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Spectrofluorometric determination of L-tryptophan in canary (*Canarium indicum* L.) seed protein hydrolysate

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ABSTRACT

Canary (*Canarium indicum* L.) is an indigenous plant of Indonesia, which mainly grows in the eastern part of Indonesia, especially in the Maluku, North Sulawesi, and Seram islands. We believe that no scientific reports have been conducted about L-tryptophan content in *Canarium indicum*. Therefore, this study was conducted to determine the presence and quantitate the aromatic amino acid (L-tryptophan) in the canary protein hydrolysate by the spectrofluorometric method. The protein hydrolysate was prepared by two hydrolysis methods, enzymatic and alkaline hydrolysis. L-tryptophan can be differentiated from tyrosine directly without using any reagent by excitation of the sample at 295 nm in order to avoid tyrosine emission. The equation of calibration curve correlation using standard in the range 0.5-5 ppm was y = 6632.3x - 845.42 and correlation coefficient of 0.9997, while the coefficient of variance in linear regression was 1.29%. The detection limit and quantification limit obtained were 0.116 ppm and 0.35 ppm respectively. The recoveries of the accuracy test were obtained in the range of 95-96%. The relative standard deviation of intra-assay precision tests were obtained in the range of 0.5-1.8%, while the intermediate precision in the range of 2.18-3.74%. L-tryptophan was detected in all samples (papain, pepsin, and alkaline hydrolysate), with concentrations 5.6, 5 and 1.53 mg/100mg of protein respectively. The used fluorometric method complied with the validation requirements and can be used to analyze L-tryptophan in samples containing tyrosine without overlapping of spectra and without the use of any specific reagent.

Keywords: *Canarium indicum; analysis; L-tryptophan; spectrofluorometric.*

1. INTRODUCTION

Canary (*Canarium indicum* L.) belongs to the family of Burseraceae, this family consists of 18 genera and around 700 species are tropical plants [1]. It is an indigenous plant in Indonesia, which mainly grows in the eastern part of Indonesia, especially in Makian Island, North Maluku Province. Canary is an oil seed, it contains lipid in high amount (66.26%) and the second largest composition is protein (13.69%).

Protein Hydrolysate is a product of protein hydrolysis using enzymes, acids and also alkalines. Hydrolysis of proteins causes the breakdown of peptide bonds (C-N) in proteins so that peptides are produced in various sizes up to amino acids. Protein hydrolysate is currently used as a supplement to increase muscle mass, and also as a support therapy for the recovery of disease [2-4]. To evaluate the quality of protein hydrolysate, a valid and robust method is needed. Since amino acids the constituents of proteins, so it can be used as a benchmark to check the content of hydrolysate protein.

Canary proteins are composed of fifteen amino acids, including eight non-essential amino acids and seven essential amino acids, namely methionine, lysine, leucine, isoleucine, threonine, phenylalanine, and valine [5]. Research on L-tryptophan content determination in *Canarium indicum* has not been conducted. L-tryptophan (Trp) is an essential aromatic amino acid for humans because it cannot be synthesized inside the human body and must be consumed in Trp-rich diet. L-tryptophan is vitally important because of its variety of biological functions. It is the precursor of the neurotransmitter serotonin, which regulates the appetite, sleep, and mood [6].

The spectrofluorometric analysis method is used in this research to detect and measure L-tryptophanin the canary protein hydrolysate. Fluorescence detection is fast and more sensitive and has fewer interfering compounds than those of ultraviolet detection (UV). In addition, L-tryptophan and tyrosine have different emission wavelength, so L-tryptophan can be differentiated from tyrosine directly without using any reagent by excitation of the sample at 295 nm [7].

Therefore, we aim to identify and determine the aromatic amino acid (L-tryptophan) content in canary protein hydrolysate by the spectrofluorometric method. The protein hydrolysate was prepared by two hydrolysis methods, enzymatic and alkaline hydrolysis method using the purple fruit (adult fruit) as a starting material.

2. MATERIALS AND METHODS

2.1. Materials.

Canary (*Canarium indicum* L) consist of purple (mature) ages 8-12 months originating from Makian Island, North Maluku, L-tryptophan (Sigma-Aldrich), Tyrosine (Sigma-Aldrich), distilled water, phosphate buffer 0.01M, Papain (Merck) and Pepsin (Merck), Bovine serum albumin (BSA) (Sigma-Aldrich), Bradford protein assay reagent (Sigma-Aldrich).

2.2. Defatted canary seed preparation.

The Canary (Nut in Testa) was peeled with hot water for 5-10 minutes in order to remove the Testa. After that, the canary seed was collected and pressed by mechanical pressing to remove the oil and the defatted canary seed was collected for further treatment.

2.3. Enzymatic hydrolysis of Canary seed protein.

Enzymatic hydrolysis of canary seeds consists of two steps, canary seed protein extraction, and hydrolysis step. For protein extraction step, the sample (defatted Canary seed) were weighed and then homogenized with 0.9% NaCl solution using a mortar to produce extracts with a concentration of 30% (w/v). Next, the homogenate was filtered using filter cloth and centrifuged at 12.000 rpm for 20 minutes, and then the supernatant was collected and adjusted the pH to reach the optimum pH of the protease enzyme (Table 1.).

While the hydrolysis step was carried out after conditioning of the supernatant by heating the sample to reach the optimum condition of the enzyme for 30 minutes, then mixing between supernatant and enzyme was done. Protein hydrolysis was carried out for 1 hour, and agitation at 300 rpm with a magnetic stirrer. After hydrolysis was completed, the enzyme was deactivated by heating the sample at ± 100ºC for 15 minutes. The protein hydrolysate was cooled to room temperature, centrifuged at 4ºC at a speed of 12,000 rpm for 20 minutes and the supernatant obtained was collected.

Table 1. Enzymatic hydrolysis condition

Enzyme	Optimum temperature	Optimum pH	Enzyme concentration	Hydrolysis time
Papain	55ºC	7	1 % (w/w_{raw})	1 hour
Pepsin	37ºC	2	1 % (w/w_{raw})	1 hour

2.4. Rapid alkaline hydrolysis of Canary seed protein.

Samples (defatted Canary seed) were weighed and then homogenized with 4 N sodium hydroxide solution using a mortar to produce extracts with a concentration of 30% (w/v). Next, the homogenate was filtered using filter cloth and centrifuged at 12.000 rpm for 20 minutes, and then the supernatant was collected.

The supernatant was sealed in hydrolysis tubes and incubated in an oven at 100ºC for 4 h. Hydrolysates were cooled down on the ice, neutralized to pH 7 using 6 N HCl to stop hydrolysis process. The protein hydrolysate was centrifuged at 10.000 rpm for 5 minutes and then the supernatant was collected and kept at a temperature below 4°C for further analysis.

2.5. Determination of protein content in the canary hydrolysate.

The determination of protein was conducted according to the method described by Bradford (1976) [8]. The measurements, 30µL samples and 1.5 mL Bradford solution were mixed and incubated for 10 min. A standard curve was made of BSA (0, 25, 125, 250, 500, 750, and 1000 µg/mL) and absorbance was read at 595 nm. The standard curve was prepared by plotting the average Blank-corrected 595 nm measurement for each BSA standard vs. its concentration in µg/mL and it was used to determine the protein concentration of each unknown sample.

2.6. Method Validation.

Validation of an analytical method is conducted to ensure that the performance characteristics have met the requirements for its intended purpose [9]. The validation process in this study includes the following parameters, linearity, specificity, accuracy, repeatability (intra-assay precision), Intermediate precision, detection limit, and quantification limits

2.6.1. Linearity.

The linearity assessed whether an analytical method generate proportional response to to the changes in analyte concentration. Both correlation coefficient (R) and Vxo is calculated from the calibration curve.

A stock solution of 100 ppm L-tryptophan 100 was prepared. Then, a replicate series of solutions consisting of six concentrations, namely 0.5, 1, 2, 3, 4, and 5 ppm of L-tryptophan that were made in phosphate buffer solution. The emission spectrum of each concentration was measured for intensity at an excitation wavelength of 295 nm.

The calibration curve was made by subtracting the fluorescence intensity of phosphate buffer from the fluorescence intensity of L-tryptophan solution. The difference in emission intensity of each concentration plotted in the calibration curve with the y-axis is the emission intensity and the x-axis is the concentration of L-tryptophan solution. The measurement of each standard solution concentration was carried out three times.

2.6.2. Specificity testing.

Specificity assessed whether an analytical method is able to generate different responses between analyte and matrix in the sample. Both L-tryptophan and tyrosine are excited at wavelength 280 nm with two overlapping spectra, which lead to inaccurately measuring of both L-tryptophan and tyrosine. Therefore, fluorescence intensity at a wavelength of 300-450 nm was measured. L-tryptophan solution and the sample was excited at 295 nm and 280 nm and compared to each other.

2.6.3. Accuracy testing.

Accuracy testing was carried out by standard additions method, the sample is analysed and a known amount of analyte was added to the sample and then analyzed again. ie each sample was added with standard L-tryptophan solution until the concentration became 80%, 100%, and 120% and the difference between the two measurements is calculated to get percent recovery.

2.6.4. Precision testing.

Precision was evaluated using repeatability, intermediate precision, and reproducibility. It is usually assessed by calculating relative standard deviation (% RSD).

a. Repeatability (intra-assay precision)

Repeatability (intra-assay precision) is evaluates precision under identical operating conditions within short time interval. It is conducted by determination of 100% test concentration for six times. Measurements are made on the same day but at three different times.

b. Intermediate precision

Intermediate precision evaluated the effect of different days, analysts and equipment on analyte measurement. It is carried out by making a protein hydrolysate solution with the addition of L-tryptophan 80, 100 and 120%. Each concentration was replicated three times for one measurement, and then each measurement was done on three different days.

2.6.5. Limit of detection and Limit of quantification testing.

Detection limits (LOD) and quantification limits (LOQ) are

determined from the calibration curve that has been made. The limit of detection and the limit of quantification can be determined by the following formula:

$$LOD = \frac{10\,\sigma}{S}\ ;\ LOQ = \frac{3.3\,\sigma}{S}$$

σ= standard deviation of analytical response

S= slope of the calibration curve

2.7. Determination of L-tryptophan in the canary protein hydrolysate.

2.7.1. 3D spectrofluorometric analysis.

For determination of L-tryptophan in canary protein hydrolysate by 3D spectrofluorometric analysis, 50 µl of protein hydrolysate was diluted in 5 ml phosphate buffer and the measurement was adjusted on 250 nm - 350 nm for excitation and from 250 nm to 500 nm for emission.

2.7.2. 2D spectrofluorometric analysis.

For 2D analysis, 150 µl of protein hydrolysate was diluted in 50 ml phosphate buffer and the measurement was adjusted on 300 nm - 450 nm for emission with excitation at 295nm.

3. RESULTS

3.1. Determination of protein content in the canary hydrolysate.

The protein content in papain, pepsin, and alkaline protein hydrolysate was determined by using Bradford assay. This method was employed due to the simplicity and applicability of food sample. In this method, protein will bind with dye (Coomassie blue), causing a shift in absorption [10]. Determination of protein content in the samples was done by calculating the equation obtained from the standard protein curve. Bovine serum albumin (BSA) was used as standard protein to make a standard curve, within series of concentration from 25 µg/ml BSA concentrations up to 1000 µg/ml. This protein is serum albumin, constructed by 583 amino acids constituents and weighed around 66 kDa. BSA is widely chosen as standard protein due to economic reasons [11]. The standard protein calibration curve is shown in Figure 1.

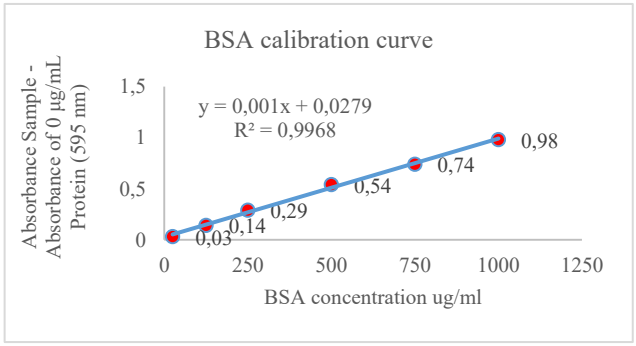


Figure 1. Bovine Serum Albumin (BSA) Calibration Curve

The samples were diluted by a dilution factor (300), two replicates of each sample were made, and three measurements of each sample have been done. Protein content in each sample is calculated from the equation (y = 0.001x + 0.0279). The analysis results of each sample are shown in Table 2. From this result, papain hydrolysate contained a smaller amount of protein compared to pepsin and alkaline hydrolysate. Reports found that strong hydrolyzing agent does not necessarily generate shorter peptide with desired bioactivity [12].

Table 2. The protein content of protein hydrolysate of defatted canary seed

Sample	Average absorbance	Protein content (mg/ml)
Papain protein hydrolysate	0.056	8.60 ± 0.62
Pepsin protein hydrolysate	0.069	12.56 ± 0.54
Alkaline protein hydrolysate	0.08	15.63 ± 0.73

3.2. Method Validation.

The validation process in this study includes the following parameters, linearity, specificity, accuracy, repeatability (intra-assay precision), Intermediate precision, detection limit, and quantification limits. These parameters are required to validate quantitative analytical method for the determination of drug components in the raw material and pharmaceutical preparations [9].

3.2.1. Linearity.

A replicate of series of solutions consisting of six concentrations, namely 0.5, 1, 2, 3, 4, and 5 ppm of L-tryptophan that were made in phosphate buffer solution. The emission spectrum of each concentration was measured for intensity at an excitation wavelength of 295 nm. There is an increase in the intensity of fluorescence as the concentration of L-tryptophan increased (Figure 2).

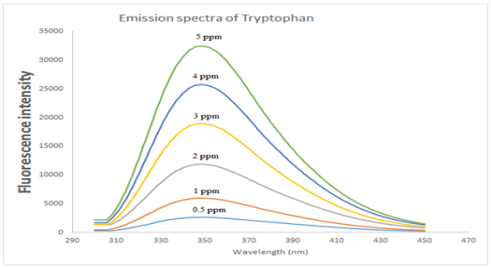


Figure 2. Emission spectra of L-tryptophan

A calibration curve then made by subtracting the fluorescence intensity of phosphate buffer from the fluorescence intensity of L-tryptophan solution. The difference in emission intensity of each concentration plotted in the calibration curve with the y-axis is the emission intensity and the x-axis is the concentration of L-tryptophan solution. The measurement of each standard solution concentration was carried out three times. From the calibration curve, the value of the correlation coefficient (r) is 0, 9997 with Vxo of 1.29% (Figure 3). Both of these values indicate that the calibration curve of L-tryptophan 0.5-5 ppm has good linearity [13].

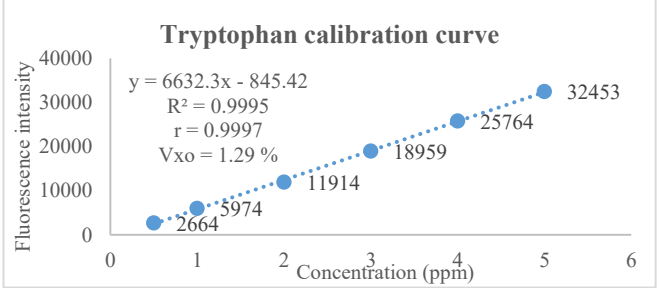


Figure 3. L-tryptophan calibration curve

3.2.2. Specificity testing.

As it is well known, when both L-tryptophan and tyrosine are excited at wavelength 280 nm, two overlapping spectra which may lead to inaccurate measurement of both L-tryptophan and tyrosine were generated. Therefore, L-tryptophan was excited at 295 nm in order to avoid tyrosine fluorescence [7, 14], and the intensity for fluorescence radiation was measured at a wavelength of 300-450, with maximum fluorescence at around 349-350 nm as shown at Figure 4.

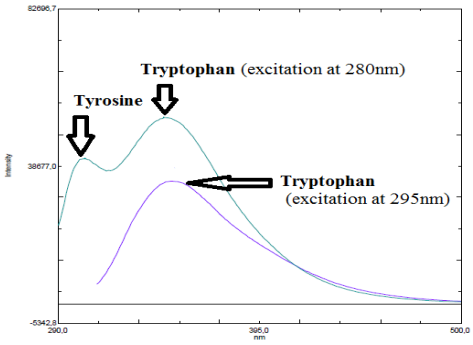


Figure 4. L-tryptophan emission after excitation at 280 and 295 nm.

3.2.3. Accuracy testing.

Accuracy of an analysis procedure illustrates the closeness of the value obtained from the method used with the actual value. Accuracy testing in this study was carried out by the standard addition method.

The standard addition method is used if a placebo is not available because the matrix is unknown. The difference between the two measurements is calculated to get a percent recovery [15]. The accuracy data of the samples can be seen in Table 3.

Table 3. Accuracy data of the protein hydrolysate

Sample	Concentration	Average	Recovery (%)
protein hydrolysate	80	20250 ± 130.07	95
	100	22183 ± 98.81	95
	120	25549 ± 118.83	96

3.2.4. Precision testing.

Precision evaluate the degree of scattering among a series of measurements of homogeneous sample under identical conditions.

a. Repeatability (intra-assay precision)

Repeatability, (intra-assay precision) result of this method is shown in (Table 4). Measurements are made on the same day but at three different times [16].

Table 4. Intra-assay precision data of the protein hydrolysate

Sample	No.	Average	Recovery (%)	RSD (%)
Protein hydrolysate	I	18775 ± 186.76	95	1
	II	17635 ± 89.66	91	0.5
	III	18209 ± 335.36	89	1.8

b. Intermediate precision

Intermediate precision of this method was carried out by making a protein hydrolysate solution with the addition of L-tryptophan 80, 100 and 120%. Each concentration was replicated three times for one measurement, and then each measurement was done on three different days (Table 5).

Table 5. Intermediate precision data of the protein hydrolysate

Sample	Concentration (%)	Average	Recovery (%)	RSD (%)
Protein hydrolysate	80	17049 ± 637.19	90	3.74
	100	19519 ± 437.15	94	2.24
	120	22954 ± 500.90	95	2.18

The acceptable level of RSD is ≤ 2%. However, this level is very flexible depending on the concentration of the analyte being examined, a number of samples, and the laboratory conditions. Relative standard deviation (RSD) increases with the decreasing level of analyte analyzed. At 1% or more, the relative standard deviation between laboratories is around 2.5% at one per thousand is 5%. On one level per million (ppm) RSD is 16%, and at the level of parts per billion (ppb) is 32% [16]. Therefore, repeatability and intermediate precision testing of this method fulfill the precision acceptance criteria (% RSD that does not exceed 16%).

3.2.5. Limit of detection and Limit of quantification testing.

The detection limit indicates the smallest amount of analyte which can be detected by an analytical method. Quantitation limit indicates smallest amount of analyte which can be determined precisely and accurately [9]. The detection limit and quantification limit are obtained from the calibration curve by statistical calculations (Table 6).

Table 6. Limit of detection and limit of quantification

Parameters	Concentration (ppm)
LOD	0.116 ppm
LOQ	0.350 ppm

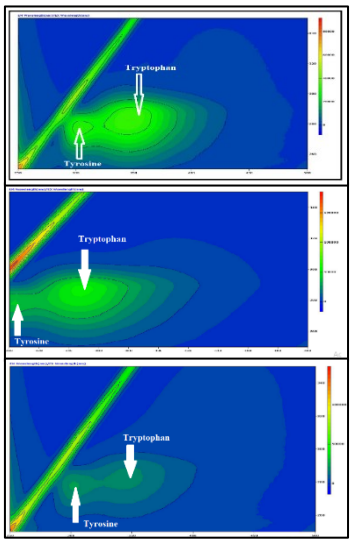
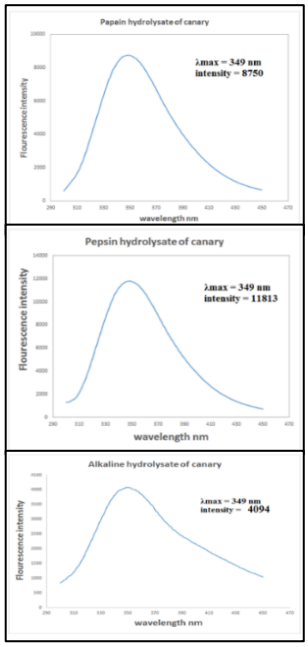


Figure 5. Excitation-emission matrix of protein hydrolysate in phosphate buffer (50 µL/5 mL) of (a) Papain protein hydrolysate, (b) Pepsin protein hydrolysate and (c) Alkaline protein hydrolysate

From the values obtained, this method has a detection limit and the quantification limit is quite small and still has a value that is below the smallest concentration of the calibration curve.



a.

b.

c.

Figure 5. 2D spectra of protein hydrolysate in phosphate buffer (150 µL/50 mL) of (a) Papain protein hydrolysate, (b) Pepsin protein hydrolysate and (c) Alkaline protein hydrolysate

3.3. Determination of L-tryptophan in the canary protein hydrolysate.

3.3.1. 3D spectrofluorometric analysis.

For determination of L-tryptophan in pepsin protein hydrolysate by 3D spectrofluorometric analysis, 50 µl of protein hydrolysate was diluted in 5 ml phosphate buffer and the measurement was adjusted on 250 nm - 350 nm for excitation and from 300-500 nm for emission as below (Figure 5). The fluorescence of L-tryptophan is affected by the environment of sample as well as protein type and conformation [17]. L-tryptophan fluorescence maxima at around 350 nm indicated that protein was dissolved in polar solvent, in this case, PBS. In nonpolar environment, the maxima will shift to a shorter wavelength (around 300 nm) [18].

3.3.2. 2D spectrofluorometric analysis.

For 2D spectrofluorometric analysis of tryptophan in pepsin protein hydrolysate, 150 µl of protein hydrolysate was diluted in 50 ml phosphate buffer and the measurement was adjusted on 300 nm - 450 nm for emission with excitation at 295 nm (Figure 6).

As shown in Table 7, L-tryptophan content decreased the most during hydrolysis using alkaline reagent, followed by pepsin and papain enzymes. This phenomenon occurred due to the non-specificity of alkaline reagent in breaking peptide bonds. Meanwhile, papain and pepsin broke peptide bond at specific sites.

Table 7. L-tryptophan content from different protein hydrolysates per 100 mg of total protein and 100ml of the protein hydrolysate

Sample	Trp content per 100 mg of total protein (mg/mg)	Trp content per 100 ml of protein hydrolysate (mg/ml)
Papain protein hydrolysate	5.6 ± 0.16	48.2 ± 1.4
Pepsin protein hydrolysate	5 ± 0.02	63.6 ± 0.3
Alkaline protein hydrolysate	1.53 ± 0.002	24 ± 0.04

4. CONCLUSIONS

The used spectrofluorometric method i.e. excitation of the samples at 295 nm, is suitable for the determination of L-tryptophan in a sample containing both L-tryptophan and tyrosine. The detection limit and quantification limit obtained with this method were 0.116 ppm and 0.35 ppm respectively. The recoveries of the accuracy test are in the range of 95-96%. The relative standard deviation of intra-assay precision tests was 1.1 ± 0.65, while the intermediate precision was 2.72 ±0 .88. L-tryptophan was detected in all samples (papain, pepsin, and alkaline hydrolysate), with concentrations of 5.6, 5 and 1.53 mg/100 mg of protein respectively.

5. REFERENCES

1. Mogana, R.; Wiart, C. Canarium L. A phytochemical and pharmacological review. *J. Pharm. Res.* **2011**, *4*, 2482-2489.
2. McCarthy, A.L.; O'Callaghan, Y.C.; O'Brien, N.M. Protein Hydrolysates from Agricultural Crops—Bioactivity and Potential for Functional Food Development. *Agriculture* **2013**, *3,* 112-130, https://doi.org/10.3390/agriculture3010112.
3. Aluko, R.E. Food protein-derived peptides: Production, isolation, and purification. In Proteins in Food Processing, 2nd ed.; Yada R.Y., Eds.; Woodhead Publishing: Cambridge, UK, 2018; pp. 389-412, https://doi.org/10.1016/B978-0-08-100722-8.00016-4.
4. Tapal, A.; Tiku, P.K. Nutritional and Nutraceutical Improvement by Enzymatic Modification of Food Proteins. In Enzyme Food Biotech.; Kuddus M., Eds.; Academic Press: Cambridge, US, 2019; pp. 471-481, https://doi.org/10.1016/C2016-0-04555-2.
5. Djarkasi, D.S.S.; Lalujan, L.; Nurali, E.J.N.; Tuju, T.J.D.; Rawung, D.; Sumual, M.F. 2017. Chemical Composition and Antioxidant Properties of Kenari (Canarium indicum) Nut. *Phar. and Chem. J.* **2017**, *4,* 79-84.
6. Jenkins, T.A.; Nguyen, J.C.D.; Polglaze K.E.; Bertrand P.P. Influence of tryptophan and serotonin on mood and cognition with a possible role of the gut-brain axis. *Nutrients* **2016,** *8*, 56, https://doi.org/10.3390/nu8010056.
7. Lakowicz, J.R. *Principles of fluorescence spectroscopy.* 3rd ed.; Springer: New York, USA, 2006.
8. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **1976**, *72*, 248-254, https://doi.org/10.1006/abio.1976.9999.
9. International Council for Harmonisation. ICH Harmonized Tripartite Guideline Validation of Analytical Procedures. 2005.
10. Rabolloud, T. Optimization of the cydex blue assay: A onestep colorimetric protein assay using cyclodextrins and compatible with detergents and reducers. *Plos One* **2018**, *13,* e0195755, https://doi.org/10.1371/journal.pone.0195755.
11. Sun, Y.; Du, P.; Lu, X; Xie, P; Qian, Z; Fan, S; Zhu Z. Quantitative characterization of bovine serum albumin thin-films using terahertz spectroscopy and machine learning methods.

Biomed. Optics Express **2018,** *9,* 2917-2929, https://doi.org/10.1364/BOE.9.002917.
12. Shu, G.; Huang, J.; Bao, C.; Meng, J.; Chen, H.; Cao, J. Effect of Different Proteases on the Degree of Hydrolysis and Angiotensin I-Converting Enzyme-Inhibitory Activity in Goat and Cow Milk. *Biomolecules* **2018,** *8* 101, https://doi.org/10.3390/biom8040101.
13. Armstrong, R.A. Should Pearson's correlation coefficient be avoided? *Ophthalmic and Phys. Optics* **2019**, *39,* 316-327, https://doi.org/10.1111/opo.12636.
14. Yammine, A.; Gao, J.; Kwan, A.H. Tryptophan Fluorescence Quenching Assays for Measuring Protein-ligand Binding Affinities: Principles and a Practical Guide. *Scientific Reports* **2019**, *9,* e3253, https://doi.org/10.21769/BioProtoc.3253.
15. Association of Official Analytical Chemist. Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals. *J. AOAC Int.* **2002**, *16*, 1-38.
16. Peris-Vicente, J.; Esteve-Romero, J.; Carda-Broch, S. Validation of Analytical Methods Based on Chromatographic Techniques: An Overview, In*: Analytical Separation Science*. edited by Anderson, J.; Alain, B.; Veronixa, P.; Apryll, S. Wiley-VCH, Weinheim, 2015; pp. 1757-1808.
17. Gambucci, M.; Gentili, P.G.; Sassi, P.; Latterini, L. A multi-spectroscopic approach to investigate the interactions between Gramicidin A and silver nanoparticles. *Soft Matter* 2019, *32,* 6571-6580, https://doi.org/10.1039/C9SM01110B.
18. Bortolotti, A.; Wong, Y.H.; Korsholm, S.S.; Bahring, N.H.B.; Bobone, S.; Tayyab, S.; Van De Weert, M.; Stella, L. On the purported "backbone fluorescence" in protein three-dimensional fluorescence spectra. *RSC Advances* **2016**, *6,* 112870-112876, https://doi.org/10.1039/c6ra23426g.

6. ACKNOWLEDGEMENTS

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