Journal of Advanced Zoology
ISSN: 0253-7214
Volume 44 Issue 4 Year 2023 Page 01-08

Fish Gelatin Detection in Edible's Bird Nest by Real-Time Polymerase Chain Reaction Method

Ratri Purwaningrum[1*], Rini Widayanti[2], Dyah Ayu Widiasih[3]

[1*]*Master of Veterinary Science, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia. email: rtrwiryakusuma@gmail.com*
[2]*Department of Biochemistry, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia*
[3]*Department of Veterinary Public Health, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia*

[*]**Corresponding Author:** *Ratri Purwaningrum*
Master of Veterinary Science, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia. email: rtrwiryakusuma@gmail.com

Article History:

Abstract

Edible's Bird Nest (EBN) is a nest made from dried bird (*Aerodramus fucuphagus*) saliva and can be consumed. The process of turning dirt into edible bird's nest involves a lengthy sequence of steps, from harvesting in the birds' houses or caves, removing dirt and sand, soaking/washing, removing feathers, reshaping, drying, and heating. In this reshaping process, gelatin can be used as the glue. Gelatin is recovered from collagen by hydrolysis. The resources of gelatin are derived from bovine, porcine, and fish sources. Indonesia is the world's largest exporter of commodities. Each export destination country has its unique requirements. Export destination countries with Muslim consumers require a product to be halal, without porcine material. In contrast, other countries require it to be free from bovine materials or pure, without gelatin. Fish gelatin has advantages as an alternative source for bovine and porcine gelatin substitutes. There is a need for a valid and fast deoxyribonucleic acid (DNA) detection method for fish to support the acceleration of exports. This study aims to detect fish DNA by real-time polymerase chain reaction and to determine the concentration of fish DNA in gelatin that can be detected by real-time PCR. Fish gelatin EBN samples and positive controls were used in this study. The primer and probe were BLAST primed before use. The results of fish gelatin amplification with graded concentrations from 50 ng/µl to 0.00005 ng/µl, and the detection limit was 0.0005 ng/µl in the presence of gelatin. The analysis of 50 samples and their results revealed that fish DNA was detected in 90% of the EBN samples, with a gelatin spike in 20% and 10%.

Keywords: EBN, Gelatin, Fish, DNA, real-time PCR

1. Introduction

Edible's Bird Nest (EBN) is consumed as both a medicine and a source of nutrition, offering numerous benefits for the body. The EBN contains protein, fat, carbohydrates, iron, calcium, phosphorus, salt, fiber, and air. Glyconutrients in swiftlet nests include sialic acid, N-acetylgalactosamine, N-acetylglucosamine, galactose,

and fructose. EBN also contains nine essential amino acids and eight nonessential amino acids (Elfita, 2015). The magnitude of SBW's benefits makes this product highly popular, despite its relatively high economic value. This presents an export opportunity for Indonesia, as it is the world's largest producer of swallow nests.
The EBN is a very strategic commodity of animal origin from Indonesia. The EBN processing industry in Indonesia is labor-intensive. Indonesian EBN exports to several countries. Based on 2017 data from the Indonesian Trade Promotion Center of the Ministry of Trade, it was recorded that as much as 78% of the world's EBN supply is from Indonesia. According to data from the Central Statistics Agency as of July 26, 2022, EBN exports totaled 1.505.5 tons in 2021.
Gelatin is a natural polymer derived from the hydrolytic degradation of collagen protein, and its distinctive amino acid structure confers several medical benefits (Kumosa et al., 2018). Hydrocolloids have been widely used as gelling agents in the food industry (Karim et al., 2008; Shyni et al., 2014). Most hydrocolloids are extracted from plants; however, gelatin is generally extracted from animals. Bovine, porcine, and fish collagens are the primary sources of gelatin. Hydrocolloids and gelatin have often been confused because some scholars support the other family of hydrocolloids, particularly plants labeled as 'veggie gelatin.' Not all hydrocolloids are plant-based, and gelatin is not a naturally occurring hydrocolloid; it must be extracted from animal parts. Therefore, the commercially viable sources of collagen are demineralized cattle bone, pig, and bovine hides (Sultana et al., 2018), which have not been genetically modified.
Many methods have been developed to measure gelatin and identify its source, such as spectroscopy, immunochemistry (Tukiran et al., 2016), mass spectrometry combined with liquid/gas chromatography (LC/GC–MS) methods (Yilmaz et al., 2013), conventional PCR, and real-time PCR.
The real-time PCR method provides quantitative estimates automatically derived from cycle monitoring using fluorescent probe labels (Asing, 2016). The real-time PCR method, targeting the primary gene cytochrome oxidase I (COI), can be used to determine the purity of a product. The rate and pattern of evolution are moderate, making the COI gene the most suitable for evaluating phylogenetic evolution at the species level. Specificity testing for primers and probes using the NCBI Primer-BLAST software.

2. Materials and Methods

2.1. Sample collection and preparation

Fish, bovine, and porcine gelatin by Sigma Aldrich, USA. Fish is obtained from a traditional market in Yogyakarta, Indonesia. EBN commercial samples were purchased from shopping outlets across Indonesia, and the samples were prepared by spiking with fish gelatin. DNA primers were designed and tested with software from the National Center for Biotechnology Information (NCBI) website and then ordered from Genetics Science Indonesia. The materials used for analysis affect the accuracy of the pro-analysis quality test results.

2.2. Instruments

Real-time PCR machine Agilent Aria Mx, biosafety cabinet BioAir A2 type, vortex mixer Biosan FVL-2400N, micropipette single channel (0.2-2 µl,1-10 µl, 10-100 µl, 100-1000 µl), PCR cabinet ESCO A2 type, vortex mixer Thermo Scientific 88880018, spindown Biosan V1 plus, and PCR plate cooler. Biosafety cabinet ESCO A2 type, vortex mixer Biosan FVL- 2400N, spindown Biosan V1 plus, PCR plate cooler, Laboratory freezer Thermo Scientific PL 6500, UV/Vis Spectrophotometer Nabi, magnetic separator, and other glass tools commonly used in the analysis found at the Laboratory.

2.3. DNA Isolation

DNA isolation from fish meat as a positive control for EBN, fish gelatin test, bovine, and porcine gelatin. Total DNA from controls and samples have been extracted using a genesig® Easy DNA/RNA Extraction Kit (Primerdesign™ Ltd). Extractions were performed under aseptic conditions to obtain good-quality DNA.
The first step is preparing control and sample materials for species testing. For meat/food, a 50 mg sample material can be homogenized in 500 µl of Sample Preparation Solution or 750 µl of Sample Preparation Solution for EBN by mixing/vortexing, and then incubated for 5 minutes in a BSC. The next step is to combine the sample preparation and lysis stages by dispensing 200 µL of the clear supernatant from the sample and the sample preparation solution, mixing, and then adding 20 µL of proteinase K. Incubate at room temperature for 15 minutes. Transfer the lysate (400 µl) to a new 1.5 ml microtube, leaving behind any particulate matter, before adding the magnetic beads. Continue the extraction step, bind nucleic acid to magnetic beads by adding 500 µl magnetic beads/binding buffer to the lysate sample, mix well by shaking, then wait 5 minutes. Be sure to mix the GeneSig Easy Extraction Beads well before removing them from the storage bottle. Vortex or shake the storage bottle briefly until a homogenous suspension has been formed. Separate the magnetic beads from

the sample by placing the tube into the magnetic separator. Wait at least 2 minutes for all the beads to be attracted to the magnet. Remove and discard supernatant by pipetting. Do not disturb the attached beads while aspirating the supernatant.
The next step is to wash with Wash Buffer 1. Remove the tube from the magnetic separator. Add 500 µL Wash Buffer 1, then shake until the beads are completely resuspended. Then wait 30 seconds. Alternatively, resuspend beads completely by repeated pipetting up and down. Separate the magnetic beads from the sample by placing the tube into the magnetic separator. Wait at least 2 minutes for all the beads to be attracted to the magnet. Remove and discard supernatant by pipetting. Do not disturb the attached beads while aspirating the supernatant. Then wash with Wash Buffer 2 and wash with 80% ethanol. The step is similar to the Wash Buffer 1 step. Air-dry the magnetic bead pellet for 20 minutes at room temperature with the tube lid open. The beads should be free from any visible liquid ethanol, but not left to dry out completely.
The final step is adding Elute DNA/RNA. Remove the tube from the magnetic separator. Add the desired volume of Elution Buffer (50–200 µL) and resuspend the beads by shaking until the beads are resuspended completely. Then wait 2 minutes. If eluting in a volume <200µl, resuspend beads completely by repeated pipetting up and down for 1 minute instead of shaking. Separate the magnetic beads from the sample by placing the tube into a magnetic separator. Wait at least 2 minutes for all the beads to be attracted to the magnet. Transfer the supernatant containing the purified DNA/RNA to a 0.5 mL tube with a flip cap for storage or use in downstream applications. Do not disturb the attracted bead whilst removing the supernatant. DNA concentration and purities were measured using a UV/Vis Spectrophotometer, Nabi. All extracted DNA samples have been frozen at -200 °C until further use.

2.4. Primer and probe design

Specific primers for fish DNA used in this research were designed using the National Centre for Biotechnology Information (NCBI) software (https://www.ncbi.nlm.nih.gov/) and designed in silico using the NCBI-BLAST website from NCBI (https://www.blast.ncbi.nlm.nih.gov/) to see the specificity of the primers obtained. The fish probe was labeled with Hex at the 5' and BHQ1 at the 3'.

2.5. Condition applied for real-time PCR

The real-time PCR assay for fish was carried out using the real-time PCR machine Agilent Aria Mx in 20 µl, reaction volume containing 10 µl Oasig lyophilized 2X reaction qPCR Master Mix (Primerdesign™), 1 µl forward primer, 1 µl reverse primer, 0,5 µl probe and 5 µl total DNA template from each sample and required quantities of nuclease-free water. Amplification was performed using an enzyme activation step at 95°C for 2 minutes, followed by 40 cycles of a denaturation step at 95°C for 5 seconds and data collection (annealing and extension) at 60°C for 5 seconds.

2.6. Limit of Detection and Limit of Quantitation

The limit of detection was determined using serial dilutions of fish DNA at 50, 5, 0.5, 0.05, 0.005, 0.0005, and 0.00005 ng/µl. The limit of detection (LOD) is the lowest concentration of analyte that can be detected and identified with certainty. LOD is also defined as the lowest concentration that can be distinguished from background noise with a certain level of confidence (Riyanto, 2014). In this assay, 5 µl of each diluted sample was added to a 15 µl reaction mixture, resulting in a final volume of 20 µl. The real-time PCR assay was performed in 10 replicates for each dilution.
The limit of Quantitation (LOQ) is the lowest amount of analyte that can be quantitatively determined with acceptable precision and accuracy in a calibration standard. The LOQ includes contamination arising from reagents, but excludes contamination from the sample preparation process. The LOD and LOQ are standard parameters in literature used to compare the sensitivity of developed analytical methods (Ershadi and Shayanfar, 2018)

2.7. Precision and accuracy

Precision is the degree of similarity between test results obtained from repeated measurements on the same samples. Precision can be measured using the formula % RSD = SD/Mean × 100%. The RSD acceptance requirement is ≤ 2%. The precision value can be determined by 100%-% RSD. Accuracy is the correspondence between the results and the true value of the analyte or the acceptable analyte reference value (Riyanto, 2014).

2.8. Sensitivity and specificity

Sensitivity and specificity can be calculated using a 2x2 Table to determine the values of true positives, true negatives, false negatives, and false positives, as well as the false positive rate (FPR) and false negative rate

(FNR). Additionally, sensitivity and specificity can be derived from these values. The validation of a method is said to be satisfactory if FPR and FNR are equal to zero, sensitivity >70% and Specificity >90% (Riyanto, 2014).

3. Results and Discussion

Authentication of gelatin, an ingredient in food and pharmaceutical products, is of interest due to specific regulations, religious guidelines, and export requirements. Determining the gelatin origin is particularly challenging because the amino acid sequences of collagen types are highly similar across species. Real-time PCR is sensitive enough to identify the origin of gelatin from traces of species-specific DNA. However, source determination is impossible when DNA is denatured or removed during the production process (Jannat et al., 2017). The basic PCR technique involves selecting a primer to be used. The specific primer will bind to the region-specific to the DNA template and amplify into a new strand. A precise primer design is required to produce specific primers that match the target amplification (Saif et al., 2012). Initially, primers were used to amplify the extracted DNA from gelatin powders of known origin (samples provided by Sigma-Aldrich Corp., St. Louis, USA). The amplification curves of the extracted DNA from standard fish gelatin samples using species-specific primers are demonstrated in Figure 1.

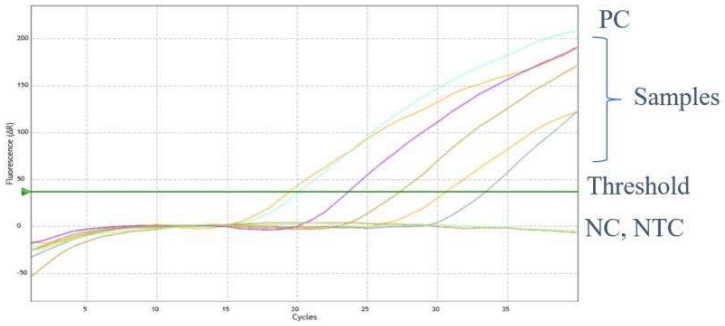

Figure 1. Amplification plots for gelatin samples

Every amplification used a positive, negative, and non-template control (NTC) to make sure the amplification was correct. Figure 1 above shows the amplification of gelatin samples with a positive control from fish meat. These fish gelatin samples will be used as a positive control in the EBN test.

3.1. Limit of Detection and Limit of Quantitation

The limits of detection (LODs) of the simplex and multiplex PCR systems were evaluated by the dilution method (Hossain et al., 2016). Analytical sensitivity was evaluated through several parameters, and each assay was repeated at least 10 times (Bru et al., 2023). The target detections use dilution methods 50, 5, 0.5, 0.05, 0.005, 0.0005, and 0.00005 ng/µl of total DNA extracted from pure fish gelatin and give the result of mean Cq 20.2090, 23.8510, 27.2000, 30.9370, 33.7160, and 37.0110, so the mean of all of them is 28.8207. The standard deviation is 0.4058, % the RSD is 1.4, the LOD is 0.0005 ng/µl, and the LOQ is 0.0015 ng/µl**.** It shows the correlation between fish DNA concentration and Cq with 10 repetitions, and the amplification plot indicates that the greater the DNA concentration, the smaller the Cq value, and vice versa: the smaller the DNA concentration, the greater the Cq value. The amplification plot above also shows that the detection limit of fish gelatin is a DNA concentration of 0.0005 ng/µl with a Cq of 37.01 ± 0.42.

3.2. Precision and accuracy

The % RSD is 1.4% and the RSD acceptance requirement is ≤ 2%. This shows that the precision value is 100% - 1.4% = 98.6%. The acceptance limit for the precision value is 60-115% (Gonzales et al., 2007), while the accuracy using the acceptance limit < CV Horwitz is calculated by the formula CV Horwitz = $2^{(1-0.5 \log C)}$. The data analyzed that the CV Horwitz is 9.6474%, so 2/3 of the CV Horwitz is 8.4676%. The limit of acceptability for accuracy is <2/3%. The accuracy for this method is 1.4% < 6.316%, so it still meets the acceptance limit of CV Horwitz.

3.3. Specificity and sensitivity

Sensitivity and specificity can be seen by using a 2x2 Table to get the value of true positive, true negative, false

negative, false positive, false positive rate (FPR), and false negative rate (FNR), sensitivity, and specificity. The validation of a method is said to be satisfactory if FPR and FNR are equal to zero, sensitivity >70% and Specificity >90% (Riyanto, 2014).

The target samples used are positive samples containing fish DNA from the isolation of fish gelatin and non-target samples derived from bovine gelatin, pork gelatin, and EBN. Ensuring the validity of the results by including positive, negative, and NTC controls in each test. The number of samples used was 3 positive samples and 3 negative samples for each non-target DNA sample, with two replicates. Sensitivity is true positive/(true positive + false negative) and specificity is true negative/(true negative + false positive) by table 2x2 (Parikh et al., 2008)

Table 1. Table 2 X 2

	Standard present	Standard absent	
Test positive	True positive (TP) a	False positive (FP) b	Total test positives a+b
Test negative	False negative (FN) c	True negative (TN) d	Total test negatives c+d
Total	a+c	b+d	Population a+b+c+d

Table 2. The true value of the target (fish) and non-target (bovine gelatin, porcine, gelatine, and EBN) samples

No	Parameters (repetition)	true positive value	true negative value	false positive value	false negative value	Total sample
1	Fish gelatin	6	0	0	0	6
2	Porcine gelatin	0	6	0	0	6
3	Bovine gelatin	0	6	0	0	6
4	EBN	0	6	0	0	6
	Total	6	18	0	0	24

Table 3. Table of 2x2 for target samples and bovine gelatin

The True Value	Result Test		Total
	Positives	Negatives	
Positives	6^a	0^b	6
Negatives	0^c	6^d	6
Total	6	6	12^n

Table 4. Table of 2x2 for target samples and porcine gelatin

The True Value	Result Test		Total
	Positives	Negatives	
Positives	6^a	0^b	6
Negatives	0^c	6^d	6
Total	6	6	12^n

Table 5. Table of 2x2 for target samples and EBN

The True Value	Result Test		Total
	Positives	Negatives	
Positives	6^a	0^b	6
Negatives	0^c	6^d	6
Total	6	6	12^n

Sensitivity is calculated by a/(a+c) x 100% with a result of 6/(6+0) x 100% = 100% while Specificity is calculated by d/(c+d) x 100% with a result of 6/(0+6) x 100% = 100%. It has a high sensitivity and specificity of 100%, giving negative results for non-target samples, bovine gelatin, porcine gelatin, and EBN. The next step was to test the suitability using Kappa as calculated for the fish target sample. Kappa for real-time PCR

testing for the detection of fish gelatin in EBN, the target sample is fish gelatin, and the non-targets are bovine (Table 3), porcine (Table 4), and EBN (Table 5), which have the same results in all three, so the Kappa can be calculated as follows:

Proportion of conformity = (a+d)/n
=1

Chance proportion of conformity = [(e x g)/n + (f x h)/n]
n
= 0,5

Proportion of suitability -chance = X
= 0,5

Maximum chance of conformity =1-chance =Y
=0,5

Kappa = X/Y
= 0,5/0,5
= 1 or 100%

The Kappa value is 100% or meets the Kappa criteria above 70%, indicating excellent agreement. Diagnostic test assessment depends on the combination of sensitivity and specificity tasks of a diagnostic tool evaluated against the test reference standard, referred to as the gold standard (Hosein et al., 2017). Real-time PCR is a well-established method for assessing the specificity and sensitivity of target DNA identification, even at low concentrations (Fajardo et al., 2010). Testing of gelatin detection using real-time PCR was carried out because it will be applied to different samples, and testing was repeated to assess sensitivity and specificity and validate the method.

The limit of detection (LOD), LOQ, accuracy, precision, specificity, and sensitivity fulfill the method validation criteria. This shows that the method is valid and can be applied for sample testing.

3.4. Edible's Bird Nest Samples

All extracted DNA samples were amplified and analyzed. A total of 50 samples were used in this study, which were purchased from shops throughout Indonesia and spiked with gelatin from fish, bovine, and porcine sources. The twenty-five samples were spiked with 1 ml of fish gelatin at concentrations of 20%, 10%, and 5%. The twenty other samples were spiked with 1 ml of bovine and porcine gelatin at concentrations of 20%, 10%, and 5%. The last five samples were not spiked. The samples were spiked with gelatin above the EBN and then allowed to dry, resulting in samples that appeared similar to the EBN.

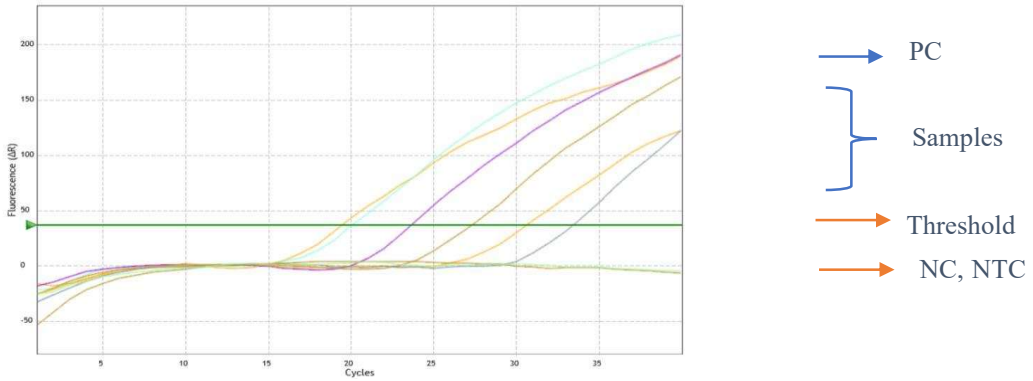

Figure 2. Several amplification plots for positive, negative, non-template control, and samples

The results of the sample testing show that 90% of the samples spiked with 20% fish gelatin and 10% are positive, while samples spiked with 5% negative. It is because fish gelatin is so clear, like EBN, and so similar to EBN that it may be imperfect for sampling, and the concentration is so low.

4. Conclusions

In this study, the real-time PCR, combined with species-specific primers targeting cytochrome oxidase I, can be used to identify fish DNA in gelatin. The minimum concentration of gelatin that can be detected is 0.0005 ng/µl. This method is specific and reliable for identifying gelatin in EBN and can be used routinely for authentication analysis.

Declaration of competing interest
The authors declare that they have no conflict of interest.

Acknowledgments
The authors thank the Faculty of Veterinary Medicine, Universitas Gadjah Mada, for providing Competitive Research Grants with Contract No. 1776/UN.1/FKH/HK4/2023, and the Yogyakarta Class II Agricultural Quarantine Service for supporting these research activities.

References

1. Ali, M. E., Sultana, S., Hamid, S. B. A., Hossain, M. M., Yehya, W. A., Kader, M. A., et al. (2016). Gelatin Controversies in Food, Pharmaceuticals, and Personal Care Products: Authentication methods, current status, and future challenges. *Crit Rev Food Sci Nutr*. 58(9):1495-1511 DOI: 10.1080/10408398.2016.1264361.
2. Asing, Ali, E., Hamid, S. B. A., Hossain, M., Ahamad, M. N. U., Hossain, S. A., Naquiah, N.,et al. (2016) . Duplex real-time PCR assay using SYBR Green to detect and quantify Malayan box turtle (Cuora amboinensis) materials in meatballs, burgers, frankfurters, and traditional Chinese herbal jelly powder. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* 33(11):1643-1659. doi: 10.1080/19440049.2016.1236403.
3. Brue, G., Candele, M. M., Romero, P., Navarro, A., & Murcia, A.M. (2023). Internal Validation of the ASFV MONODOSE dtec-qPCR Kit for African Swine Fever Virus Detection under the UNE-EN ISO/IEC 17025:2005 Criteria. *Vet Sci*. 2023, 10,564 DOI: 10.3390/vetsci10090564.
4. Elfita, L . (2015). Analisa Protein dan Asam Amino Sarang Burung Walet (Collocalia fuchiphaga) Asal Painan. *Jurnal Sains Farmasi & Klinis*. 1(1): 1-22. http://doi.org/10.29208/jsfk.2014.1.1.22
5. Ershadi, A. and Shayanfar, A.,(2018). Are LOD and LOQ reliable parameters for sensitivity evaluation of spectroscopic methods? *Journal of AOAC International.* Vol 101. No 4,
6. Fajardo, V, Gonzales. I., Rojas M, T., Martin R. (2010). Determination of animal origin of meat and gelatin by MALDI-TOF-MS. *Journal of Food Composition and Analysis*, 41, 104-112.
7. GMIA 2012 Gelatine Handbook. USA: Gelatin Manufacturers Institute of America. GMIA
8. Gonzalez, A.G. and Herrador, M.A. (2007). A practical guide to analytical method validation, including measurement uncertainty and accuracy profiles. *Trends in Analytical Chemistry* 26: 227 –238.
9. Hosein, H.I., Rouby, S.R., Menshawy, A., Ahmed, E. A., (2017), Sensitivity and specificity the commonly used diagnostic procedures of Bovine Brucellosis. *Veterinary Science Vol.* 3: 45
10. Jannat, B., Ghorbani, K., Shafieyan, H., Kouchaki, S., Behfar, A., Sadeghi, N., Beyramysoltan, S., Rabbani, F., Dashtifard, S., & Sadeghi, M. (2018). Gelatin Speciation Using Real-time PCR and Analysis of Mass Spectrometry-based Proteomics Datasets. *Food Control 87*: 79–87. https://doi.org/10.1016/j.foodcont.2017.12.006
11. Karim, A.A. R. Bhat, (2008). Gelatin alternatives for the food industry: recent developments, challenges, and prospects, *Trends Food Sci. Technol*. 19. (2008).644– 656,https://doi.org/10.1016/j.tifs.2008.08.001
12. Kumosa,L.S., V. Zetterberg, J. Schouenborg, (2018). Gelatin promotes rapid restoration of the blood-brain barrier after acute brain injury, Acta Biomater. 65 (2018)137–149,https://doi.org/10.1016/j.aCqbio.2017.10.020 NCBI https://www.ncbi.nlm.nih.gov/
13. Parikh R., Mathai A., Parikh S., Chandra S.G, Thomas R. (2008). Understanding and using sensitivity, specificity, and predictive values. *Indian J Ophthalmol*. Jan-Feb;56(1):45-50. doi: 10.4103/0301-4738.37595. PMID: 18158403; PMCID: PMC2636062.
14. Riyanto. (2014). *Validasi & Verifikasi Metode Uji: Sesuai dengan ISO/IEC 17025 Laboratorium Penguji dan Kalibrasi*. Ed. 1, Cetakan 1. Deepublish. Yogyakarta.
15. Salamah, N., Erwanto, Y., Martono, S., & Rohman, A. (2022). The Employment of Real-Time Polymerase Chain Reaction for the Identification of Sapi Gelatin in Gummy Candy. *Indonesian J Pharm* 33(3): 448-454. http://blast.ncbi.nlm.nih.gov/

16. Shyni, K. G.S. Hema, G. Ninan, S. Mathew, C.G. Joshy, P.T. Lakshmanan. (2014). FoodHydrocolloids Isolation and characterization of gelatin from the skins of skipjack tuna (Katsuwonus pelamis), dog shark (Scoliodon sorrakowah), and rohu (Labeo rohita), *Food Hydrocoll*. 39 2014 68–76,https://doi.org/10.1016/j.foodhyd.2013.12.008
17. Sultana, S. M.E. Ali, M.N.U. Ahamad, (2018). Gelatine, Collagen, and Single Cell Proteins as a Natural and Newly Emerging Food Ingredients, *Elsevier* Ltd,https://doi.org/10.1016/b978- 0-08-101892-7.00011-0.
18. Sultana, S., Hossain, M. A. M., Azlan, A., Johan, M. R., Chowdhury, Z. Z., & Ali, M. E.(2020). TaqMan Probe Based Multiplex Quantitative PCR Assay for Determination of Bovine, Fish and Fish DNA in Gelatin Admixture, Food Products and Dietary Supplements. *Food Chemistry*, 325. https://doi.org/10.1016/j.foodchem.2020.126756
19. Tukiran, N. A., Ismail, A., Mustafa, S., & Hamid, M. (2016). Development of Anti Peptide Enzyme-linked Immunosorbent Assay for Determination of Gelatin in ConfeCqionery Products. *Journal of Food Science & Technology*, 51:54–6. DOI:10.1111/IJFS.12971.
20. Yilmaz, M. T., Kesmen, Z., Baykal, B., Sagdic, O., Kulen, O., Kacar, O., & Baykal, A. T. (2013). A Novel Method to Differentiate Bovine and Fish Gelatins in Food Products: Nano UPLC-ESI-Q-TOF-MS E based data independent acquisition technique to detect marker peptides in gelatin. *Food Chem* 141(3):2450-8. doi 10.1016/j.foodchem.2013.05.096.