RESEARCH ARTICLE

Solubility Profiling and UV-Spectrophotometric Determination of Bioactive Peptides from Ragi

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ABSTRACT

Objective: The primary aim was to determine the solubility of isolated bioactive peptides (BPs) from Ragi (*Eleusine coracana L.*) A review of the literature revealed that there is no UV-visible method to measure bioactive peptides is developed yet. Therefore, it is necessary to design and authenticate an accurate, simple, exact, durable and economical UV visible spectrophotometric method of the quantification of bioactive peptides derived from Ragi.

Method: The solubility of bioactive peptides derived from Ragi was evaluated in DMSO, methanol, and buffers at pH levels of 1.2, 4.5 (Acetate and phosphate), 6.8, 7.4 and 10, using the saturated solubility determination technique. A standard solution was prepared using the selected solvent, and the maximum absorbance wavelength (λmax) was identified. Subsequently, a calibration curve was constructed based on absorbance measurements. The method's analytical performance was assessed by evaluating parameters including linearity, range, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), and robustness, in accordance with the International Conference on Harmonisation (ICH) Q2 (R2) guidelines.

Result: Bioactive peptides isolated from *Eleusine coracana* (ragi) exhibited highest solubility in phosphate buffer at pH 7.4, followed by buffers at pH 6.8, pH 10, and organic solvents such as dimethyl sulfoxide (DMSO) and methanol. The peptides showed maximum absorbance at a wavelength of 379 nm. A linear response consistent with the Beer-Lambert law was observed over the concentration range of 100–1000 µg/ml, yielding a correlation coefficient (R^2) of 0.993. The method demonstrated a limit of detection (LOD) of 2.89 µg/ml and a limit of quantification (LOQ) of 8.76 µg/ml. Precision and repeatability assessments were within acceptable regulatory thresholds, with relative standard deviation (RSD) values below 2%. Recovery studies indicated an accuracy range between 98.23% and 100.03%.

Conclusion: The study concluded that the isolated BPs demonstrate diverse solubility in various solvents. The established process was accurate, exact, reproducible, easy, ecologically sustainable, repeatable, and cost-effective, rendering it appropriate for quantifying BPs in pharmaceutical dosage forms.

Keywords: Bioactive peptides isolated from Ragi, UV visible spectrophotometry, Method development and Validation, *Eleusine coracana L*

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INTRODUCTION

Bioactive peptides (BPs), short chains of amino acids derived from dietary proteins, are known for a broader range of biological capabilities, including antioxidant, angiotensin-converting enzyme (ACE) inhibition, antibacterial, and anti-inflammatory activity. Finger millet (*Eleusine coracana* L.), widely known as ragi, has garnered considerable interest among other plant sources owing to its elevated protein content and health advantages. These peptides, particularly those generated during germination or enzymatic proteolysis, exhibit considerable bioactivity due to "active" amino acid sequences[1].

Successful application of these bioactive peptides in nutraceuticals or therapeutic contexts necessitates a comprehensive understanding of their solubility characteristics in many solvents and buffer systems, which is intrinsically linked to their bioavailability, stability, and formulation potential. The solubility determination is essential for advancing delivery innovations in oral, topical, or injectable formulations and for selecting an appropriate solvent solution for conducting accurate analytical assays. The outcome is contingent upon the peptide's structural attributes, such as hydrophobicity, charge, sequence length, and environmental factors like pH and ionic strength[2].

Standardization and quality control of these peptides and their associated analytical procedures are crucial. The simplicity, cost-effectiveness, and appropriateness for regular analysis render UV-spectrophotometry a universally applicable technology. Method development must consider the peptide's λ max, linearity, precision, accuracy, and specificity to provide consistent quantification across various matrices[3].

While extracts and peptides from Ragi have been partially extracted and structurally described, there is less prior

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Table 1: Method Development Study

Method	Important Parameters	Considerations and reference
Stock Solution	100 mg of isolated BPs in 100 ml of solvent	Prepared using volumetric flask to make 1000 ppm.
Selection of wavelength (λ max)	Analyzed in the range of 800 - 200 nm	Using double beam UV-visible spectrophotometer
Calibration Curve	Dilutions from 100 – 1000 µg/ml; Recording absorbance at λ max	A graph was plotted with concentration on the X-axis along with absorbance on the Y-axis, resulting in the derivation of a linear equation[8,9].

Table 2: Method Validation parameters

Parameter	Reason to perform	Performance parameters
Specificity and selectivity	Remove solvent absorbance (noise) from solution and plot overlay spectrum.	Blank scan from 800 – 200 nm, followed by different conc. of sample, to get an overlay spectrum[8].
Linearity	To confirm that the analytical method produces results those are precisely proportional to the sample's concentration.	Preparation of dilutions; determination of absorbance at λ max; plot graph with linear equation[8-10].
Limit of Detection and Quantification (LOD & LOQ)	To calculate the minimal concentration of analyte that can be consistently detected (LOD) and quantified (LOQ).	Calculate SD(σ) and slope (s) from the linearity data and calculate LOD and LOQ using the mentioned formula[11-13].
Precision	To access consistency and reproducibility of analytical method under same conditions	Three conc. i.e. 100, 500, and 1000 µg/ml were prepared; In intraday study absorbance was taken at 3 different times in a single day; in interday study absorbance was taken at 3 consecutive days; response was quantified by calculation %RSD across tested conc. levels [10,14,15].
Accuracy	To determine how accurate the method is by determining how much of the analyte can be recovered.	Addition of known amounts spiked into the sample at three levels (i.e. 80%, 100% and 120%); standardization process was used to analyze the solutions and calculate % recovery from each sample[15,16].
Ruggedness	To analyze reproducibility of the analytical condition under varied conditions such as different analyst	Three conc. i.e. 100, 500, and 1000 µg/ml were prepared; two different analysts tested samples separately; % RSD was calculated to demonstrate repeatability of the method[15,17].
Robustness	To access reliability of the analytical method then small, deliberate changes to the analytical parameters are made	Three conc. i.e. 100, 500, and 1000 µg/ml were prepared; deviation in the absorption wavelength was made (± 5 nm); absorbance was measured and reliability was determined based on % RSD calculations[15,18].

knowledge concerning their relative solubility in various physiologically and pharmaceutically applicable solutions. Furthermore, there is no validated UV-spectrophotometric method for estimating these peptides. Addressing this gap will significantly enhance the utilization of Ragi-based bioactive peptides in functional foods and therapeutic applications.

This study evaluates the solubility of bioactive peptides extracted from Ragi across several solvents and buffers, employing a proven UV-spectrophotometric method for precise measurement. This synergistic method establishes a foundation for preparing Ragi-peptide-based products, enhancing delivery and ensuring quality assurance.

MATERIALS AND METHODS

Materials

Chemicals and Reagents

Bioactive peptides were extracted from dried seeds of Ragi (*Eleusine coracana*), procured from from M.S. Dagdu Teli Chandwadkar Trading Company, Nashik, Maharashtra, India, and taxonomically authenticated by the Botanical Survey of India, Pune (Department of Botany), under verification report number BSI/WRC/100-1/Tech./2020 according to the procedure demonstrated by Fernández - Quintela, et. al[4]. Buffers in the range of 1.2 to 10 were prepared as per the procedures mentioned in the Indian Pharmacopoeia 2018[5]. Dimethyl Sulphoxide and methanol were purchased and procured from S D Fine-Chem Limited, Mumbai, Maharashtra, India. All the chemicals utilized were of analytical grade.

Instrument

Spectral measurements were carried out using a Shimadzu UV-1800 double-beam spectrophotometer integrated with UV Probe software.

Methods

Saturated Solubility Study

The study is carried as per the process mentioned in the fig. 1[6]

Method Development

The solvent exhibiting the highest solubility for the bioactive peptide, determined through a saturated solubility study of the isolated peptide, will be utilized to determine

Table 3: Saturated Solubility Study

Solvent	Mean Absorbance ± SD	Conc. Of Unknown (µg/ml)	Dilution factor	Conc. of Undiluted (µg/ml)	Solubility (mg/ml)
Methanol	0.7837 ± 0.0045	1170.38	5	5851.90	5.8519
DMSO	0.6527 ± 0.0015	1457.91	5	7289.55	7.2896
pH 1.2 Buffer	0.2185 ± 0.0008	624.912	5	3124.56	3.1245
pH 4.5 Buffer (Acetate)	0.265 ± 0.0026	715.723	5	3578.615	3.5786
pH 4.5 Buffer (Phosphate)	0.4066 ±0.0002	971.099	5	4855.795	4.8554
pH 6.8 Buffer	0.8457 ± 0.0035	1602.99	5	8014.97	8.0150
pH 7.4 Buffer	0.9780 ± 0.0062	2075.69	5	10378.44	10.3784
pH 10 Buffer	0.6921 ± 0.0003	1564.618	5	7823.091	7.8230

Table 4: Linearity data of isolated bioactive peptide

Conc. (ppm)	Abs. Mean	% RSD	Regressed Absorbance
100	0.0423 ± 0.0007	1.6664	0.0587
200	0.1017 ± 0.0015	1.5025	0.1093
300	0.1646 ± 0.0003	0.1856	0.1599
400	0.2291 ± 0.0002	0.0667	0.2106
500	0.2740 ± 0.0004	0.1475	0.2612
600	0.3192 ± 0.0002	0.0652	0.3118
700	0.3569 ± 0.0002	0.0428	0.3624
800	0.3954 ± 0.0004	0.0888	0.413
900	0.4566 ± 0.0002	0.0456	0.4636
1000	0.5183 ± 0.0004	0.0803	0.5142

the optimal detection wavelength and to establish the appropriate concentration range for standard dilutions.[7] Method developed with all the parameters involved is mentioned in the Table 1.

Method Validation

The International Conference on Harmonisation (ICH) provides standardized guidelines for the validation of analytical procedures, classifying such methodologies as standard performance measures verified through laboratory evaluation. In accordance with these guidelines, the proposed method was further validated following the ICH Q2 (R2) guideline titled *"Validation of Analytical Procedures: Text and Methodology*[7]. All the parameters along with procedure adopted is listed in Table 2.

RESULT AND DISCUSSION

Saturated Solubility Study

The phosphate buffer at pH 7.4 demonstrated superior solubility (~10.38 mg/ml) compared to the solvents analyzed, while DMSO, methanol, pH 4.5 (phosphate and acetate) and pH 1.2 buffer exhibited lower solubility values. The study conducted by Kim et al. demonstrated that the bioactive peptides derived from perilla seed meal exhibited a notable enhancement in solubility at alkaline pH levels (pH 7), attributed to the polar ion of the peptide in physiological pH conditions. Based on the analysis, a pH 7.4 buffer is utilized as a solvent for conducting the UV method development and validation studies[19].

Method Development

A UV-Visible spectrophotometric method was developed utilizing a Shimadzu UV-1800 spectrophotometer, with phosphate buffer (pH 7.4) employed as the solvent system.

Method Validation

The method that was developed underwent Validation in accordance with ICH guidelines. The preparation of the drug solution followed the method outlined in the methodology section. The proposed method exhibited acceptable specificity, selectivity, linearity, precision, robustness, ruggedness, and reproducibility, confirming its suitability for reliable analytical application.

Specificity and Selectivity

The UV spectrum of the isolated bioactive peptide revealed a maximum absorbance (λ max) at 379 nm, affirming the method's specificity and selectivity (Figure 2). No absorbance was observed at this wavelength in the blank solution, further confirming the absence of interfering substances and thus supporting the method's selectivity. Overlay spectral analysis demonstrated consistent λ max values with no significant spectral shifts across the tested concentration range of 200–1000 µg/ml (Figure 3). These findings validate the UV-Visible spectrophotometric method as a precise, selective, and reliable analytical approach for the identification and quantification of bioactive peptides in isolated samples.

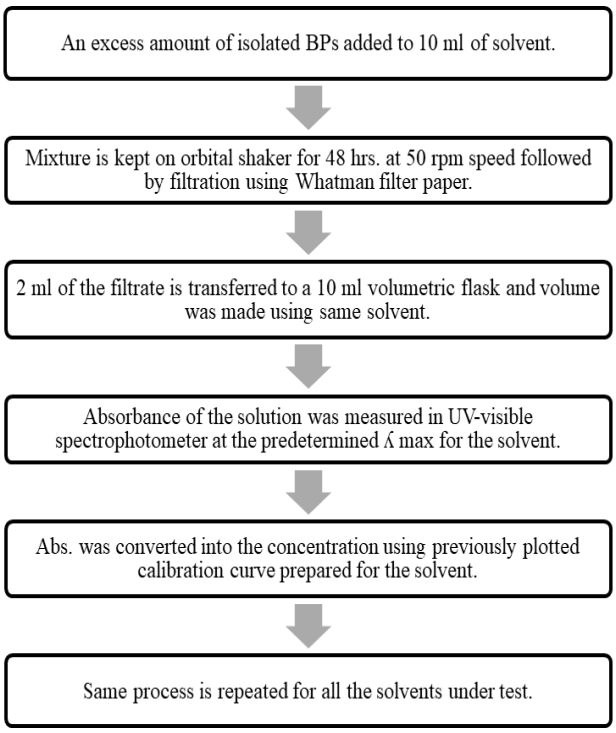

Figure 1: Process to carry Saturated Solubility Study

Table 5: Intraday Precision data of isolated bioactive peptide

Conc.	Morning Abs.	Afternoon Abs.	Evening Abs.	Mean Abs.	± SD	% RSD
100	0.0455	0.0449	0.0452	0.0452	0.0003	0.6967
	0.0452	0.0452	0.0451	0.0452	0.0001	0.1776
	0.0449	0.0457	0.0443	0.0450	0.0007	1.6117
500	0.2681	0.2765	0.2741	0.2729	0.0043	1.5854
	0.2712	0.2695	0.2686	0.2698	0.0013	0.4894
	0.2739	0.2736	0.2719	0.2731	0.0011	0.3949
1000	0.5241	0.5194	0.5216	0.5217	0.0024	0.4508
	0.5149	0.5187	0.5204	0.5180	0.0028	0.5436
	0.5213	0.5183	0.5205	0.5200	0.0016	0.2987

Table 6: Interday Precision (Repeatability) data of isolated bioactive peptide

Conc. (µg/ml)	Abs. Day 1	Abs. Day 2	Abs. Day 3	Mean Abs.	± SD	% RSD
100	0.0446	0.0451	0.0449	0.0449	0.0003	0.5684
	0.0446	0.0449	0.0450	0.0448	0.0002	0.5233
	0.0450	0.0451	0.0449	0.0450	0.0001	0.2528
500	0.2715	0.2729	0.2708	0.2717	0.0011	0.3935
	0.2726	0.2731	0.2722	0.2726	0.0005	0.1654
	0.2709	0.2738	0.2718	0.2722	0.0015	0.5454
1000	0.5143	0.5161	0.5136	0.5147	0.0013	0.2506
	0.5124	0.5149	0.5129	0.5134	0.0013	0.2577
	0.5129	0.5144	0.5141	0.5138	0.0008	0.1545

Table 7: Accuracy and Recovery Study

% Std. Spiked	Sample	Total drug including spiked (µg/ml)	Abs	Mean ± SD	Conc. Of drug recovered (µg/ml)	% Recovery
80	500	900	0.4561 0.4549 0.4557	0.4556 ± 0.0006	884.0837	98.23
100	500	1000	0.5148 0.5139 0.5146	0.5144 ± 0.0005	1000.338	100.03
120	500	1100	0.5569 0.5674 0.5679	0.5641 ± 0.0062	1098.911	99.90

Linearity

Samples with concentrations ranging from 100 to 1000 µg/ml were analyzed, and absorbance values were recorded in triplicate. The mean absorbance values (n = 3) were calculated and ranged from 0.042 ± 0.0007 to 0.5183 ± 0.0004, with relative standard deviation (RSD) values consistently remaining below 2%, as detailed in Table 4. A calibration curve was constructed by plotting concentration versus absorbance, and linear regression analysis revealed a strong linear relationship within the tested concentration range. The resulting regression equation was $y = 0.0005x + 0.008$, with a correlation coefficient (R^2) of 0.993 (Figure 4).

The high degree of linearity observed confirms the method's capability to accurately quantify bioactive peptides within the evaluated concentration range. Furthermore, the elevated R^2 value underscores the model's excellent fit to the experimental data, validating the reliability and robustness of the UV-spectrophotometric method for quantitative analysis of the isolated peptide.

Limit of Detection and Limit of Quantification

The extracted bioactive peptide's LOD and LOQ were determined to be 2.89 µg/ml and 8.76 µg/ml, respectively. These are the BP concentrations that the technology can detect and quantify as legitimate with a respectable degree of accuracy and precision.

This work's low LOD and LOQ values demonstrate how effective and sensitive the technology is for detecting and quantifying BPs in complicated sample matrices at trace concentrations. This demonstrates that the suggested approach can determine BPs in product pharmacokinetic and quality control analyses.

Precision

Three isolated bioactive peptide samples (100 µg/ml, 500 µg/ml, and 1000 µg/ml) were produced independently for intraday and interday studies. The absorbance of each sample at 379 nm was measured three times on the same day to evaluate system accuracy during the intraday research. As indicated in Table 5, the computed %RSD was less than 2%. To assess interday precision, the absorbance of each concentration level was measured over three consecutive days under identical experimental conditions.

The percentage relative standard deviation (%RSD) was calculated for each concentration to evaluate variability over time (Table 6). The percentage RSD values were less than 2% at all tested concentrations.

These findings indicate strong reproducibility of measurements across different days, demonstrating the

Table 8: Ruggedness study by change in analyst

Conc. (ppm)	Analyst 1				Analyst 2			
	Abs.	Mean	± SD	% RSD	Abs.	Mean	± SD	% RSD
100	0.0442	0.0442	0.0000	0.0598	0.0443	0.0443	0.0000	0.1019
	0.0443				0.0443			
	0.0442				0.0442			
500	0.2715	0.2715	0.0005	0.1661	0.2726	0.2722	0.0004	0.1485
	0.2710				0.2718			
	0.2719				0.2723			
1000	0.5192	0.5187	0.0008	0.1506	0.5187	0.5193	0.0006	0.1072
	0.5178				0.5194			
	0.5191				0.5198			

Table 9: Ruggedness study of the developed method

Conc.	374 nm			379 nm			384 nm		
	Abs.	Mean ± SD	% RSD	Abs.	Mean ± SD	% RSD	Abs.	Mean ± SD	% RSD
100	0.0413	0.0417 ±	1.0167	0.0452	0.0451 ±	0.1573	0.0411	0.0414 ±	0.5323
	0.0419	0.0004		0.0450	0.0001		0.0415	0.0002	
	0.0420			0.0451			0.0415		
500	0.2596	0.2596 ±	0.4817	0.2733	0.2736 ±	0.0967	0.2491	0.2493 ±	0.3283
	0.2608	0.0013		0.2738	0.0003		0.2486	0.0008	
	0.2583			0.2737			0.2502		
1000	0.4981	0.4987 ±	0.1116	0.5168	0.516 ±	0.1940	0.4913	0.4924 ±	0.2237
	0.4992	0.0006		0.5172	0.0010		0.4935	0.0011	
	0.4988			0.5153			0.4923		

method's stability and feasibility for routine analysis. The low %RSD values reflect minimal variability among replicates conducted on separate days, thereby confirming the high precision of the developed UV spectrophotometric method for the determination of bioactive peptides (BPs).

Accuracy

The analytical procedure's dependability was evaluated using recovery tests conducted at 80%, 100%, and 120% of the spiked sample concentrations. Standardisation was used to prepare and analyse sample solutions at each level. A reasonable degree of accuracy was indicated by the percent recoveries, which ranged from 98.23 to 100.03% (Table 7), with a mean recovery of 99.39%. The overall accuracy of the procedure demonstrates its dependability in accurately and precisely determining the amount of BPs present in pharmaceutical formulations. Further optimization of the strategy could reduce variation and improve its adaptability.

Ruggedness

The ruggedness and repeatability of the proposed method were evaluated by independent analysts under varying experimental conditions. Repeatability was demonstrated with %RSD values consistently below 2% (Table 8), indicating high methodological consistency. The uniformity in analytical response across different evaluators confirms the method's resilience to minor variations in laboratory conditions and operator handling. These results underscore the robustness of the method, supporting its suitability as a reliable and reproducible analytical tool for the precise quantification of individual bioactive peptides.

Robustness

The robustness of the method was further evaluated by introducing deliberate variations in the detection wavelength. Absorbance measurements were conducted at wavelengths deviating by ±5 nm from the established λ

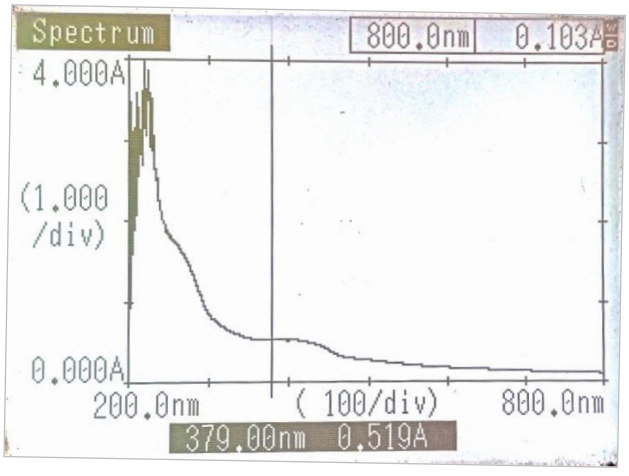

Figure 2: λ max of isolated bioactive peptide

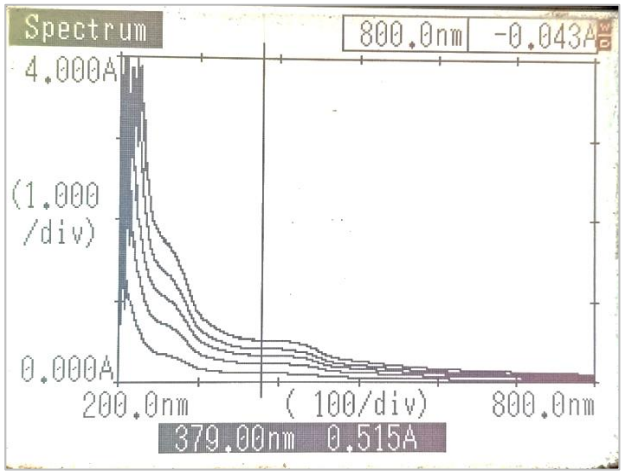

Figure 3: Overlay UV spectra of isolated bioactive peptide

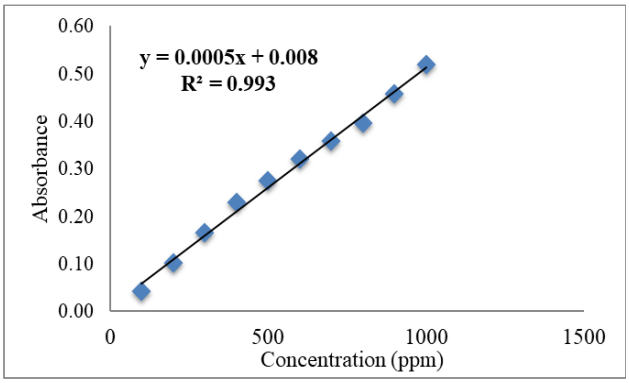

Figure 4: Calibration curve for isolated bioactive peptide

max. Despite these intentional modifications, the method consistently produced reproducible results. The %RSD values for absorbance readings at the varied wavelengths remained within acceptable limits, generally below 2% (Table 9). These findings confirm the robustness of the method, demonstrating its ability to deliver reliable analytical performance under minor deviations in experimental conditions.

CONCLUSION

The UV-spectrophotometric method developed and validated for quantifying isolated bioactive peptides exhibited simplicity, precision, accuracy, and robustness. This method demonstrated reliable performance across various metrics, including precision, accuracy, sensitivity, ruggedness, and robustness, making it suitable for regular use in quality control laboratories. The simplicity and cost-effectiveness enhance its practical applicability, particularly in environments with restricted access to advanced analytical instruments. This method warrants further exploration regarding its efficacy in assessing isolated bioactive peptides in complex formulations, such as pharmaceuticals, cosmeceuticals, nutraceuticals, or dietary supplements. Moreover, integrating this method with advanced analytical techniques like HPLC or LC-MS could enhance its sensitivity and selectivity, thereby aiding in identifying trace phytochemical constituents. Investigating its scalability for industrial applications and integration into regulatory frameworks may establish this technology as the standard for isolated bioactive peptide analysis and quality control of bioactive peptide-based products.

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