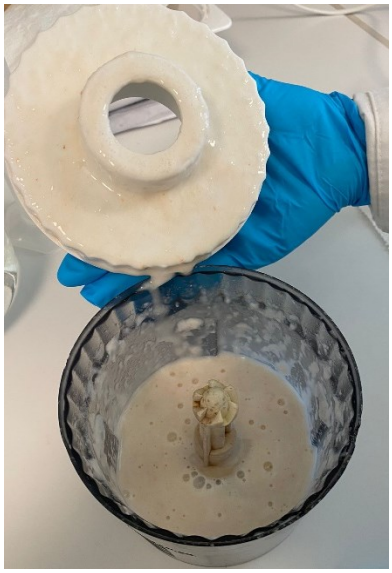

Figure 10- Grinded sample with 100 mL of 5% ethanol.

Figure 11- H2SO4 titration with NaOH, changing the red from methyl red to yellowish colour.

Table 3- Results from Phase I of sulfite concentration on boiled shrimps with different treatments from supplier X, collected on 30/11/2022. The expiration date of the product is 08/12/2022.

Product	Moment of analysis	pH	Legal Limit	Result Conformity
30/50 KG- Fresh- Collection Day	02/12/2022	7.1±0.09	135	Compliant
30/50 KG- Fresh- 24h after collection	02/12/2022	7.7±0.1	135	Non-Compliant
30/50 KG- Frozen/ Partially Thawed/ with defrosting liquid	06/12/2022	7.3±0.09	135	Non-Compliant
30/50 KG- Frozen/ Partially Thawed/ without defrosting liquid	06/12/2022	7.4±0.1	135	Compliant
30/50 KG-Frozen/ Fully defrosted/ with defrosting liquid	07/12/2022	7.3±0.09	135	Compliant
30/50 KG- Frozen/ Fully defrosted/ without defrosting liquid	09/12/2022	7.4±0.1	135	Compliant
30/50 KG- Frozen (1 month)/ Partially Thawed/ with defrosting liquid	27/12/2022-02/01/2023	7.3±0.09	135	Compliant
30/50 KG- Frozen (1 month)/ Partially Thawed/ without defrosting liquid	27/12/2022-04/01/2023	7.4±0.1	135	Compliant
30/50 KG- Frozen (1 month)/ Fully defrosted/ with defrosting liquid	27/12/2022-04/01/2024	7.6±0.1	135	Compliant

30/50 KG- Frozen (1 month)/ Fully defrosted/ without defrosting liquid	27/12/2022-05/01/2025	7.4±0.1	135	Compliant
(60-80) KG- Fresh-Collection Day	02/12/2022	7.3±0.09	135	Compliant
(60-80) KG- Fresh-24h after collection	02/12/2022	7.7±0.1	135	Non-Compliant
(60-80) KG- Frozen/ Partially Thawed/ with defrosting liquid	06/12/2022	7.3±0.09	135	Compliant
(60-80) KG- Frozen/ Partially Thawed/ without defrosting liquid	06/12/2022	7.4±0.1	135	Compliant
(60-80) KG- Frozen/ Fully defrosted/ with defrosting liquid	07/12/2022	7.6±0.1	135	Compliant
(60-80) KG- Frozen/ Fully defrosted/ without defrosting liquid	09/12/2022	7.8±0.1	135	Compliant
(60-80) KG- Frozen (1 month)/ Partially Thawed/ with defrosting liquid	27/12/2022-02/01/2023	7.5±0.1	135	Compliant
(60-80) KG- Frozen (1 month)/ Partially Thawed/ without defrosting liquid	03/01/2023-04/01/2023	7.4±0.1	135	Compliant
(60-80) KG- Frozen (1 month)/ Fully defrosted/ with defrosting liquid	27/12/2022-04/01/2023	7.4±0.1	135	Compliant
(60-80) KG- Frozen (1 month)/ Fully defrosted/ without defrosting liquid	27/12/2022-05/01/2023	7.4±0.1	135	Compliant

Table 4- Results from Phase I of sulfite concentration on boiled shrimps with different treatments from supplier Y, collected on 20/12/2022. The expiration date of the product is 28/12/2022.

Product	Moment of analysis	pH	Legal Limit	Result Conformity
30/50 KG- Fresh-Collection Day	27/12/2022	7.6±0.1	135	Non-Compliant
30/50 KG- Fresh-24h after collection	27/12/2022	7.7±0.1	135	Non-Compliant
30/50 KG- Frozen/ Partially Thawed/ with defrosting liquid	27/12/2022	7.6±0.1	135	Non-Compliant

30/50 KG- Frozen/ Partially Thawed/ without defrosting liquid	27/12/2022	7.5±0.1	135	Non-Compliant
30/50 KG-Frozen/ Fully defrosted/ with defrosting liquid	27/12/2022	7.6±0.1	135	Non-Compliant
30/50 KG- Frozen/ Fully defrosted/ without defrosting liquid	27/12/2022-30/12/2022	7.7±0.1	135	Non-Compliant
30/50 KG- Frozen (1 month)/ Fully defrosted/ without defrosting liquid	27/12/2022-01/02/2023	7.7±0.1	135	Non-Compliant
30/50 KG- Frozen (1 month)/ Fully defrosted/ with defrosting liquid	27/12/2022-01/02/2023	7.6±0.1	135	Compliant
30/50 KG- Frozen (1 month)/ Partially defrosted/ with defrosting liquid	30/12/2022-01/02/2023	7.3±0.09	135	Non-Compliant
30/50 KG- Frozen (1 month)/ Partially defrosted/ without defrosting liquid	27/12/2022-01/02/2023	7.6±0.1	135	Non-Compliant
60-80 600G- Fresh-Collection Day	27/12/2022	7.4±0.1	135	Non-Compliant
60-80 600G - Fresh-End of the Expiration date	27/12/2022-30/12/2022	7.4±0.1	135	Non-Compliant
60-80 600G - Frozen/ Partially Thawed/ with defrosting liquid	27/12/2022	7.5±0.1	135	Non-Compliant
60-80 600G - Frozen/ Partially Thawed/ without defrosting liquid	27/12/2022	7.4±0.1	135	Non-Compliant
60-80 600G - Frozen/ Fully defrosted/ with defrosting liquid	27/12/2022	7.5±0.1	135	Non-Compliant
60-80 600G - Frozen/ Fully defrosted/ without defrosting liquid	27/12/2022-30/12/2022	7.6±0.1	135	Non-Compliant
60-80 600G - Frozen (1 month)/ Partially Thawed/ with defrosting liquid	27/12/2022-01/02/2023	7.2±0.09	135	Non-Compliant
60-80 600G - Frozen (1 month)/ Partially	27/12/2022-01/02/2023	7.6±0.1	135	Non-Compliant

Thawed/ without defrosting liquid				
60-80 600G - Frozen (1 month)/ Fully defrosted/ without defrosting liquid	27/12/2022-01/02/2023	7.6±0.1	135	Non-Compliant
60-80 600G - Frozen (1 month)/ Fully defrosted/ with defrosting liquid	27/12/2022-01/02/2023	7.5±0.1	135	Non-Compliant
30-50 600G - Fresh-Collection Day	27/12/2022	7.2±0.09	135	Compliant
30-50 600G - Frozen/ Partially Thawed/ with defrosting liquid	27/12/2022	7.1±0.09	135	Compliant
30-50 600G - Frozen/ Partially Thawed/ without defrosting liquid	27/12/2022	7.2±0.09	135	Compliant
30-50 600G - Frozen/ Fully defrosted/ without defrosting liquid	27/12/2022-30/12/2022	7.3±0.09	135	Compliant
30-50 600G - Frozen/ Fully defrosted/ with defrosting liquid	27/12/2022	7.6±0.1	135	Compliant
30-50 600G - Frozen (1 month)/ Fully defrosted/ with defrosting liquid	27/12/2022-01/02/2023	7.3±0.09	135	Compliant
30-50 600G - Frozen (1 month)/ Fully defrosted/ without defrosting liquid	27/12/2022-01/02/2023	7.3±0.09	135	Compliant
30-50 600G - Frozen (1 month)/ Partially defrosted/ without defrosting liquid	27/12/2022-01/02/2023	7.3±0.09	135	Compliant
30-50 600G - Frozen (1 month)/ Partially defrosted/ with defrosting liquid	27/12/2022-01/02/2023	7.0±0.09	135	Compliant

Table 5- Results from Phase II of sulfite concentration on boiled shrimps with different treatments from supplier X, collected on 02/05/2023. The expiration date of the product is 09/05/2023.

Product	Moment of analysis	pH	Legal Limit	Result Conformity
30/50 KG- Fresh-Collection Day	03/05/2023	7.6±0.11	135	Compliant

30/50 KG- Fresh- 24h after collection	04/05/2023	7.5±0.11	135	Compliant
30/50 KG- Frozen/ Partially Thawed/ with defrosting liquid	12/05/2023	7.5±0.11	135	Compliant
30/50 KG- Frozen/ Partially Thawed/ without defrosting liquid	12/05/2023	7.6±0.11	135	Compliant
30/50 KG-Frozen/ Fully defrosted/ with defrosting liquid	16/05/2023	7.4±0.11	135	Compliant
30/50 KG- Frozen/ Fully defrosted/ without defrosting liquid	17/05/2023	7.5±0.11	135	Compliant
30/50 KG- Frozen (1 month)/ Partially Thawed/ with defrosting liquid	14/06/2023	7.5±0.11	135	Compliant
30/50 KG- Frozen (1 month)/ Partially Thawed/ without defrosting liquid	18/06/2023	7.6±0.11	135	Compliant
30/50 KG- Frozen (1 month)/ Fully defrosted/ with defrosting liquid	18/06/2023	7.6±0.11	135	Compliant
30/50 KG- Frozen (1 month)/ Fully defrosted/ without defrosting liquid	18/06/2023	7.6±0.11	135	Compliant
60-80 600G - Fresh- Collection Day	03/05/2023	7.8±0.12	135	Compliant
60-80 600G - Fresh- End of the Expiration date	11/05/2023	7.4±0.11	135	Compliant
60-80 600G - Frozen/ Partially Thawed/ with defrosting liquid	12/05/2023	7.8±0.12	135	Compliant
60-80 600G - Frozen/ Partially Thawed/ without defrosting liquid	12/05/2023	7.8±0.12	135	Compliant
60-80 600G - Frozen/ Fully defrosted/ with defrosting liquid	16/05/2023	7.3±0.11	135	Compliant
60-80 600G - Frozen/ Fully defrosted/ without defrosting liquid	17/05/2023	7.5±0.11	135	Compliant
60-80 600G - Frozen (1 month)/ Partially	14/06/2023	7.6±0.11	135	Compliant

Thawed/ with defrosting liquid				
60-80 600G - Frozen (1 month)/ Partially Thawed/ without defrosting liquid	18/06/2023	7.8±0.12	135	Compliant
60-80 600G - Frozen (1 month)/ Fully defrosted/ with defrosting liquid	18/06/2023	7.7±0.12	135	Compliant
60-80 600G - Frozen (1 month)/ Fully defrosted/ without defrosting liquid	18/06/2023	7.7±0.12	135	Compliant
30/50 600G - Fresh-Collection Day	03/05/2023	7.7±0.11	135	Compliant
30/50 600G - Fresh-End of the Expiration date	08/05/2023	7.3±0.11	135	Non-Compliant
30/50 600G - Frozen/ Partially Thawed/ with defrosting liquid	12/05/2023	7.6±0.11	135	Non-Compliant
30/50 600G - Frozen/ Partially Thawed/ without defrosting liquid	12/05/2023	7.1±0.11	135	Non-Compliant
30/50 600G -Frozen/ Fully defrosted/ with defrosting liquid	16/05/2023	7.1±0.11	135	Compliant
30/50 600G - Frozen/ Fully defrosted/ without defrosting liquid	17/05/2023	7.3±0.11	135	Compliant
30/50 600G - Frozen (1 month)/ Partially Thawed/ with defrosting liquid	14/06/2023	7.5±0.11	135	Compliant
30/50 600G - Frozen (1 month)/ Partially Thawed/ without defrosting liquid	18/06/2023	7.7±0.12	135	Non-Compliant
30/50 600G - Frozen (1 month)/ Fully defrosted/ with defrosting liquid	18/06/2023	7.6±0.11	135	Non-Compliant
30/50 600G - Frozen (1 month)/ Fully defrosted/ without defrosting liquid	18/06/2023	7.6±0.11	135	Compliant

Table 6- Results from Phase II of sulfite concentration on boiled shrimps with different treatments from supplier Y, collected on 09/05/2023. The expiration date of the product is 15/05/2023.

Product	Moment of analysis	pH	Legal Limit	Result Conformity
30/50 KG- Fresh- Collection Day	10/05/2023	7.3±0.11	135	Compliant
30/50 KG- Fresh- 24h after collection	11/05/2023	7.3±0.11	135	Compliant
30/50 KG- Frozen/ Partially Thawed/ with defrosting liquid	26/05/2023	7.6±0.11	135	Compliant
30/50 KG- Frozen/ Partially Thawed/ without defrosting liquid	26/05/2023	7.5±0.11	135	Compliant
30/50 KG-Frozen/ Fully defrosted/ with defrosting liquid	26/05/2023	7.4±0.11	135	Compliant
30/50 KG- Frozen/ Fully defrosted/ without defrosting liquid	26/05/2023	7.5±0.11	135	Compliant
30/50 KG- Frozen (1 month)/ Partially Thawed/ with defrosting liquid	26/06/2023	7.5±0.11	135	Compliant
30/50 KG- Frozen (1 month)/ Partially Thawed/ without defrosting liquid	26/06/2023	7.5±0.11	135	Compliant
30/50 KG- Frozen (1 month)/ Fully defrosted/ with defrosting liquid	26/06/2023	7.5±0.11	135	Compliant
30/50 KG- Frozen (1 month)/ Fully defrosted/ without defrosting liquid	26/06/2023	7.5±0.11	135	Compliant
60-80 600G - Fresh- Collection Day	10/05/2023	7.5±0.11	135	Compliant
60-80 600G - Fresh- End of the Expiration date	16/05/2023	7.1±0.11	135	Non-Compliant
60-80 600G - Frozen/ Partially Thawed/ with defrosting liquid	26/05/2023	7.7±0.12	135	Compliant
60-80 600G - Frozen/ Partially Thawed/ without defrosting liquid	26/05/2023	7.7±0.12	135	Compliant

60-80 600G - Frozen/ Fully defrosted/ with defrosting liquid	26/05/2023	7.7±0.12	135	Compliant
60-80 600G - Frozen/ Fully defrosted/ without defrosting liquid	26/05/2023	7.7±0.12	135	Compliant
60-80 600G - Frozen (1 month)/ Partially Thawed/ with defrosting liquid	26/06/2023	7.7±0.12	135	Compliant
60-80 600G - Frozen (1 month)/ Partially Thawed/ without defrosting liquid	26/06/2023	7.6±0.11	135	Compliant
60-80 600G - Frozen (1 month)/ Fully defrosted/ with defrosting liquid	26/06/2023	7.6±0.11	135	Compliant
60-80 600G - Frozen (1 month)/ Fully defrosted/ without defrosting liquid	26/06/2023	7.7±0.12	135	Compliant
30/50 600G - Fresh-Collection Day	10/05/2023	7.3±0.11	135	Non-Compliant
30/50 600G - Fresh-End of the Expiration date	16/05/2023	7.0±0.11	135	Non-Compliant
30/50 600G - Frozen/ Partially Thawed/ with defrosting liquid	26/05/2023	7.5±0.11	135	Non-Compliant
30/50 600G - Frozen/ Partially Thawed/ without defrosting liquid	26/05/2023	7.5±0.11	135	Non-Compliant
30/50 600G -Frozen/ Fully defrosted/ with defrosting liquid	26/05/2023	7.3±0.11	135	Non-Compliant
30/50 600G - Frozen/ Fully defrosted/ without defrosting liquid	26/05/2023	7.5±0.11	135	Non-Compliant
30/50 600G - Frozen (1 month)/ Partially Thawed/ with defrosting liquid	26/06/2023	7.4±0.11	135	Non-Compliant
30/50 600G - Frozen (1 month)/ Partially Thawed/ without defrosting liquid	26/06/2023	7.5±0.11	135	Non-Compliant
30/50 600G - Frozen (1 month)/ Fully defrosted/ with defrosting liquid	26/06/2023	7.5±0.11	135	Non-Compliant

30/50 600G - Frozen (1 month)/ Fully defrosted/ without defrosting liquid	26/06/2023	7.5±0.11	135	Non-Compliant

Table 7- Evolution of sulfites in the fresh product, Phase I.

Fresh Product (Phase I)					
Calibre	**Collection Day (mg/kg)**	**24h (mg/kg)**	**Final (mg/kg)**	**Increase/ Decrease**	
Supplier X:					
30-50/kg	<135	>135	-	93%	Increase
60-80/kg	<135	>135	-	100%	Increase
Supplier Y:					
30-50/kg	>135	>135	-	35%	Increase
60-80/600g	>135	-	>135	9%	Decrease

Table 8- Evolution of sulfites in the fresh product, Phase II.

Fresh Product (Phase II)					
Calibre	**Collection Day (mg/kg)**	**24h (mg/kg)**	**Final (mg/kg)**	**Increase/ Decrease**	
Supplier X:					
30-50/kg	<135	<135	-	24%	Decrease
60-80/600g	<135	-	<135	10%	Decrease
30-50/600g	<135	-	>135	18%	Increase
Supplier Y:					
30-50	<135	<135	-	24%	Increase
60-80/600g	<135	-	>135	65%	Increase
30-50/600g	>135	-	>135	18%	Increase

Table 9- Statistical analysis of samples with increased residual sulfites from the collection day to the final expiration date.

Supplier	**Calibre**	**Collection Day (mg/kg)**	**Final (mg/kg)**	**Normality**	**Sig. (95% confidence level)**
X	30-50/600g	<135	>135	0.98	0.045
Y	60-80/600g	<135	>135		
Y	30-50/600g	>135	>135		

Table 10- Statistical analysis of the pH of fresh samples from phase I.

	Collection Day	**24h**	**Sig.** (95% confidence level)
Supplier X:	pH		0.064
30-50/kg	7.1±0.09	7.7±0.10	
60-80/kg	7.3±0.09	7.7±0.10	
Supplier Y:			
30-50/kg	7.6±0.10	7.7±0.10	
60-80/600g	7.4±0.10	-	

Table 11- Statistical analysis of the pH of fresh samples from phase II.

	Collection Day	**Final**		
Supplier X:	pH		Normality	Sig. (95% confidence level)
30-50/kg	7.6±0.11	-	0.89	<0.001
60-80/600g	7.8±0.12	7.4±0.11		
30-50/600g	7.7±0.12	7.3±0.11		
Supplier Y:				
30-50/kg	7.3±0.11	-		
60-80/600g	7.5±0.11	7.1±0.11		
30-50/600g	7.3±0.11	7.0±0.11		

Table 12- Statistical Analysis of the defrosting liquid for the different methodologies applied in frozen samples, Phase I.

Different Frozen Methodologies						**Statistical Analysis**	
	Calibre	**Partially Without Liquid** (mg SO_2/ kg)	**Partially With Liquid** (mg SO_2/ kg)	**Fully Without Liquid** (mg SO_2/ kg)	**Fully With Liquid** (mg SO_2/ kg)	**With Liq./ Without Liq.**	
Supplier X	30-50/ kg	<135	>135	<135	<135	Partially	Fully
	60-80/ kg	<135	<135	<135	<135	Normality	
Supplier Y	30-50/ kg	>135	>135	>135	>135	0,80	0,69
	60-80/ 600g	>135	>135	>135	>135	Sig. (95% confidence level)	
	30-50/ 600g	<135	<135	<135	<135	0,335	0,498

Table 13- Statistical Analysis of the thawing method for the different methodologies applied in frozen samples, Phase I.

Different Frozen Methodologies						Statistical Analysis	
	Calibre	**Partially Without Liquid (mg SO_2/ kg)**	**Partially With Liquid (mg SO_2/ kg)**	**Fully Without Liquid (mg SO_2/ kg)**	**Fully With Liquid (mg SO_2/ kg)**	**Fully/ Partially**	
Supplier X	30-50/ kg	<135	>135	<135	<135	With Liq.	Without Liq.
	60-80 /kg	<135	<135	<135	<135	Normality	
Supplier Y	30-50/ kg	>135	>135	>135	>135	0,79	0,69
	60-80/ 600g	>135	>135	>135	>135	Sig. (95% confidence level)	
	30-50/ 600g	<135	<135	<135	<135	0,319	0,477

Table 14- Statistical Analysis of the defrosting liquid for the different methodologies applied in frozen samples, Phase II.

Different Frozen Methodologies						Statistical Analysis	
	Calibre	**Partially Without Liquid (mg SO_2/ kg)**	**Partially With Liquid (mg SO_2/ kg)**	**Fully Without Liquid (mg SO_2/ kg)**	**Fully With Liquid (mg SO_2/ kg)**	**With Liq./ Without Liq.**	
Supplier X	30-50/ kg	<135	<135	<135	<135	Partially	Fully
	60-80/ 600g	<135	<135	<135	<135	Normality	
	30-50/ 600g	>135	>135	<135	<135	0,72	0,86
Supplier Y	30-50/ kg	<135	<135	<135	<135	Sig. (95% confidence level)	
	60-80/ 600g	<135	<135	<135	<135		
	30-50/ 600g	>135	>135	>135	>135	0,472	0,344

Table 15- Statistical Analysis of the thawing method for the different methodologies applied in frozen samples, Phase II.

Different Frozen Methodologies						Statistical Analysis	
	Calibre	**Partially Without Liquid (mg SO_2/ kg)**	**Partially With Liquid (mg SO_2/ kg**	**Fully Without Liquid (mg SO_2/ kg)**	**Fully With Liquid (mg SO_2/ kg)**	**Fully/ Partially**	
Supplier X	30-50/ kg	<135	<135	<135	<135	With Liq.	Without Liq.
	60-80/ 600g	<135	<135	<135	<135	Normality	
	30-50/ 600g	>135	>135	<135	<135	0,15	0,20

Supplier Y	30-50/ kg	<135	<135	<135	<135	Sig. (95% confidence level)	
	60-80/ 600g	<135	<135	<135	<135		
	30-50/ 600g	>135	>135	>135	>135	0,249	0,338

Table 16- Statistical analysis of sulfite concentration throughout frozen storage time, Phase I.

	Calibre	**Frozen-Average**	**1 month Frozen-Average**	**Statistical Analysis**
Supplier X	30-50/ kg	<135	<135	Normality
	60-80/ kg	<135	<135	0,86
Supplier Y	30-50/ kg	>135	>135	Sig. (95% confidence level)
	60-80/ 600g	>135	>135	0,202
	30-50/ 600g	<135	<135	

Table 17- Statistical analysis of sulfite concentration throughout frozen storage time, Phase II.

	Calibre	**Frozen-Average**	**1 month Frozen-Average**	**Statistical Analysis**
Supplier X	30-50/ kg	<135	<135	Normality
	60-80/ 600g	<135	<135	0,83
	30-50/ 600g	>135	>135	
Supplier Y	30-50/ kg	<135	<135	Sig. (95% confidence level)
	60-80/ 600g	<135	<135	0,295
	30-50/ 600g	>135	>135	