Extraction and Method Validation for Collagen Obtained from the Jellyfish Rhizostoma Pulmo Found on the Romanian Black Sea Coast

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Abstract

The biological qualities that collagen provides have been the basis for the continuous research and development of medicines, dematocosmetics, nutraceuticals, and food supplements. Fibrillar collagen is the most common type of protein. The interest in the study of jellyfish is justified by their increasing numbers and the biological structural and functional properties of the biocompounds they contain. Extraction, purification, characterization of collagen by known experimental methods, and determination of collagen yield for the jellyfish *Rhizostoma pulmo* species found in the Black Sea basin were extracted, purified, characterized, and determined. The validation of the method is also demonstrated by determining the collagen content by the hydroxyproline method. Validation is the confirmation, through examination and the provision of objective evidence, that particular requirements aimed at a defined and specific purpose are achieved.

Keywords: collagen, jellyfish, *Rhizostoma pulmo*, collagen extraction, method validation, Black Sea

Introduction

Collagen is the most ubiquitous biomacromolecule in the animal kingdom and is commonly used as a biomaterial in regenerative medicine therapies and biomedical research. Marine collagen is found in several invertebrate organisms such as porifera (sea sponges), molluscs (byssus and cephalopods), crustaceans (mantis shrimp),

echinoderms (starfish and sea urchin) and coelenterates (jellyfish). Porifera, or marine sponges, have a structure similar to the cancellous architecture of bone tissue and are a major source of collagen. A number of studies have highlighted the availability of type I collagen in byssus [1] and cephalopods [2], indicating their suitability as potential sources of raw materials for cosmetic products. The extraction method in their case is more difficult due to the resistance to acid extraction due to the chemical composition and specific structure of the collagen in crustaceans.

Therefore, the enzymatic method is often used to isolate collagen from crustaceans, this method is more suitable for breaking down the hard collagen fibers and preserving their integrity. Enzymatic extraction, in particular, involves the use of specific enzymes that can break down collagen without damaging its properties. Although the percentage of collagen content is relatively low, ranging from 0.015% - 0.4878%, crustaceans can show considerable variation in collagen content, as well as differences in the types of collagen they produce.

Other marine animals from which collagen was extracted were starfish, species such as *A. pectinifera* and *A. rubens*, which had yields between 1.44% - 6.1% [3], [4], [5]. Another study by Li [6] reported type I collagen extracted from sea cucumber (*H. cinerascens*) with a high collagen percentage content of up to 72 %. There are several methods of collagen extraction, the main categories of marine collagen isolation are: acid soluble collagen (ASC), pepsin soluble collagen (PSC), salt soluble collagen (SSC) and ultrasound assisted collagen (UAC).

New methods that can be used fol collagen extraction are supercritical fluid extraction, ultrasound extraction and deep eutectic solvent (DES). Establishing and choosing the extraction method can significantly influence both the yield and the physico-chemical properties of collagen, the importance of selecting the appropriate technique being vital for the success of the preparation with potential biomedical, dermato-cosmetic, nutraceutical or food industry applications.

Material and methods

The materials used for collagen extraction include a jellyfish from the Black Sea basin, measuring approximately 30 cm in length and weighing 877 g in wet tissue. This paper aims to extract marine collagen from the *Rhizostoma pulmo* (**Figure 1**) species, with a primary objective of utilizing marine resources from the Black Sea basin. The methods for collagen extraction from jellyfish, as outlined in the literature, involve various biotechnologies that differ based on the treatments applied. These treatments include alkaline and acid processes, acid treatments alone, enzymatic methods, and combined approaches.

Chemicals. All reagents used were of analytical reagent grade and were purchased from Sigma-Aldrich, Germany.

Figure 1. *Rhizostoma pulmo,* jellyfish from Black Sea

Acetic acid extraction method. Samples were chosen from jellyfish in a wet state for collagen isolation. The jellyfish collagen was obtained from the Black Sea species *Rhizostoma pulmo* by a biotechnological acid extraction process. The mesogel pieces were crushed with a tissue homogenization machine. The crushed mesoglea was added to a 0.5 M acetic acid solution with continuous stirring at 4˚C for 72 h. The mixture was filtered through cloth to remove water-insoluble components. Then, solid NaCl was added to the filtrate to a final concentration of 0.9 M, and the precipitate was harvested by centrifugation at 4 000 g for 15 min. After centrifugation, the precipitate was termed acid-soluble collagen (ASC). The steps of the collagen extraction process are shown in **Figure 2.**

Stages of Jellyfish Collagen Extraction Asc Process

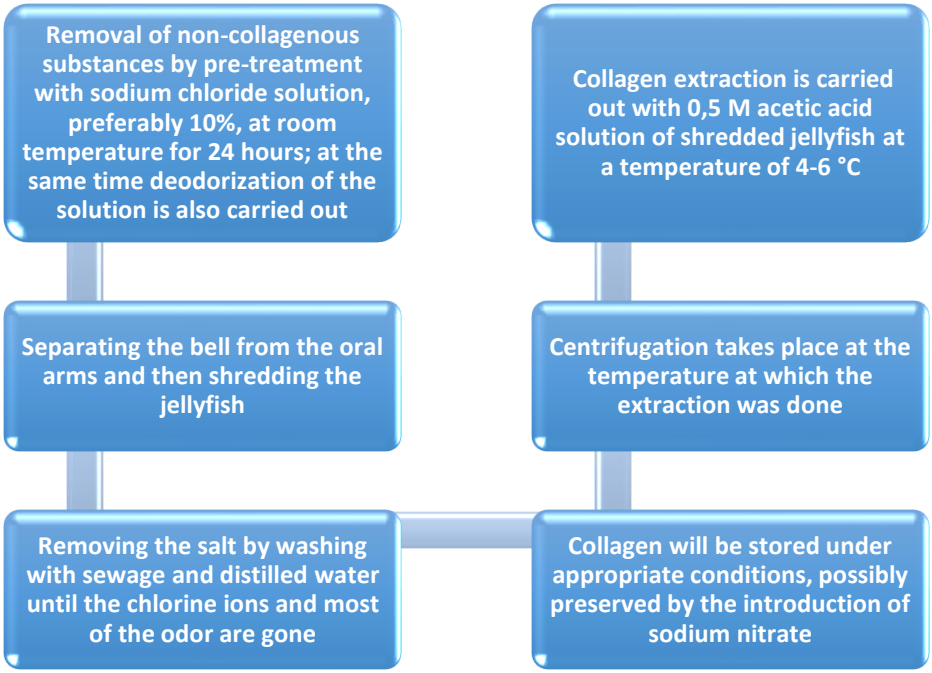

Figure 2 Schematic of collagen extraction from jellyfish *R. pulmo*

Figure 3 shows the *Rhizostoma pulmo* jellyfish which was collected from the Black Sea.

Figure 3. *The length of the Rhizostoma pulmo jellyfish taken for collagen extraction*

Table 1 shows two different extraction methods for obtaining collagen from the jellyfish *Rhizostoma pulmo*: acid soluble collagen (ACS) and pepsin soluble collagen (PCS).

Table 1 Different extraction methods for obtaining collagen from the jellyfish *Rhizostoma pulmo*

ACID SOLUBLE COLLAGEN (ACS)	**PEPSIN-SOLUBLE COLLAGEN (PCS)**
Pre-washed and shredded jellyfish is left in 0.5M acetic acid for 3 days	Prepare by dispersing AC in a mixture of 0.5M acetic acid containing 1% pepsin
Removal by dialysis against a solution of NA_2HPO_4	Incubation for 24 hours at 4 ^oC
The precipitated collagen is separated by centrifugation	Centrifugation - 10,000 rpm for 30 minutes
Then solubilized in acetic acid	The supernatant is dialyzed against a Na_2HCO_3 solution to inactivate pepsin
Purification by reprecipitation with 0.9M NaCl	Purification is by reprecipitation with 0.9M NaCl
Previously washed and shredded jellyfish is left in 0.5M acetic acid for 3 days	Prepare by dispersing AC in a mixture of 0.5M acetic acid containing 1% pepsin
Elimination by dialysis against a solution of NA_2HPO_4	Incubate for 24 hours at 4 ^oC

Purification of collagen from *Rhizostoma pulmo*. Perform at 4 °C from frozen jellyfish tissues. A minimum of 10 g of tissue is used for extraction in each experiment with 10 mL extraction solution/g tissue. Tissue powders were mixed with 0.5 M acetic acid, and acid-soluble collagen was extracted overnight under continuous shaking. This mixture was centrifuged (15.000 g for 1 h), and the acid-soluble collagen was precipitated from the supernatant by adjusting the final concentration of NaCI to 0.9 M.

The resulting precipitate was recovered by centrifugation. Acid-soluble collagen was resuspended in 0.5 M acetic acid and dialyzed against 0.1 M acetic acid. For the extraction of collagen from the jellyfish, several parts of the jellyfish, such as the bell and oral bracts, were used. The acid-treatment extraction biotechnology was utilized with 0.5 M acetic acid (Figure 3). In order to obtain good yields and undenatured fibrillar type I collagen from jellyfish, several preparation, extraction, and purification operations were performed. The results of jellyfish collagen yield by acid treatment with 0.5 M acetic acid are shown in **Figure 4**.

Figure 4. Collagen extracted from *Rhizostoma pulmo* in acetic acid 0,5M

Yields obtained from collagen extraction from the jellyfish *Rhizostoma pulmo.* The best yield was obtained from the oral arms of the jellyfish. The extraction yield is done with at least 10g of tissue (wet weight) suspended in 10mL extraction solution/g of tissue. The yield varied as follows: 0.83 - 3.15 mg/g - from the umbrella and 2.61 - 10.3 mg/g - from the oral arms [7]. The ASC yield was calculated from the percentage of dry weight of extracted collagen (Mo) compared to wet weight of jellyfish used (M):

$$\text{Yield } \% = Mo/M * 100 \quad (1)$$

Validation of Method for Determination of the Hydroxyproline Content of Collagen Extracted from Jellyfish

Method validation shall be carried out on the basis of a plan, which shall include: purpose, characteristics, performance, acceptance limits, limit of detection and limit of quantification, accuracy, precision, selectivity, linearity, concentration and application range, experimental conditions, and statistical calculation. Hydroxyproline is an amino acid that is synthesized by the post-translational hydroxylation of proline by prolyl hydroxylase. Hydroxyproline in Type I collagen is found in about 11.3%. Hydroxyproline determination is used to determine a number of conditions involving collagen lysis, such as bone metastases, liver fibrosis, or prostate carcinoma. The determination of hydroxyproline is also frequently used in the food industry for quality control of meat and meat products [8].

To determine the extracted collagen content, a quantitative analysis method based on the determination of hydroxyproline content was optimized [9]. The basic principles for spectrophotometric determination of hydroxyprolin are listed in the ISO 3496/1994 standard and have been used by Macovescu et al. in the validation of the method for the determination of hydroxyproline in collagenic medical biomaterials [10].

Equipment. For the determination of the hydroxyproline content (percentage) a Hellios Omega Thermo Scientific 171008 series Omega Thermo Scientific Hellios spectrometer was used at a wavelength of 558 nm ± 2 nm. The glassware used consisted of class A volumetric flasks and pipettes, certified by the manufacturer and internally verified. Also, an oven adjustable to 105 °C ± 2 °C, an analytical balance accurate to 0.0001 g and a water bath.

Methods. Hydroxyproline is determined according to the ISO standard for meat and meat products, ISO 3496/1994. This value is used to measure the extraction yield of collagen, calculated as the extracted hydroxyproline relative to its initial concentration in the jellyfish. The method is the spectrophotometric determination of hydroxyproline after reaction with Ehrlich reagent. The sample to be analyzed is subjected to hydrolysis with sulfuric acid at 105°C, when the collagen is converted to hydroxyproline; the hydrolysate is filtered and diluted, and hydroxyproline is then oxidized in the presence of chloramine T [10].

The oxidation product is decarboxylated to pyrrole; then, in the presence of p-dimethylaminobenzaldehyde, a red compound is formed. The chemical mechanism of this process was described as follows: The pyrrolidine ring in the hydroxyproline structure can oxidatively undergo dehydrogenation to a pyrrolyl ring, which can be identified using an Ehrlich reagent reaction.

The resulting quinoid compound is intensely colored (color ranges from orange to lilac) [9]. Hydroxyproline is used to determine the amount of collagen in a given tissue type. The presence of hydroxyproline and the determination of its amount specifically certifies the existence of collagen, which can then be calculated.

The absorbance at a wavelength of 558 nm is measured using a cuvette with a path length of 1 cm. The spectrophotometric method allows the percentage of collagen to be calculated based on the percentage content of hydroxyprolin. The method was found to be valid for the determination of hydroxyproline in collagen isolated from jellyfish. The percentage collagen is calculated by multiplying the percentage hydroxyproline content by 8. The percentage of collagen was calculated based on the percentage of hydroxyproline according to the relation [8]:

$$\text{Collagen content\%} = W_{\text{Hyp content}}\% * 8 \qquad (2)$$

0,121*8=0.96% collagen

where: $W_{\text{Hyp content}}$ is the Hydroxyproline content, calculated as mass percentage, while 8 is the transformation factor [10].

Analytical method validation. Analytical validation is the basis of laboratory quality assurance. Validation is the confirmation, by examination and provision of objective evidence the particular requirements aimed at a defined and specific purpose are fulfilled [11]. The main purpose of validation is to verify that the method is correct, specific, and can produce the expected results. In order to validate the method, a number of performance factors, such as:

Linearity. The calibration curve must be linear in the concentration range established. The correlation coefficient r must be at least 0.995 and the information value PG< F=4.54 for a curve in N = 6 points (5 degrees of freedom) [12]. The coefficient of variation of the method imposed in the laboratory is Vx_0<10%.

Limit of detection and quantitation. Application of the calculation formula for the values obtained when testing the established standard solution (LOD= 3SD and LOQ=10*SD). Absorbance of reagent blanck solution = max. 0.40 a.u.a (λ = 538nm) according to SR ISO 3496/1994 [8]. After performing the calculation, it can be checked and judged, if the detection limit is numerically equal to three tenths of the limit of quantification. The following criteria are required for LOQ: Recovery = 80 - 120%; RSD = max 20%.

Accuracy/Reliability. Fidelity - in the case of a certified reference material, the deviation between the experimentally determined mean content and the certified

value must be within ± 10%. Recovery data are acceptable within ± 10% of the target value. Fidelity (expressed as a percentage) must be in the range 90 -110%.

Stability/Robustness. Internal control chart plotting over time according to Shewhart charts for setting intervention limits ±3*SR and warning limits ±2*SR (internal reproducibility).

Repeatability. Aims to trace the accuracy of the method under repeatability conditions, r = 0.0131 + 0.0322 X, according to SR ISO 3496/1994 [8].

Estimation of measurement uncertainty. It was recommended that the uncertainty be expressed in percent and correspond to the concentration level for which it has been calculated. It will be estimated if the expanded measurement uncertainty, calculated on the basis of a coverage factor of 2 (at 95% confidence level), exceeds the value of the maximum standard uncertainty given by the formula Uf, where Uc ≤ Uf for the calculation of the maximum limit of standard uncertainty taking into account with Commission Regulation (EC) No. 333/ 2007 [13].

Results and Discussion

Linearity is an analytical method that expresses the proportionality between the intensity of the analytical signal and the concentration or activity of the analyte. It expresses the ability of an analytical method to provide within a limited concentration range analytical signals proportional to the activity or concentration, this results in acceptable quality results. It is represented by a linear regression curve of the measured value as a function of increasing analyte concentration. The linearity can be expressed graphically or by mathematical evaluation.

The validation of the method of analysis for the determination of hydroxyproline content was carried out in accordance with the validation guidelines for analytical methods recommended by EURACHEM. The established working concentration range, represented by the range between the lower and upper concentrations of the analyzed sample, was established, proving that the procedure has an adequate level of precision, accuracy, and linearity.

Linearity by evaluation of the calibration curve: according to ISO 8466-1/2022, the equation of the straight line is given by the relation: y=a+bx. The characteristics of the obtained straight line are shown in **Table 2** and **Figure 5**.

Table 2 Standard curve values for the determination of hydroxyproline by molecular absorption

Sample number	1	2	3	4	5	6
Concentration mg/mL	0.500	1.000	1.500	2.000	2.500	3.500
Absorbance (a.u.)	0.103	0.189	0.291	0.401	0.489	0.659

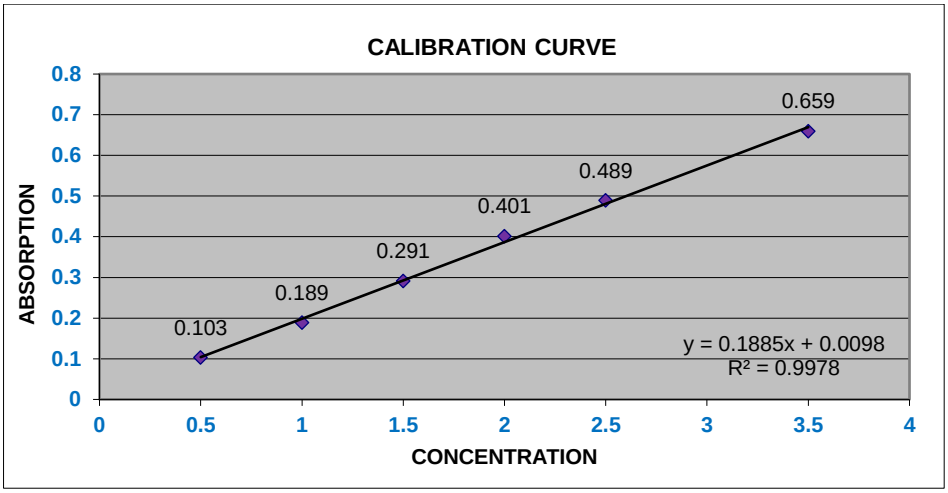

Figure 5. Calibration curve representation of the first order equation determining the linearity of the extraction method

Performance criteria were presented in **Table 3**. The calibration curve must be linear in the stability concentration range between 0.5 and 3.5 µg/mL. The correlation coefficient r must be at least 0.995 and the information value PG< F= 5.54 for a curve in N= 6 points (5 degrees of freedom) (according to SR ISO 8466:1/2022). The coefficient of variation of the working method imposed in the laboratory is Vx_0<10%. For the calibration curve, solutions with concentrations ranging from 0.5 mg/L to 0.5 mg/L have been taken in work.

Table 3 Characteristics for Linearity assessment

Characteristic data	Characteristic definition of the calibration curve	Result obtained	Performance requirement
a	The ordinate at the origin (or intercept), refers to the value of the output magnitude when the input magnitude is 0 and characterizes the calculated reagent margin.	a=0	
b	Regression slope of the straight line, refers to the significant change in analytical response to a small change in analyte concentration and characterizes sensitivity.	b=0.1884	

x_i	The concentrations of the standard solutions used to plot the curve.	0.5; 1 ; 1.5; 2; 2.5; 3.5µg/mL	
y_i	The absorbance values measured by the equipment for the standard solutions.		
Sy	The residual standard deviation, represents the dispersion of the measured values around the linear regression line; it is a performance indicator describing the accuracy of the calibration.	Sy=0.0140	
Sx_0	The standard deviation of the method = Sy/b, is an absolute measure of the precision of the method.	$Sx_{0=}$ 0.074	1.8333
Vx_0	The coefficient of variation of the method =Sx_0 /x medium*100, is a relative measure of the precision of the method.	Vx_0=4.03	Vx_0<10%
r	Correlation coefficient.	r= 0.9977	R≥0.995
r^2	Coefficient of determination. r and r2 describe.	r^2=0.9954	
Value of information PG	Quantitatively the linearity of the calibration function.	DS^2/Sy_2^2 PG=3.7768	PG<F3.77<4.54

The calibration data are used to calculate a linear function of the calibration: y=a+bx, the information value: PG= DS2/Sy22= 3.77. It was compared with the tabulated value of the factor F= 5.54 (according to SR ISO 8466-1/2022).

If PG<F then the nonlinear calibration function does not give an adjustment and the calibration function is linear.

If PG>F then the calibration function is nonlinear and the working range must be reduced to obtain a linear calibration function. A calibration curve was obtained with correlation coefficient r = 0.997 and PG = 3.77 < 5.54.

Decision: calculated F is smaller than F1/F2; 99% - in this case the function is linear in the range of the examined working range, also it is linear according to the Mandel domain - for linearity check (according to SR ISO 8466-1/2022). The coefficient of variation of the method was obtained, Vx_0= 4.03 < 10%.

In **Table 4** the calibration curve data after the first order equation is presented.

Table 4 Calibration curve data after a first order equation

Data required to draw up the calibration line n=6/22.08.2024					
No.	Concentration	Instrumental response, y (u.a.)	x mediu=1.83 ug/mL	$Sx_0=Sy/b=0.074$	
			Y= linear unction	Δy	Δy^2
1	0.5	0.103	0.0942	0.0088	0.000077
2	1.0	0.189	0.1884	0.0006	0.000000
3	1,5	0.291	0.2826	0.0084	0.000070
4	2,0	0.401	0.3768	0.0242	0.000586
5	2,5	0.489	0.471	0.0180	0.000324
6	3,5	0.659	0.6594	-0.0004	-0.000001
				$\Sigma\Delta y^2=0.001056$	
y=0.1884x + 0.0000 - parameters are calculated from the equipment software				Standard deviation residual	$Sy_1=\sqrt{\Sigma\Delta y^2}/n-1$ $Sy_1=0.0140$
					$Sy_1^2=0,00019746$
$Vx_0=Sx_0*100/Xmediu=4.03\%$					

Limit of detection and limit of quantitation. The detection limit is the point at which a measured value is greater than the accompanying uncertainty, representing the smallest amount of analyte sample that can be detected [14]. In **Table 5** are presented LOD and LOQ values.

Table 5 Limits of detection and quantification of hydroxyproline

No. repetitions	Hydroxyproline concentration c (μg/mL), on the calibration curve	Hydroxyproline concentration %, Collagen for m = 5.00 g sample and V = 5 mL hydrolyzed, with d.f. = 5.05
1	0.0329	0.005
2	0.0332	0.08
3	0.0668	0.08
4	0.0662	0.007
5	0.0547	0.012
6	0.0828	0.011
7	0.0825	0.01
8	0.0828	0.006
9	0.0307	0.01
10	0.0763	0.01
11	0.9220	0.01
12	0.0558	0.006

13	0.0366	0.007
14	0.0834	0.01
15	0.0623	0.01
16	0.0725	0.009
17	0.0685	0.006
18	0.0541	0.01
19	0.0636	0.011
20	0.0541	0.006
21	0.0784	0.005
MEDIA		0.014863
DEV. STAND.		0.021195
LOD collagen=3*STDEV		0.06
LOQ collagen = 10*STDEV		0.21
LOD=3*LOQ/10		0.06

Performance criteria: application of the calculation formula for the values obtained when testing the established standard solution (LOD=3*SD and LOQ=10*SD). Absorbance of the reagent blank sample = max.040 a.u.a (λ = 538nm) according to SR ISO 3496/1997. Following the calculation performed, if the detection limit is equal to three tenths of the limit of quantification, then the performance is verified. The following criteria were imposed for LOQ: Recovery = 80-120%; RSD = max 20%. Experiments: 21 replicates were analyzed for the 0.05 μg/mL (0.01% hydroxyproline) standard solution according to the procedure. LOQ was subsequently checked under repeatability conditions to assess accuracy and precision at this level. Recovery = 105.0%; RSDr = 16.82%. The results fall within the established performance criteria.

Recovery refers to the percentage of the true concentration of a substance that is recovered during the analytical procedure [14]. Before analyzing the samples, a reagent blank test was conducted as part of the spectrophotometry process. Instead of using a blank solution, a standard hydroxyproline solution with decreasing concentrations (ranging from 0.1 to 0.025 μg/mL) was used. The absorbance values were recorded in sequence, with each reading taken after measuring the absorbance of the reagent blank solution (which was less than 0.040 a.u.) against distilled water. The spectrophotometer was calibrated to zero before taking measurements.

Repeatability refers to the ability to obtain consistent results when using the same procedure on identical samples, in the same laboratory, with the same operator and equipment, within a short time interval. A minimum of six determinations must be performed at 100% concentration of the solution being tested [14]. The precision of the method will be evaluated under repeatability conditions, with a maximum allowable difference (r_max) calculated as 0.0131 + 0.0322Xm, according to SR ISO 3496/1994. This represents the value below which the absolute difference between

two test results, under repeatability conditions, is expected to fall within a 95% probability interval (see equation 3).

$$s_r = \sqrt{\frac{\sum (x_{ij} - x_{j\ mediu})^2}{n-1}} \quad (3)$$

Where: r = repeatability limit, s_r - standard deviation at repeatability, x_{ij} - value determined for each experiment, mean x_j - average value of the experiments, n - number of repetitions.

Evaluation of samples. To evaluate duplicate samples in the laboratory, the absolute difference Δ= Ix1-Ix2 between the duplicate samples of routine determinations is compared with the repeatability limit r_{max}:

if Ix_1-$Ix_2 \leq r_{max} \rightarrow$ values fall within the requirement and the result is reported as the average of the two values;

if Ix_1-$Ix_2 > r_{max} \rightarrow$ 3rd determination is performed, the result is reported as the median of the 3 values. The calculated values are: rmax=0.013%, for the median value of 0.21 Hydroxyproline (LOQ) and Ix_1-Ix_2=0.017, for the laboratory prepared hydroxyproline standard solution. The results fall within the established performance criteria. **Table 6** shows experimental data for repeatability for both hydroxyproline and satndard hydroxyproline samples.

Table 6 Experiment data for the repeatability of samples and for hydroxyproline standard

Type of test	Double test		Hydroxyproline standard
	Sample 1	Sample 2	Hidroxiproline (%)
Parameter	Hydroxyproline (%)	Hydroxyproline (%)	
Date of execution	02.08.2024	28.08.2024	29.08.2024
R1	0.397	0.166	0.028
R2	x1=Max=0.399	x2=Min=0.156	0.029
R3	0.386	0.165	0.032
R4	0.387	0.168	x2=Min=0.028
R5	x2=Min=0.378	x1=Max=0.169	x1=Max=0.045
R6	0.392	0.167	0.034
Media results	0.388	0.165	0.032
Standard deviation after repeatability, Sr	0.007765307	0.005244044	0.006501
Ix1- Ix2	0.021	0.013	0.017
rmax =0,0131 +0,0322*Xmed	Absolute difference between two	Absolute difference between two	Absolute difference between two

	independent results =0.025%	independent results =0.018%	independent results =max.0.013%
RSDr, %			16.82%<20%
Recovery%			105.0% Verify LOQ = 0.21%

Stability and Control Chart. Stability of an analytical method is the degree of reproducibility of results obtained on the same sample under different conditions [14]. The control chart is a visual tool for checking the analytical measurement process, developed by W. Shewhart as a primary internal control tool. It should be mentioned that hydroxyproline collagen is a natural component that is stable over time and does not undergo transformations during thermal and technological processing.

Performance criteria. Jellyfish collagen hydrolysate was taken and the hydrolysate was tested to verify the stability of hydroxyproline in the hydrolyzed extract stored at T = 4°C for use in method verification and control chart. **Figure 6** shows the control map data.

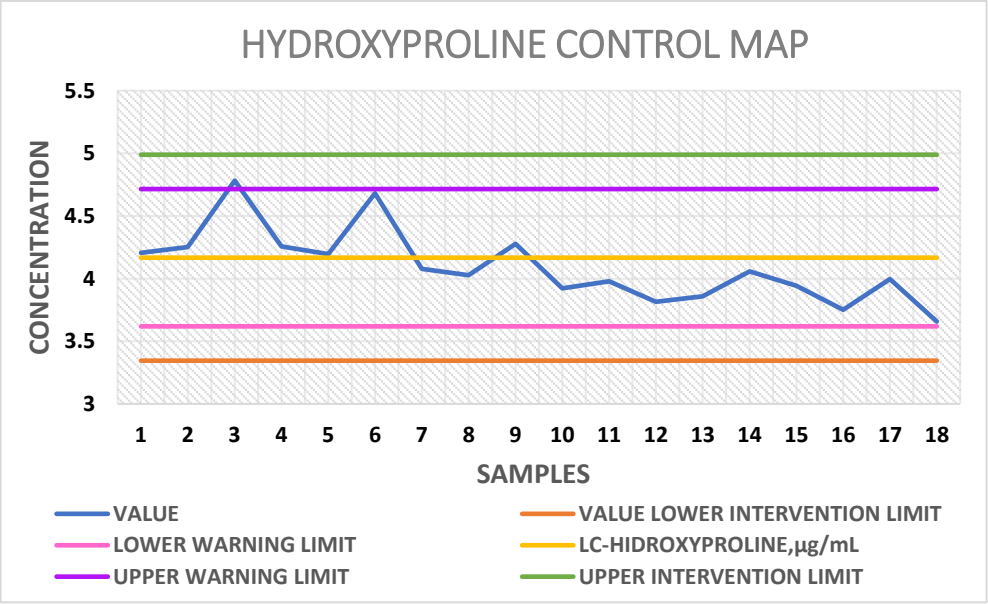

Figure 6. Graphical representation of control map data for the determination of hydroxyproline in jellyfish collagen extract

The hydroxyproline control map shows the lower, upper intervention and warning limits as well as the concentration at which the recording was made and the

corresponding value of the quantitative hydroxyproline recording expressed in micrograms/mL.

The results of the recordings made determined the center line (mean of the recorded values), the intervention limits (mean of three determinations ±3sD) and the warning limits (mean of 2 determinations ±2sD). Signals indicating that the analytical process is undergoing disturbances are: - 7 consecutive values on the same side of the centerline; - 7 consecutive values indicating increasing concentration trends; - 7 consecutive values indicating decreasing concentration trends; - the value above the intervention limits (mean ±3sD). Comment: - 3 values in the range between the warning limit and the action limit are acceptable, but not consecutive.

Accuracy. Accuracy is a systematic error expressed as the difference between the mean value for a large number of repeated determinations and the true value according to the equation [15]. **Table 7** shows the results for Hydroxiproline content, collagen content, LOD, and LOQ.

Table 7 Results of Hydroxyproline (*Hpro*) content measured spectrophotometrically on collagen hydrolysate extracted from *Rhizostoma pulmo*

Collagen type	Hpro content %	Collagen content %	LOD	LOQ
Acetic Acid Extraction 0.5 M	0.121%	0.96%	0.018%	0.21%

The method was found to be valid for the determination of hydroxyproline in collagen extracted from jellyfish. The percent collagen is calculated by multiplying the percent hydroxyproline content by 8, according to the relationship, see equation (4):

Collagen content % = HPro % content * 8 (4)

The spectrophotometric method used to determine the content of hydroxyproline in jellyfish protein products has been validated according to existing performance criteria. The obtained standard curve is linear, the coefficient of variation of the method is $Vx_0 = 4.03 < 10\%$, the correlation coefficient $R = 0.997 > 0.995$, and the value of the comparative informant $F = 5.54$, The linearity was respected over the whole established working range, in this case the calibration curve meets the performance criteria (detection and quantification limit, accuracy, precision, fidelity, stability, robustness, repeatability and measurement uncertainty). The performance criteria meet the requirements, which shows that the method is viable for analyzing the content of hydroxyproline in the extract obtained from the jellyfish species *Rhizostoma pulmo*.

Analysis of denaturation temperature of collagen from *Rhizostoma pulmo* jellyfish. By means of thermogravimetric analysis, the denaturation temperature can be determined, which is also a good indicator of collagen stability that varies directly

proportional to the hydroxyproline content, namely the higher it is, the more stable the helical structure of collagen is.

The thermogravimetric analysis showed that the thermal denaturation temperature (Td) was estimated at 28.9^{o}C, which agrees with the data from the specialized literature, demonstrating the presence of collagen in the hydrolyzed solutions obtained. Following the extraction of collagen from the *Rhizostoma pulmo* jellyfish carried out in the laboratories of the Ovidius University in Constanța, the following results were obtained:

Yield obtained after extraction with acetic acid from oral arms **– 5.32 mg/g.**

Yield obtained after extraction with acetic acid from the bell **– 1.20 mg/g.**

A series of physico-chemical characteristics of the collagen extracted from *Rhizostoma pulmo* were also determined, including the appearance, color, pH value, denaturation temperature, but also the spectrophotometric analysis of the extract for the demonstration and dosage of hydroxyproline present in type I collagen. **Table 8 shows** the results for physico-chemical of collagen extracted, and **table 9** shows the hydroxyproline content from collagen hydrolizate extracted from *Rhizostoma pulmo.*

Table 8 Physico-chemical characteristics of collagen extracted from *Rhizostoma pulmo*

Characteristics	**Collagen extracted from *Rhizostoma pulmo* with acetic acid 0.5 m**
Appearance	Gelatinous white appearance
Color	Translucent-White
pH	6
Denaturation temperature (Td)	28.9^{0}C

Table 9 Results of hydroxyproline content measured spectrophotometrically on collagen hydrolyzate extracted from *Rhizostoma pulmo*

Type Jellyfish Collagen Extraction with Acetic Acid 0.5 M	**Hydroxyproline content W%**	**Collagen content W%**	**Limit of detection LOD**	**Limit of quantification LOQ**
	0.121%	0.96%	0.018%	0.21%

Hydroxyproline is an amino acid that is irreversibly synthesized from the post-translational hydroxylation of proline by prolyl hydroxylase, it is determined according to the guidelines of an ISO standard for meat and meat products - ISO 3496:1994, this value is used to measure the extraction yield of collagen.

Hydroxyproline measurement is also useful to identify certain diseases involving collagen lysis. By determining the spectrophotometrically measured hydroxyproline content of hydrolyzed collagen of the jellyfish, *Rhizostoma pulmo* species, the method was validated, the collagen content was calculated by multiplying the percentage value of hydroxyproline by 8, according to the relation (4). The limits of detection as well as the limits of quantification of hydroxyproline were also systematized in **Table 9**.

Although additional research is needed to better understand extraction methods, obtain higher yields, and create industrial lines to exploit the full potential of jellyfish collagen, this emerging trend for jellyfish collagen will be increasingly researched.

A limitation is still the relatively modest extraction yields, but once improved and industrialized marine collagen extractions, in particular jellyfish collagen, will open new directions for consumption, exploitation and production of jellyfish collagen products.

Conclusions and Future Directions

The collagen extraction yield from the jellyfish *Rhizostoma pulmo* was consistent with findings reported in the literature, using methods specifically designed for marine collagen extraction. Physico-chemical analysis confirmed the presence of fibrillar type I collagen, derived from *Rhizostoma pulmo* specimens collected from the Black Sea basin. This confirms that collagen from this invertebrate is a viable marine biomaterial, suitable for use in pharmaceuticals, food supplements, and nutraceuticals.

In the future, the global jellyfish biomass will increase due to the extremely visible change in climatic conditions from year to year, therefore, the development of new jellyfish collagen products is a potential solution to increase the demand for jellyfish, in this regard it would be useful to study the extraction of collagen from several species of jellyfish inhabiting the Black Sea. Particular attention should be given to improving the extraction process, obtaining higher yields and optimizing an industrial technological process flow for extracting collagen from jellyfish found in the Black Sea basin. Jellyfish are an excellent source of high quality, mammalian-like collagen, which can be enzymatically or chemically hydrolyzed to obtain high quality marine collagen products.

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