Measurement of Histamine, *Listeria monocytogenes,* and Mercury (Hg) in Steak Tuna Product

Mahaldika Cesrany[1], *Siluh Putu Sri Dia* Utari [1*], and *Ida Bagus Kade Vhisnu* Manuaba[1]

[1]Marine Product Processing Study Program, Marine and Fisheries Polytechnic of Jembrana, Pengambengan Village, 82218 Jembrana, Bali, Indonesia

Abstract. The export of tuna fish is an essential activity, with major destinations being the United States, China, Japan, ASEAN, and the European Union. The export of fishery products is subject to strict regulations on the levels of histamine, mercury (Hg), and the presence of *Listeria monocytogenes*. In this study, we aimed to determine the levels of histamine and mercury, as well as the presence of *L. monocytogenes*, in steak tuna products intended for export. The samples were analyzed for histamine levels using Enzyme-Linked Immunosorbent Assay (ELISA), mercury levels using Atomic Absorption Spectrophotometer (AAS), and the presence of *L. monocytogenes* using Real Time-PCR. Results from our study indicated that all samples with temperatures ranging from -0.2 to -0.3°C had a histamine content of less than one ppm, which meets the acceptance standard for histamine on tuna exports as stipulated by European Commission (EC) number 2073/2005. Additionally, the mercury level in all samples was less than one ppm, which also meets the acceptance standard for mercury on tuna exports as stipulated by EC number 617/2022. Furthermore, *L. monocytogenes* were absent in all the samples analyzed. Based on our findings, we conclude that steak tuna products are suitable for export to various countries, especially those in the European Union.

1 Introduction

Data from the Directorate General of Strengthening the Competitiveness of Marine and Fishery Products (2022) indicates that tuna exports have been instrumental in driving the growth of fishery exports from January to November 2022, registering a 10.66% increase compared to the corresponding period of the previous year [1]. Tuna is one of the most exported fish commodities, with major destinations including the United States, China, Japan, ASEAN, and the European Union.

In the export market, ensuring top-notch quality assurance and the highest level of safety for export products is of utmost importance. The presence of histamine content, heavy metal contamination, and harmful bacteria are the primary challenges leading to the rejection of export products.

* Corresponding author: putudia15@gmail.com

In this regard, histamine levels are a crucial parameter in the export market, as the allowable levels are tightly regulated due to the risk of human poisoning. The Indonesian National Standard (SNI) sets the threshold for histamine levels in fish at 100 mg/kg (100 ppm), while the Codex Alimentarius specifies a maximum histamine content of 200 ppm [2, 3]. Consumption of histamine beyond 100 mg/kg can lead to poisoning, making its detection, which is usually performed using the Enzyme-Linked Immunosorbent Assay (ELISA) technique, critical.

Apart from histamine, heavy metal contamination is another critical export market parameter. Tuna, being an ocean-dwelling fish, has the potential to accumulate heavy metals from ocean pollution. Heavy metal contamination in tuna can be harmful to human health if consumed. For instance, the European Union has set a strict standard requirement threshold of 1 ppm for heavy metal contamination, which is usually identified using Atomic Absorption Spectrophotometry (AAS) [4].

The presence of pathogenic bacteria also needs to be considered, especially tuna products exported to the United States, China, Japan and the European Union. *Listeria monocytogenes* is one of concern pathogen bacteria in seafood export poducts [5]. *L. monocytogenes* is a gram-positive bacterium that can thrive in both fresh and saltwater environments [6, 7]. Physiologically, *L. monocytogenes* can survive at freezing temperatures and NaCl concentrations up to 28% [7, 8, 9, 10]. Listeriosis, a severe disease caused by *L. monocytogenes*, has a high mortality rate of approximately 20-30% [11]. Contamination can occur through various means, including environmental exposure and processing [12]. In 2016, *L. monocytogenes* was detected in fish samples, causing 2536 cases of listeriosis [13]. According to an European Food Safety Authority (EFSA) investigation, the highest percentage of non-compliance with EU criteria was observed in 2016 in the fish and fisheries product categories, with 5,6% and 4,7% of fish and fisheries goods, respectively [13]. The standard requirement for *Listeria monocytogenes* contamination in fish is typically 'negative/25 g', and it is identified using culture and Real-Time PCR [14].

Given the strict standards and regulations governing histamine, heavy metal contamination, and bacterial presence in tuna fish exports, the research titled 'Identification of Histamine, *Listeria monocytogenes*, and Mercury (Hg) in steak tuna products.

2 Research method

2.1 Sample preparation

Tuna steak samples were provided by five different companies with codes A1, B1, C1, D1, and E1. The samples were initially thawed for 3-4 hours. Once the samples reached the thawed state, for histamine testing, 30 g of muscle tissue from the tuna steak sample was weighed; for *Listeria monocytogenes* testing, 25 g were weighed from both the middle and edges of the tuna steak samples, and; for mercury testing, 25 g were weighed from any part randomly of the tuna steak samples.

Each of the prepared items was then homogenized by grinding using a wooden rolling pin and cutting board. It's important to note that for histamine testing, compositing was done directly during the preparation, whereas for the other tests, it was not done directly during sample preparation.

2.2 Histamine testing

This methods refers to Veratox Quantitative Histamine Tuna Test Pack Neogen Corporation, 620 Lesher Place, limsing, MI 48912, USA, Base in AOAC Official Methods [1]. This test

refers to the Manual Book Programing Histamine (0 ppm, 5 ppm, 20 ppm, 40 ppm and 100 ppm) Test Parameters For The Neogen®321 Microwell Reader. Histamine content testing in tuna steaks involved three stages: sample preparation, sample extraction, and histamine level testing. For sample extraction, 10 g of the composite was mixed with 90 mL of distilled water. The extraction process was done by shaking the distilled water and sample mixture for 20 seconds, followed by a 5-minute incubation. This process was repeated three times. The resulting extraction was then filtered using a cotton filter and collected in a tube.

Subsequently, the filtered results were transferred into a 10 mL phosphate-buffered saline (PBS) using a 100 µL micropipette. Once the extraction process was complete, histamine-level testing proceeded. In the next step, red and white wells were prepared. 100 µL of Veratox conjugate solution reagent was added to the red well. An additional 100 µL of the sample in PBS was also added to the red-well containing the reagent. The sample was homogenized by gently moving it up and down in the red well containing the Veratox conjugate solution reagent.

After achieving homogeneity, 100 µL of the sample extract was transferred to the white well and incubated for 10 minutes. Following incubation, the solution in the white well was discarded, and a wash buffer solution was used to wash the well. The white well was then dried with a tissue to ensure no residual liquid remained.

Next, 100 µL of Veratox substrate solution reagent was added to the white well using a 100 µL micropipette, and the mixture was incubated for an additional 10 minutes. To stop the reaction in the white well, 100 µL of Veratox stop solution reagent was added by gently raising and lowering the micropipette three times.

The final step involved reading the histamine levels using a Neogen-Statfax machine. The readings were taken at a wavelength (λ) of 650 nm and repeated twice for accuracy and precision. The histamine testing included five quantitative control levels: 0 ppm, 2.5 ppm, 10 ppm, 20 ppm, and 50 ppm. The determination of histamine levels was based on color changes observed in the controls.

In this study, the temperature of the 5 tuna samples used was also checked. The check is carried out using a thermocouple tool. The tool is inserted at an angle of 45° into the fish flesh. The identified temperature is then recorded.

2.3 Mercury testing

The sample were weighed to obtain a mass range of 40-50 mg. Subsequently, the sample were coated with additive M (USGS Mercury Research Laboratory, 2016), followed by a layer of additive B (USGS Mercury Research Laboratory, 2016), and then topped with another layer of additive M. After this preparation, the boat should be placed onto the *Mercury Analyzer-2000 Atomic Absorption Spectrophotometer (SSA)*.

2.4 Realtime-PCR test for *Listeria monocytogenes*

Testing for *L. monocytogenes* on tuna steaks using the RT-PCR method went through several stages, including pre-enrichment, enrichment, DNA extraction, interpretation, and RT-PCR preparation. The primers used are the *L.monocytogenes* master mix bio primer kit and assay mix. The parameters used are FAM and ROX controls, which are seen based on the FAM and ROX dyes. If FAM has numbers and ROX also has numbers, the result is positive, and vice versa. If there are no numbers on FAM and ROX, then the result is negative. The pre-enrichment process began by weighing a 25 g sample for *L. monocytogenes* testing and placing it in a stomacher bag. The sample was then homogenized by grinding it using a wooden rolling pin and a cutting board. Next, 225 mL of buffered peptone water was prepared to homogenize the ground 25 g sample, and it was incubated in an incubator for

approximately 18 hours at 35 °C.

The DNA extraction process involved combining four samples by transferring 375 µL of samples that had been incubated for approximately 18 hours into a microtube. The composite was then centrifuged at a speed of 12,000 rpm for 15 minutes. After centrifugation, the liquid (supernatant) and solid (pellets) phases were separated. The upper liquid (supernatant) was discarded to retain the solid pellet. 100 µL of lysis buffer was added to the pellet and homogenized by pipetting up and down 7-15 times. The microtubes were incubated in a water bath for 30 minutes at 95 °C. Then, the tubes were centrifuged again at a speed of 12,000 rpm for 15 minutes.

Once the samples were centrifuged, the next step was to prepare the PCR bio primer kit reagents. For testing *L. monocytogenes*, each reaction required a master mix and an *L. monocytogenes* assay mix. In one reaction, 16 µL of master mix and 2 µL of *L. monocytogenes* assay mix were combined in a microtube. The mixture was homogenized by pipetting up and down 7-15 times. The PCR tubes, positive control, and negative control for *L. monocytogenes* were prepared. 18 µL of PCR mix was placed into each PCR tube (sample tube, negative control tube, and positive control tube). Then, 2 µL of the negative control, 2 µL of the DNA sample, and 2 µL of the positive control were added to their respective prepared PCR tubes. The PCR tubes were sealed, spun down, and then loaded into the RT-PCR machine.

3 Results and discussion

3.1 Histamine level testing results

The tuna steak samples underwent histamine testing using the Enzyme-Linked Immunosorbent Assay (ELISA) method. The ELISA method for histamine testing comprises several stages. The first stage involves sample preparation, where the objective is to prepare the sample for testing in composite form. In this study, the composite was created by combining three different samples; each was weighing 10 g.

The histamine test readings, obtained using the ELISA method, were compared to a standard curve to assess the color change in the sample and determine histamine levels. Figure 1 illustrates the standard curve used for this purpose.

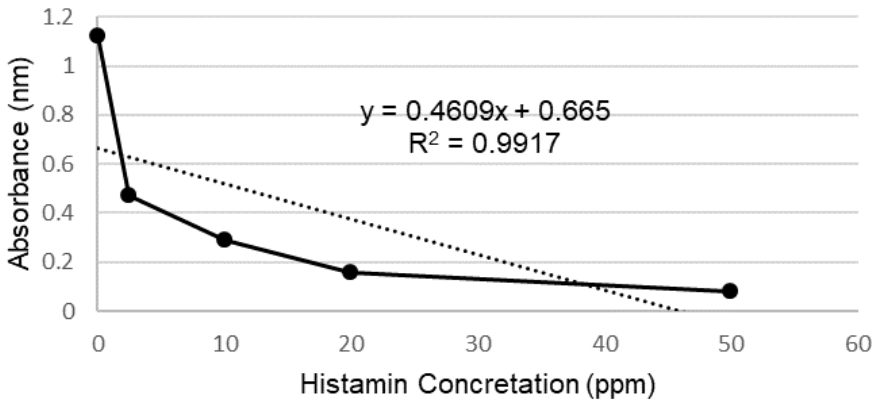

Fig. 1. Histamine standard curve.

Based on the standard curve, it can be observed that the y-axis represents the reading results from the ELISA reader in the form of absorbance values, while the x-axis represents the concentration values of the standard solution. According to [15], the R-value will indicate a strong relationship when it is close to -1 for a negative correlation or +1 for a positive correlation. Figure 1 shows that the R^2 value is 0.9917, indicating a strong positive relationship between concentration and absorbance. A standard curve should consist of a minimum of five data points (duplicates) and have an R-value greater than 0.9 [16].

Samples tested for histamine levels using the Enzyme-Linked Immunosorbent Assay (ELISA) method from five different companies yielded a total of five samples with histamine levels below 1 ppm. As per Europe Commision Regulation No. 2073/2005 standard, the acceptance limit for fisheries products is 100 ppm [17]. The results of the histamine testing are depicted in Figure 2.

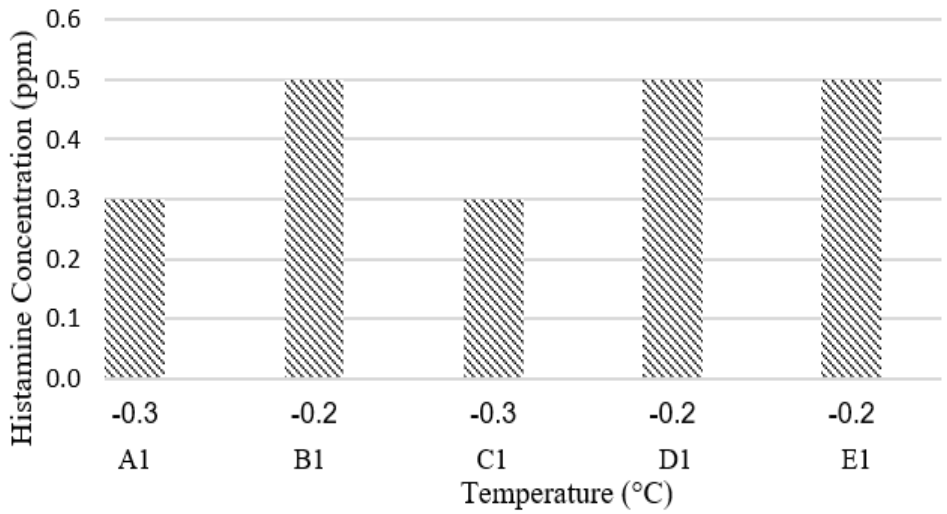

Fig. 2. Histamine level testing results.

Based on the results of histamine testing conducted at five different companies with sample codes A1, B1, C1, D1, and E1, histamine levels were found to be between 0.3 and 0.5 ppm. Low temperatures can control histamine-forming bacteria, but the histidine decarboxylase enzyme that has already formed will continue to produce histamine even if the bacteria responsible for its formation are inactive [18]. The temperature range recorded upon sample arrival was -0.2°C to -0.3°C, allowing for the identification of histamine content. This is because the histamine content had already formed when the sample was received frozen.

3.2 Mercury testing results

The working principle of the mercury machine involves thermally decomposing the sample using a heater within the machine. This process utilizes the cold atomic absorption method at a wavelength of 253.7 nm within the detector's absorption cell.

The samples submitted for mercury testing included samples from five different companies, originating from both Bali and other regions. These samples were frozen steaks, totaling five samples tested for mercury content. According to [19], the maximum permissible limit for mercury content in fish is < 0.500 ppm.

During the mercury testing conducted with the Mercury Analyzer-2000, the results revealed the lowest concentration of mercury in tuna steak as 0.180 ppm (A1) and the highest

concentration as 0.603 ppm (D1). Among these results, one sample, specifically D1, exceeded the maximum limit set by SNI, reaching 0.603 ppm. However, it's worth noting that these samples can still be exported to the European Union because the applicable standard requirement threshold in the European Union is 1 ppm [4]. The histogram illustrating the results of mercury testing with tuna steak samples can be found in Figure 3.

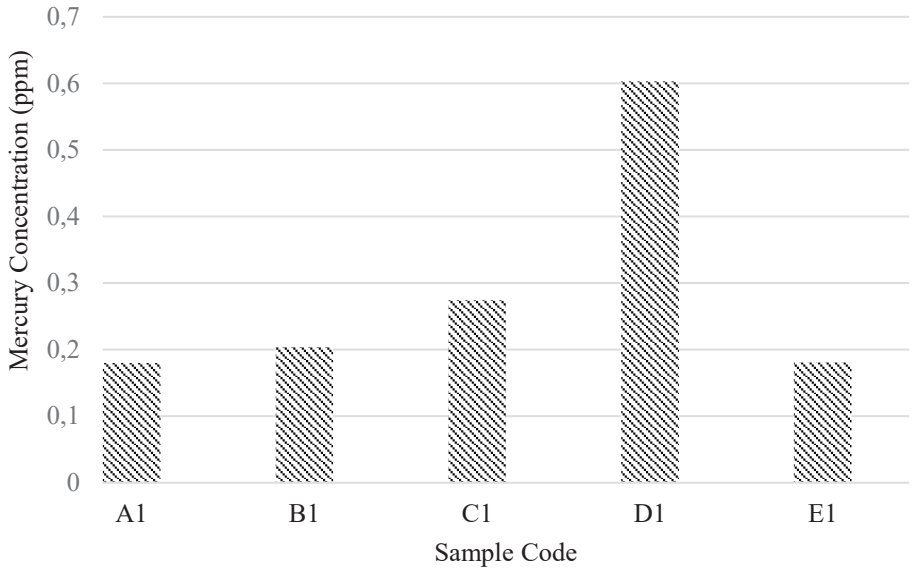

Fig. 3. Mercury testing results.

Mercury (Hg) is one of the most toxic heavy metals, and exposure to high concentrations can lead to various symptoms. Typically, the initial symptoms at elevated mercury levels include paresthesia or a narrowed field of vision. At even higher concentrations, individuals may experience ataxia, deafness, and, ultimately, fatal outcomes [20].

According to [21], fish have the capacity to accumulate heavy metals, including mercury, due to its high toxicity even at low concentrations. This bioaccumulation process initiates within the aquatic food chain, starting with plankton that absorbs mercury from the water. Subsequently, small fish consume the plankton, and small and large predatory fish, such as tuna, consume these smaller fish in turn [22].

3.3 *Listeria monocytogenes* testing results

The samples submitted for *L. monocytogenes* testing via Real Time-PCR (RT-PCR) consisted of five samples from five different companies. These samples were sourced from various areas within Bali and outside of Bali. The results of the *L. monocytogenes* RT-PCR test, as shown in Table 1, indicate that no *L. monocytogenes* bacterial contamination was detected in any of the composites from the five different companies.

The PCR process consists of repeated cycles of denaturation, annealing, and extension. In this study, during denaturation, the temperature was set to 95 ºC for 30 seconds, and this step involved the separation of DNA strands using high temperatures.

In the annealing process, a temperature range of 55-60 ºC was employed for 30 seconds. Annealing means the stage where primers attach to specific DNA sequences. The annealing temperature is crucial, as it influences the successful attachment of primers and plays a

critical role in obtaining optimal DNA binding[23].

The final stage in this study involved DNA extension facilitated by the DNA polymerase enzyme. DNA extension occured at a temperature of 72 ºC for a specified time, which depended on the length of the DNA to be amplified.

During the amplification stage, an efficacy curve was generated as the amplification process commences. This curve served the purpose of determining whether amplification was occurring within the thermal cycler. DNA amplification was assessed based on fluorescent intensity, where a higher production of amplification products corresponded to a greater accumulation of fluorescent readings. The increase in fluorescence is depicted by a sigmoidal curve, which results from the intersection with the predefined threshold baseline set by the program.

Table 1. Results of RT-PCR testing for *Listeria monocytogenes*.

No	Flour	Composite Code	Cq	Cq mean	Cq Std Dev	Status	[8]
1	FAM	NC LM		0.00	0.000	Negative Control	Negative/25 g
2	ROX	NC LM	21.25	21.25	0.000		
3	FAM	PC LM	22.85	22.85	0.000	Positive Control	
4	ROX	PC LM	20.09	20.09	0.000		
5	FAM	A1		0.00	0.000	Negative	
6	ROX	A1	17.74	17.74	0.000		
7	FAM	B1		0.00	0.000	Negative	
8	ROX	B1	18.56	18.56	0.000		
9	FAM	C1		0.00	0.000	Negative	
10	ROX	C1	20.14	20.14	0.000		
11	FAM	D1		0.00	0.000	Negative	
12	ROX	D1	21.48	21.48	0.000		
13	FAM	E1		0.00	0.000	Negative	
14	ROX	E1	16.86	16.86	0.000		

According to [24], ROX is an inert fluorescent dye that can be incorporated as a component in the RT-PCR master mix. In contrast to reporter dyes like SYBR Green or FAM dyes, the fluorescence of ROX remains unaffected by the amplification of PCR products. In the *L. monocytogenes* test that has been conducted, only ROX dye numbers were observed, with no indication of numbers on the FAM dye. Consequently, all tested samples were negative for the presence of *L. monocytogenes* bacteria. This indicates that the processing and sample capture procedures were carried out correctly in accordance with the company's Standard Operating Procedures (SOP). As per SNI 7388:2009 standards, *L. monocytogenes* contamination in fish must yield negative results [14]. This also applies to Europe Commision Regulation number 2073/2005, *L. monocytogenes* contamination must yield negative results [17].

Consuming food contaminated with *L. monocytogenes* can lead to listeriosis, which poses particular risks to individuals with compromised immune systems, the elderly, pregnant women, and unborn babies or newborns. Listeriosis can result in complications affecting the uterus in pregnant women, as well as the central nervous and circulatory systems [25].

4 Conclusion

Based on the research conducted, histamine testing using the ELISA method revealed that all tuna steak samples had a histamine content of less than 1 ppm. The sample temperatures were recorded within the range of -0.2 to -0.3°C. The level of mercury was obtained less than 1 ppm. These results are in accordance with European Commission (EC) number 2073/2005 and 617/2022 about standards for an acceptance standard for histamine and mercury on tuna exports, respectively. Furthermore, *L. monocytogenes* were absence in tuna steak samples.

We thank PT Seafood Inspection Laboratory, Denpasar, Bali, for tuna steak samples as well as histamine , mercury (Hg), and *Listeria monocytogenes* testing.

References

1. [DJPDSPKP], Direktorat Jendral Penguatan Daya Saing Produk Kelautan dan Perikanan (Jakarta, 2022)
2. [BSN], Badan Standardisasi Nasional (Jakarta, 2013)
3. [FAO], Food Agricultural Organization of the United Nations (USA, 2012)
4. [EC], European Commission, Regulation (Brussels, 2022)
5. M. Jami, M. Ghanbari, M. Zunabovic, K. J. Domig, W. Kneifel, Compr. Rev. Food. Sci. F. **13**, 1-16 (2014)
6. L. Gram, J. Food. Sci. **66**, S1072-81 (2001)
7. M. Zhu, M. Du, J. Cordray, D. U. Ahn, Compr. Rev. Food. Sci. F. **4**, 34-42 (2005)
8. A. Beaufort, S. Rudelle, N. Gnanou-Besse, M. T. Toquin, A. Kerouanton, H. Bergis, G. Salvat, M. Cornu, Letter Applied Microbiology. **44**, 406-11 (2007)
9. P. Calo-Mata, S. Arlindo, K. Boehme, T. de Miguel, A. Pascoal, J. Barros-Velzaquez, Food Bioprocess Tech. **1**, 43-63 (2008)
10. M. Ghanbari, M. Jami, K. J. Domig, W. Kneifel, LWT – Food Science Tech-Brazil. **50**, 2 (2013)
11. R. A. Anggraeni, *Sensitivitas dan selektivitas pilator (rapid Salmonella detector): biosensor kolorimetri berbasis immunosensor sebagai pendeteksi Salmonella* (Brawijaya University, Malang, 2018)
12. R. Tocmo, K. Krizman, W. J. Khoo, L. K. Phua, M. Kim, H. Yuk, Compr. Rev. Food Sci. F. **13**, 172-189 (2014)
13. [EFSA], European Food Safety Authority and European Centre for Disease Prevention and Control, EFSA J. **15**, 12 (2017)
14. [BSN] Badan Standardisasi Nasional (Jakarta, 2009)
15. K. Santoso, U. K. Herowati, D. A. Rotinsulu, S. Murtini, M. Y. Ridwan, D. W. Hikman, E. Sukmawinata, *Jurnal Veteriner Maret*. **22**, 79-85 (2021)
16. P. G. Naully, G. Khairinisa, *Panduan analisis laboratorium imunoserologi untuk D3 teknologi laboratorium medis* (Stikes Jenderal Achmad Yani, Cimahi, 2018)
17. [EC], European Commission, Regulation (EC) No.2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs (Europe Commision. Brussels, 2005)
18. N. R. Prasetiawan, T. W. Agustini, W. Farid, JPHPI. **16** (2013)
19. [BSN] Badan Standardisasi Nasional (Badan Standardisasi Nasional, Jakarta, 2009)
20. A. Salsabila, *Analisis kandungan merkuri (Hg) pada ikan tongkol (Euthynnus affinis) di TPI Bom Kalianda Lampung Selatan* (UIN Raden Intan, Lampung, 2023)

21. L. Lensoni, A. Nurdin, Z. I. Zaini, *Jurnal Aceh Medika*. **4**, 102-112 (2020)
22. E. P. Wijaya, *Validasi metode pengujian merkuri Hg pada ikan tuna Thunnus* sp. *menggunakan Mercury Hg Analyzer Type Ma-3 Solo dan Dma-80* (UIN Syarif Hidayatullah, Jakarta, 2023)
23. A. Ludyasari, *Pengaruh suhu anealing pada program PCR terhadap keberhasilan amplifikasi DNA udang jari (Metapenaeus elegans De Man, 1907) Laguna Segara anakan Cilacap, Jawa Tengah, Malang* (UIN Malang, Malang, 2016)
24. S.R. Hong, and S. Kyoung-Jin. Bisulfite-converted DNA quantity evaluation: a multiplex quantitative real-time PCR system for evaluation of bisulfite conversion (Yonsei University College of Medicine, Seoul, 2021)
25. C. R.Tjampakasari, *Cermin Dunia Kedokteran*. **48**, 20-24 (2021)