



DOI: <https://doi.org/10.56936/18290825-1.v19.2025-87>

SEX DETERMINATION USING CONFOCAL RAMAN MICROSCOPE WITH CHEMOMETRIC METHOD FROM DENTAL SAMPLE AND CONFIRMATION BY AMELOGENIN GENE

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Received 15.10.2024; Accepted for printing 11.02.2025

ABSTRACT

Lack of evidence in DNA analysis for sex determination of dental samples, with burned teeth. Resulting in the field of forensic dentistry looking for alternatives in sex determination for personal identification efforts. One alternative is to use spectral analysis from the Raman Microscope Confocal with the chemometric method. This alternative method uses samples of 20 teeth from 10 male and female dental samples, which are then analyzed on the enamel, dentin, and pulp surfaces with Raman spectra and also gold standards in the form of amelogenin DNA analysis, accompanied by chemometric tests using machine learning in the form of principal component analysis, restricted Boltzmann machines, support vector machine and artificial neural network, and comparative analysis based on the dental surface and signal fragments in the form of full width at half maximum.

The results obtained the accuracy value of the artificial neural network and support vector machine comparison, namely 75% on the Dentin surface using artificial neural network principal component analysis and Enamel using support vector machine, radial basis function, restricted Boltzmann machines. This means that the enamel and dentin surfaces are potential areas that can be used to predict gender using the chemometric method. Analysis of signal fragments at wave numbers 920-1080 cm⁻¹ with the main molecule (PO₄) symmetric stretching (960 cm⁻¹) analyzed in the form of full width at half maximum has a p value of 0.001, this means that there is a significant difference between men and women based on signal fragments. The examination of the gold standard DNA amelogenin has the same results between men and women in each layer of teeth. It can be concluded that the analysis of the confocal raman microscope in sex determination with the chemometric method of dental samples confirmed by DNA amelogenin gene can be used as an alternative method of gender identification.

KEYWORDS: Raman, artificial neural network, support vector machine, restricted Boltzmann machines Amelogenin, dental sample.

CITE THIS ARTICLE AS:

Kristanto R., Junitha K., Suyanto H., Pharmawati M., Yudianto A. (2025). Sex determination using confocal Raman microscope with chemometric method from dental sample and confirmation by amelogenin gene. The New Armenian Medical Journal, vol.19(1), 87-94; DOI: <https://doi.org/10.56936/18290825-1.v19.2025-87>

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INTRODUCTION

In criminal cases, there is usually no evidence that can be used as a source for DNA (deoxyribonucleic acid) examination in large quantities, this makes DNA samples require careful handling. DNA analysis can use bones and teeth which are the strongest tissues of all human organs, which can be used as a source of forensic identification [Yudianto A, dan Setiawan F, 2020]. In dental samples, more accurate sex determination methods based on dental samples include Barr identification of dental pulp and amplification of specific DNA sequences of the X or Y chromosome using polymerase chain reaction or identification of sex-specific differences in the enamel matrix protein, amelogenin [Gamulin O et al., 2021]. Some literature shows that DNA examination of dental samples has shortcomings, most researchers have reported that it is very difficult to extract DNA from teeth that have been exposed to temperatures of 200°-400°C, and no consensus has been reached on the level of cremation at which teeth will still produce nuclear DNA signals [Peral D et al., 2021].

Forensic odontologist uses various analytical tools for sex determination, one alternative for sex determination can also be done using Raman spectral analysis [Banjsak L et al., 2023; Kristanto R et al., 2023]. Raman spectroscopy and Fourier Transform InfraRed analysis, which was shown in the study of Sekhaneh W. et al. (2021), namely Raman spectroscopy can identify and classify several elements of content in ancient teeth with burnt conditions or those that have been buried for a long time. Raman mapping can help to explore archaeological samples for well-preserved organic material, thereby identifying the best candidates for further analysis (DNA extraction) [Sekhaneh W et al., 2021].

This is also proven in the study of Rubio L. et al. (2018) that Spectrophotometry can be used to measure the color of 40 teeth heated at temperatures of 100°, 200°, and 400°C for 60 minutes. Spectrophotometric analysis of the color of burned teeth can predict the feasibility of human DNA extraction for identification purposes [Rubio L et al., 2018]. Raman Spectroscopy is a sophisticated analytical method that provides detailed and specific information at the molecular level. In terms of its versatility, this method can provide information that may be below the capabilities of other

spectroscopic methods. Raman spectroscopy has properties that are useful for forensic applications [Kristanto R et al., 2023].

Raman Spectroscopy can provide fast, accurate, sensitive, and in situ detection analysis. It can sensitively and accurately reflect changes in the composition and structure of the material. With the advent of nanotechnology, advanced optical microscopes and miniaturized lasers have been developed, and problems such as weak signals, low signal-to-noise ratios, and strong autofluorescence backgrounds have also been overcome to enable Raman Spectroscopy to be gradually applied in the biomedical field. Raman Spectroscopy has been used to detect bacteria and the composition of cells, tissues, and biofluids, and in recent decades has received increasing interest for medical prognosis and diagnosis [Zhang Y et al., 2022].

This study aims to determine the accuracy of the spectral analysis results of confocal Raman microscopy in sex determination using human tooth samples with chemometric methods and data confirmation with the amelogenin gene.

MATERIAL AND METHODS

This study used 20 human dental samples from tooth extraction, consisting of 10 male tooth samples and 10 female tooth samples. The tooth elements used were random, consisting of incisors, premolars, and molars. This study has obtained ethical clearance from the ethics committee of the Faculty of Dentistry, Airlangga University No: 634/HRECC.FODM/V/2023.

Research samples with permanent teeth conditions, no caries, or fractures. The samples were then vertically sectioned using a disk diamond bur, so that 2 sides of the teeth were obtained. Then the results of the first side of the tooth section were subjected to spectral analysis using Confocal Raman Microscope and while the other side was subjected to confirmation analysis using the amelogenin gene.

The Spectral Analysis Examination using Confocal Raman Microscope was carried out in the Chemical Engineering Lab, Faculty of Chemical Engineering, Gadjah Mada University, Yogyakarta. The Raman Spectra tool used was the HORIBA brand (Kyoto, Japan) with a laser length of 785 nm (Max 100 mW). Laser with a wavelength of 100-

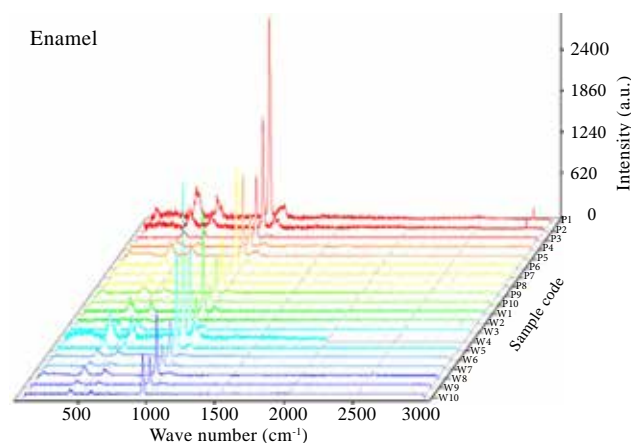


FIGURE 1. Results of Raman Spectra Graph on the enamel surface in the wave number range of 100-3400 cm^{-1} from all samples between men and women

3400 cm^{-1} was fired at 3 areas of the tooth surface consisting of enamel, dentin, and tooth pulp. The results of the recorded spectra examples are shown in figure 1.

Amelogenin Gene Analysis Examination at the Institute of Tropical Disease, Universitas Airlangga, Surabaya. This study used Sigma primers with leaders X: 542 *bp* and Y: 358 *bp* which were analyzed on extra samples from the enamel, dentin, and tooth pulp tooth surfaces.

Chemometric analysis using principal component analysis, restricted Boltzmann machines, support vector machine and artificial neural network using the Matlab application, and continued comparative analysis using SPSS Statistics 27.

RESULTS

The results of the spectral analysis of Confocal Raman Microscope showed the highest spectral peak between the wave numbers 920-1080 cm^{-1} with the molecule (PO_4) symmetric stretching (960 cm^{-1}). This can be done by cutting the signal fragments at these numbers to carry out more detailed analysis based on the enamel, dentin, and pulp tooth surfaces, as well as the signal fragments in figure 2.

The results of the Chemometric analysis in this study used principal component analysis and restricted Boltzmann machines on the 920-1080 cm^{-1} wavenumber signal fragment shown in figure 3. Principal component analysis is a method designed to reduce the spatial dimensions of data to find and interpret dependencies between vari-

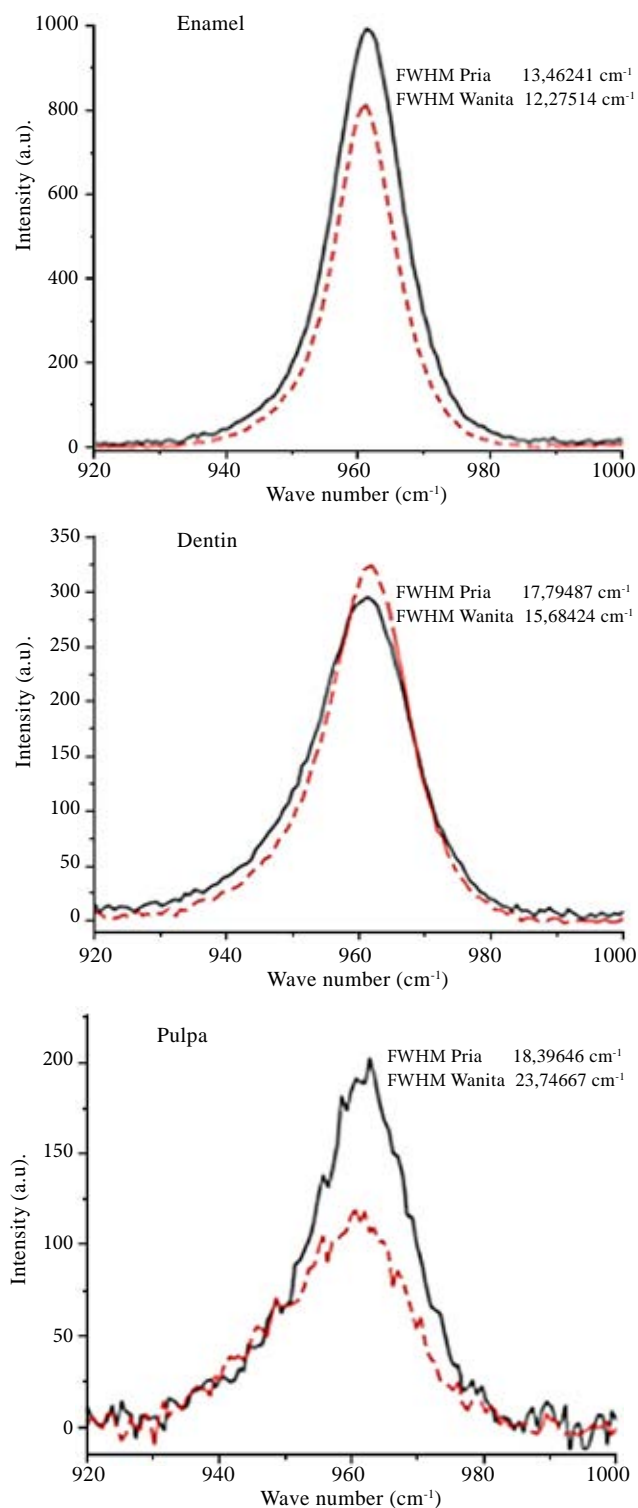


FIGURE 2. Spectral graph results of signal fragments with wave numbers 920-1080 cm^{-1} from enamel, dentin and dental pulp samples

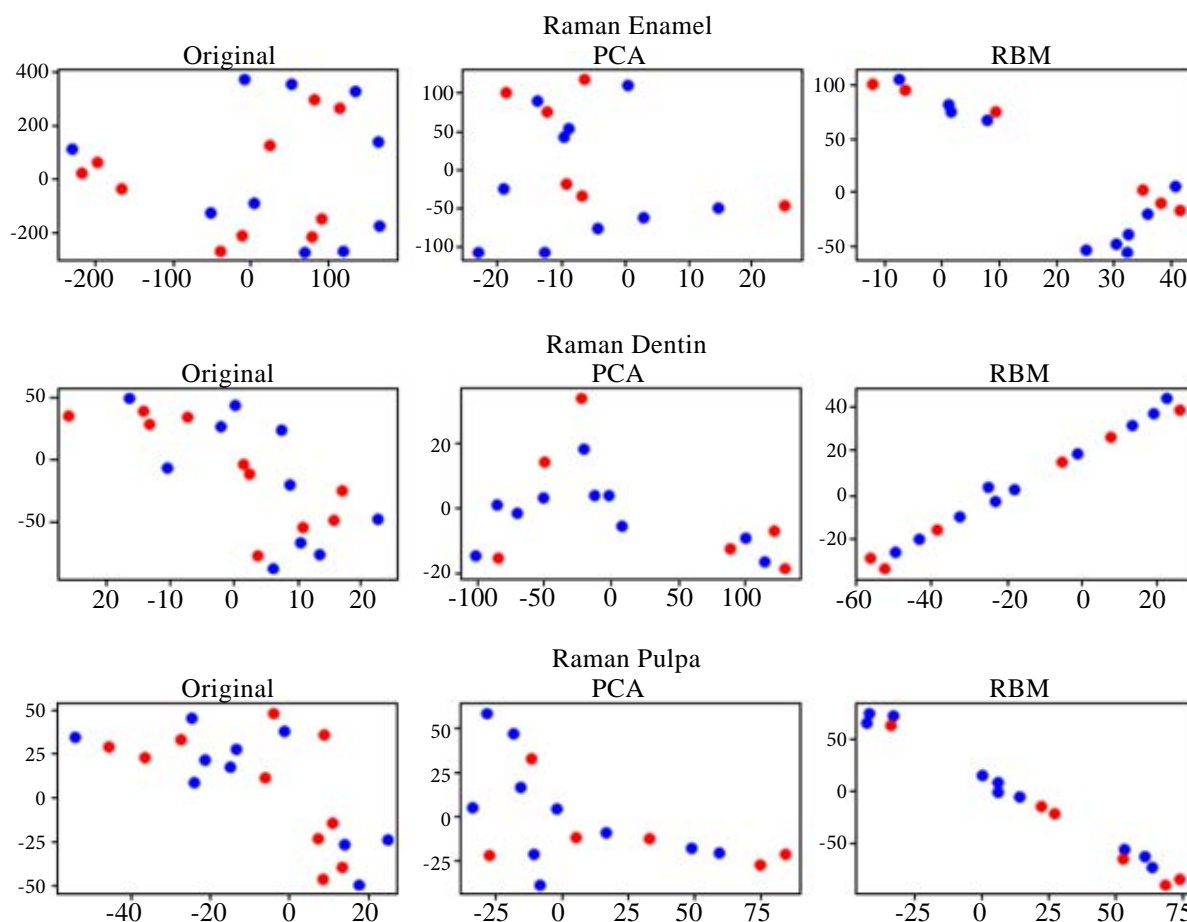


FIGURE 3. Clustering results on Raman data using principal component analysis and restricted Boltzmann machines on the enamel, dentin, and pulp surfaces with blue indicating male gender and red indicating female gender

ables or to help stabilize measurements in statistical analysis such as regression analysis or cluster analysis [Haryati A, Sugiyarto S, 2021]. Restricted Boltzmann machines are unsupervised energy-based generative models (neural networks), which are directly inspired by statistical physics. Restricted Boltzmann machines attempts to represent complex interactions (or correlations) in the visible layer (data) by introducing new hidden (latent) variables [Vrabel et al, 2020].

Comparison results of artificial neural network and support vector machine analysis accuracy, and confusion matrix of dental samples using confocal Raman microscope

Table 1 shows the artificial neural network principal component analysis using 1 principle component that has 95% accuracy has the highest value in the Dentin surface area with a value of 75%, while the support vector machine restricted Boltzmann machines analysis using 8 principle

TABLE 1

Comparison results of artificial neural network and support vector machine accuracy

Area	Artificial neural network		Support vector machine, radial basis function,	
	principal component analysis	restricted Boltzmann machines	principal component analysis	restricted Boltzmann machines
	1 PCA 95%	1 PCA 95%	8 PCA 95%	8 PCA 95%
Enamel	25%	50%	50%	75%
Dentin	75%	25%	50%	25%
Pulp	50%	0%	50%	0%

NOTES: PCA - principal component, accuracy 95%

components that have 95% accuracy has the highest value in the Enamel area with a value of 75%. This means that gender determination with Raman Micro Confocal can be used in the Enamel and Dentin areas. The results of the artificial neural network and support vector machine are based on the Confusion Matrix Analysis shown in table 2.

Statistical Analysis SPSS version 27 using non-parametric Kruskal Wallis Test based on gender with enamel, dentin, and pulp tooth surfaces (Table 3). Based on gender with fragment-signal size analysis of molecules (Area, center of gravity, max height, and full width at half maximum). Showing have different result in parameter of fragment signal, that can be using to sex determination of male and female by confocal Raman spectra.

DNA analysis of amelogenin

The results of the confirmation of the amelogenin gene analysis showed significant results on the enamel surface of each sample between men and women. This shows that the sigma primer with a large leader has good accuracy as a gold standard in determining male and female sex.

DISCUSSION

Raman spectroscopy has been shown to be important in the forensic analysis of trace evidence [Tague T, Leona M, 2013], and Raman Spectrometry has proven its value in the field of forensic dentistry. This is a very practical method of tissue chemical analysis because it can be performed on living specimens and “ex vivo” [Ionita I, 2009].

Based on the results of the Raman study, it is predicted that teeth contain inorganic (PO_4 and CO_3) in the wave number range of 400 and 1100 cm^{-1} [Gamulin O et al., 2021]. The data obtained in this study which are in the wave number range are 853 cm^{-1} (CCH) aromatic and (CC) proline; 876 cm^{-1} (CC), (CO_3) hydroxypoline; 960 cm^{-1} (PO_4) symmetric stretching.

Based on the spectral data produced in this study, the (PO_4) symmetric stretching molecule (960 cm^{-1}) is the strongest signal as in figures 1 and 2, which will be used in this study to analyze the differences between male and female gender. This is supported

TABLE 2
Results of Confusion Matrix Analysis from Confocal Raman Microscope in male and female gender

Analysis	Confusion Matrix			Confusion Matrix			Confusion Matrix			Confusion Matrix		
	TRUE	M	F	TRUE	M	F	TRUE	M	F	TRUE	M	F
Raman Enamel	M	0	2	M	0	2	M	1	1	M	2	0
	F	1	1	F	0	2	F	0	2	F	2	0
Raman Dentin	M	0	2	M	0	2	M	0	2	M	0	2
	F	1	1	F	0	2	F	1	1	F	0	2
Raman Pulp	M	0	2	M	0	2	M	0	2	M	0	2
	F	0	2	F	0	2	F	2	0	F	2	0

by the results of research conducted by Banjsak L. et al. (2023), showing that the average Raman spectrum of male and female teeth shows the main differences at wave numbers 964 cm^{-1} (PO_4), 1006 cm^{-1} (PH_3), 1453 cm^{-1} (CH_2). However, there is no clear characteristic of these types of molecules as a differentiator between male and female gender, so it is necessary to conduct a non-parametric statistical test of differences as seen in table 3. The results of the analysis show that the enamel, dentin and pulp surfaces of men and women do not have significant differences with a p value. 0.605 ± 0.271 , which is significantly greater than $p > 0.05$.

Based on the signal fragments on the enamel, dentin and pulp surfaces, there is a significant difference with a p value of 0.001, which is smaller than $p < 0.05$. This indicates a significant difference between male and female gender based on full width at half maximum. In accordance with the research of Greco et al (2023), the wave number 960 cm^{-1} shows the main spectroscopic parameters that can be obtained from dentin samples. Based on the signal fragment (full width at half maxi-

TABLE 3
Analysis of differences in non-parametric Kruskal Wallis test

Group	Comparison	
	Layer	Fragment signal
Chi Square	Male – Female	Male - Female
Asymp. Sig	1.005 ± 2.610	99.568 ± 90.518
P	0.605 ± 0.271	0.001
	> 0.05	< 0.05

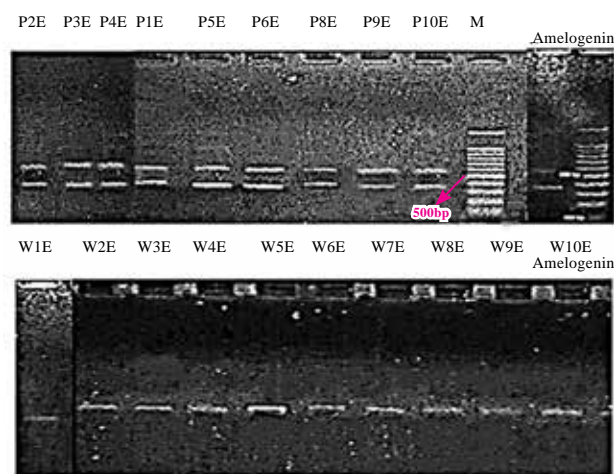


Figure 4. The result of Amelogenin Gen analysis of male and female gender use dental sample (Enamel)

mum) of the band located at around 960 cm^{-1} , and associated with the ν_1 vibration of PO_3^{4-} , it can be used to estimate the crystallinity of the hydroxyapatite phosphate group.

To find out which areas are significant in the analysis of gender on the enamel, dentin, and pulp surfaces, this study was continued using chemometrics with the support vector machine and artificial neural network algorithms to classify male and female gender [Mohammad N et al., 2022]. For the purposes of support vector machine and artificial neural network analysis, where the wavelength and wave number data are limited to a certain Raman range ($920 - 1080\text{ cm}^{-1}$). This limitation is based on the content of the main elements and main molecules owned by teeth called host elements [Birzhandi P et al., 2022]. Apart from that, this limitation is also intended to reduce over-fitting when classifying with the chemometric algorithm [Trisnawati N et al., 2019].

Before being analyzed with support vector machine and artificial neural network, the data underwent initial data processing (preprocessing) in order to eliminate variables that were very different from the average or to reduce/reduce the data dimension (data sets with large numbers into simpler data sets) when classifying [Yang H et al., 2015]. Initial data processing used principal component analysis and restricted Boltzmann machines in each section (enamel, dentin, and pulp) and the results are as in figure 3.

Figure 3 shows the distribution of the initial Raman data (original) and the results of the transformation with principal component analysis and restricted Boltzmann machines in the enamel, dentin, and pulp sections. In the enamel and dentin sections, the distribution of principal component analysis results is better than the distribution of the initial data, while in the pulp section, the principal component analysis distribution is still mixed between gender and women like the initial distribution and data groups have not yet formed. The results of the restricted Boltzmann machines transformation in the enamel and pulp sections show that data groups are formed for each gender. However, for the dentin section, the data distribution tends to form a linear line with the data still mixed. The results of this study show that the support vector machine restricted Boltzmann machines value on the enamel surface has a good accuracy of 75%, this result is different from the research conducted by Gamulin O. et al. (2021) and Banjsak L. et al. (2023) which showed that the dentin surface is more accurate with a value of 70-75%.

The results of this study were confirmed by DNA analysis of the amelogenin gene, showing the suitability of samples on the enamel surface of men and women with the amelogenin gene marker $X = 542\text{ bp}$ and $Y = 358\text{ bp}$ with the Elmrghni S. and Kaddura M. method (2019). In this study, the polymerase chain reaction results showed 2 bands which meant male gender and 1 band which meant female gender. In the polymerase chain reaction analysis of amelogenin, if the male control DNA produced two clear peaks, while in the female control DNA, there was only one peak [Ahmad A, Khan S, 2022]. Basically, amelogenin is the main protein component found in the enamel matrix and is involved in amelogenesis, which is the development of enamel. Amelogenin is a type of extracellular matrix protein that, together with ameloblastin, enamelin, and tuftelin, directs enamel mineralization to form a highly organized matrix of rods, interstem crystals, and proteins [Bansal A et al., 2012; Habelitz S, Bai Y, 2021]. The amelogenin system is very important in forensic science, especially in the context of genetic sex determination [Cardoso I et al., 2024].

CONCLUSION

Sex determination can be done using a Confocal Raman Microscope with 75% accuracy on support vector machine and artificial neural network

on the surface of enamel and dentin. All samples showing 100% accuracy or significance with gold standard analysis use DNA Amelogenin Gene either enamel, dentin, or pulp area.

ACKNOWLEDGEMENT: The authors thank to Prof Junitha, Prof Hery, Prof Pharma and Prof Yudi, for their kind contribution to this project. Institute of Tropical Disease for helping me to do research there.

FUNDING: This research was funded by Research Udayana University, No: B/255.569/UN14.4.A/PT.01.03/2024.

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