Validation of a Quantitative Analysis Method for Collagen Extracted from Grey Mullet Marine Fish

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The Black Sea offers numerous harnessing possibilities for the medical and pharmaceutical, agricultural, food industry and cosmetic fields. Collagen extraction from the Black Sea fish is a research area of great interest. The purpose of this paper is to optimize the collagen quantitative analysis method based on hydroxyproline reagent through visible molecular absorption spectrometry. The adapted method was validated, achieving the following performance criteria: linearity, detection and quantification limits, accuracy/fidelity, stability/sturdiness, repeatability, and measurement uncertainty. The validated method was applied for the quantitative determination of collagen content in Grey Mullet fish and for the evaluation of collagen extraction output.

Keywords: fish collagen, quantitative analysis validation, spectrophotometry, hydroxyproline

The presence of collagen in almost all vital organs and tissues of living organisms, as well as its structural and biochemical particularities have drawn the attention of researchers worldwide regarding the importance of this protein. Collagen is a natural polymer, consisting of a characteristic sequence of 20 amino acids with a complex conformational structure, organized on four levels, also called a primary structure [1]. Collagen contains higher amounts of certain amino acids. Almost a third of collagen is represented by glycine, while another third consists of proline and hydroxyproline. The most recent data regarding collagen composition show that hydroxyproline residue is present in the major phenotype I collagen in a proportion of 11.3% of its weight, while in the type II collagen of cartilages and type IV collagen of basal membranes the proportions vary between 12.9% and 14.3%, respectively, while in type III collagen it measures to approximately 15% [2]. Hydroxyproline measurements have been used as markers to quantify levels of collagen and/or gelatin (knowing that the partial hydrolysis of collagen leads to a mixture of proteins and peptides) [3].

Collagen has multiple uses, especially in the pharmaceutical and biomedical fields [4-7]. The most commonly found source of collagen comes from terrestrial mammals. However, due to the risk of bovine spongiform encephalopathy and aphthous fever, from recent years the marine sources of collagen have also come into the spotlight [8-11]. Globally, in order to extract collagen from saltwater fish, or fresh water fish the followings have been spotlight [8-11]. Globally, in order to extract collagen from saltwater fish, based on the determination of hydroxyproline content through visible spectrophotometry molecular absorption. The adapted method has been validated, by respecting the following performance criteria: linearity, detection and quantification limits, accuracy/fidelity, stability/sturdiness, repeatability, and measurement uncertainty. The validated method was applied for the quantitative determination of collagen content in Grey Mullet fish and for the evaluation of collagen extraction output.

Experimental part
In order to determine the extracted collagen content, it was necessary to optimize a quantitative analysis method, based on the determination of hydroxyproline content [24, 25]. The basic principles for the spectrophotometric determination of hydroxyproline are listed in the ISO 3496/1994 standard [23].

Reagents
Only reagents with a renowned analytical quality were used (Sigma Aldrich) as well as distilled, denionized or water equivalent in purity. H2SO4, 3 mol/L solution and buffer solution pH = 6.8 (prepared from 26 g of monohydrate citric acid; 14 g NaOH; 78 g sodium acetate). The reagents are diluted in 500 mL and then passed into a 1 L graded flask. 250 mL of 1-propanol are added and brought to the graduation mark, chloramine-T solution (1.41 grams of sodium salt of N-chloro-p-toluene sulfonamide in 100 mL of prepared buffer solution), color reagent, prepared in the day of the use (10 g of p-dimethylaminobenzaldehyde in 35 mL solution of perchloric acid, 60% (m/m) and 65 mL isopropanol). Hydroxyproline standard solution was obtained from a stock solution prepared in a 100 mL volumetric flask by dissolving 50 mg of hydroxypropiolidine-

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α-carboxylic - hydroxyproline - in water. A drop of sulphuric acid is added and brought to graduation mark. This solution is maintained stable for a month at 4°C. On the day of the use, 5 mL from the stock solution are introduced into a 500 mL volumetric flask, after which four standard solutions of 0.5 \( \mu \text{g/mL} \), 1 \( \mu \text{g/mL} \), 1.5 \( \mu \text{g/mL} \) and 2 \( \mu \text{g/mL} \) respectively are prepared by taking 10 mL, 20 mL, 30 mL and 40 mL in 100 mL flasks, completed to graduation mark. The reference material used was TET003 RM from FAPAS.

Equipments
A Hellios Omega Thermo Scientific series 1771008 spectrometer was used, suitable for use at a wave length of 558 nm ± 2 nm. Glassware consisted in volumetric flasks and class A pipettes, certified by the producer and internally verified. Also, an adjustable oven at 105°C ± 2°C, an analytical scale with an accuracy of 0.001 g and a water bath were used.

Methods
The method is represented by the spectrophotometric determination of hydroxyproline after reaction with the Ehrlich reagent [3, 24]. The sample to be analyzed is subjected to hydrolysis with sulphuric acid at 105°C, when collagen is transformed into hydroxyproline; the hydrolysate is filtered, diluted and the hydroxyproline is then oxidized in the presence of chloramine T, and the oxidation product is decarboxylated to pyrrole; next, in the presence of p-dimethylaminobenzaldehyde, a red colored compound is formed. Absorbance at a wavelength of 558 nm is measured using a 1 cm pathlength cuvette.

The spectrophotometric method allows calculating the percentage of collagen based on the percentage content of hydroxyproline, using the following equation (1):

\[
\text{Content of collagen} = 8 W\% \quad (1)
\]

where: W is Hyp content, calculated as mass percentage, while 8 is the transformation factor [23].

Sample preparation
Method validation was performed on collagen extract obtained with 0.5 M acetic acid. The samples were homogenized. The analysis sample was had 4g weighted with an accuracy of 0.0001 g and a water bath used.

Equipment
A Hellios Omega Thermo Scientific series 1771008 spectrometer was used, suitable for use at a wave length of 558 nm ± 2 nm. Glassware consisted in volumetric flasks and class A pipettes, certified by the producer and internally verified. Also, an adjustable oven at 105°C ± 2°C, an analytical scale with an accuracy of 0.001 g and a water bath were used.


ew

\[
\text{W %} = \frac{6.25c}{V} \quad (2)
\]

where: c is the hydroxyproline concentration of the diluted hydrolysate read in \( \mu \text{g/mL} \) on the standard curve, and V is the volume of the part of the hydrolysate collected for dilution at 250 mL. The result is expressed with an accuracy of 0.01% [23].

Results and discussions
Validation of the analysis method
Validation of the method for hydroxyproline content determination was performed according to validation guidelines for analytical methods recommended by EURACHEM [26-28]. Linearity test was performed according to the requirements of the SR ISO 8466-1/199 standard [29]. The work concentration domain was established, represented by the interval between the inferior and superior concentrations of the analyzed sample, through which it was proven that the procedure has an adequate level of precision, exactness and linearity.

The hydroxyproline standard curve was drawn for 6 samples. In the linearity statistical test, standardization data was processed either through a linear function according to a \( y = ax + bx \) equation or a non-linear function according to a polynomial equation such as \( y = ax^2 + bx + c \). The absorbance values obtained for various concentration of hydroxyproline are presented in table 1 and the standard curve for the linear function is shown in figure 1.

| Concentration, mg/mL | 0.500 | 1.000 | 1.500 | 2.000 | 2.500 | 3.500 |
|----------------------|-------|-------|-------|-------|-------|-------|
| Absorbance (u.a.)    | 0.101 | 0.179 | 0.279 | 0.379 | 0.480 | 0.633 |
Spectrometry for all 6 Samples

The standardization data have the following characteristics, which are also presented in tables 2 and 3.
- For the linear standardization function: \( y = 0.1817x + 0.0072 \) with \( R^2 = 0.9971 \), with a residual standard deviation \( S_y = 0.0105 \);
- For the non-linear standardization function: \( y = -0.0041x^2 + 0.1983x - 0.0052 \) with \( R^2 = 0.9976 \), with a residual standard deviation \( S_y = 0.00965 \);

Because the two values of residual standard deviations \( S_y \) differ, the difference of dispersions (DS) must be evaluated with the following equation:

\[
DS = (N-2)S_y^2 - (N-3)S_y^2
\]

(3)

where: \( N = 6 \) is samples number and DS is difference of dispersions [29].

- PG information values was calculated using the following equation:

\[
PG = \frac{DS}{S_y^2}
\]

(4)

Where: PG is the test value calculated for comparison with the values of Fisher distribution (testul F Fisher-Snedecor) [29]. The results obtained depict a standard curve with the correlation coefficient \( R = 0.9985 \) and PG = 1.7399 < 10.97. It is compared with the F factor = 10.97 (according to SR ISO 8466-1:1999, pt.4.1.3).

**Dispersions homogeneity test**

In order to appreciate the homogeneity of dispersions, it was evaluated whether the differences at the ends of the work domain (the first and last concentration point on the standard curve) are significant [29]. In order to do so, the PG informant value is subjected to an F test which takes into account the number of freedom degrees \( f = n - 1 \). For a six-point curve, 5 values were used for each of the two ends of the domain, and, in this case, for \( f = n - 1 = 4 \) degrees of freedom. The value in the table 4 is \( F = 15.98 \). The results of the homogeneity test are presented also in table 4. Because \( S_6^2 > S_1^2 \) PG informant values are calculated from the condition given by the equation (5):

\[
PG = S_6^2/S_1^2
\]

(5)

As it is outlined in table 4, an informant value PG = 11.27 < 15.98 was obtained; this indicates the fact that the deviation between dispersions at the ends of the working domain is not significant and that the dispersions are thus homogenous. The working domain is adequate due to the fact that it adheres to linearity alongside the entire chosen work domain and the calibration curve meets the established performance criteria.
Limit of detection (LOD) and limit of quantification (LOQ)

LOD limit of detection is equal with three times the standard deviation of the mean of the determinations for the sample blank, and has been calculated for 21 determinations.

LOQ limit of quantification is equal with ten times the standard deviation of the mean of the determination for the sample blank (21 determinations), taken into account that both accuracy and precision are constant for an interval of concentrations around the detection limit. Results are presented in table 5.

Recovery

Before reading the samples, the blank reagent test was performed for the spectrophotometry. In the absence of a sample blank, standard hydroxyproline solution with decreasing concentrations (0.1; 0.05; 0.025 \( \mu \)g/mL) and their absorbances were registered sequentially. Readings were performed after registering the absorbance of the blank reagent solution (< 0.040 u.a) compared to distilled water and setting the spectrophotometer to zero with this value. The reagent blank sample was obtained by replacing the diluted hydrolysate with 4 mL of distilled water. Thus, 6 repetitions for 0.1 \( \mu \)g/mL hydroxyproline standard samples prepared in the laboratory, 6 repetitions for 0.05 \( \mu \)g/mL standard samples and 2 repetitions for 0.025 \( \mu \)g/mL standard samples were analyzed. Due to the fact that absorbances registered for the 0.025 \( \mu \)g/mL standard were identical with those registered for the 0.05 \( \mu \)g/mL standard, while for those registered for the 0.1 \( \mu \)g/mL were visibly higher, it was convened that LOD will be tested for 0.05 \( \mu \)g/mL (0.01% Hyp). 21 repetitions for the 0.05 \( \mu \)g/mL standard solution (0.01% Hydroxyproline) were analyzed according to the work procedure (data are presented in table 6). LOQ was then verified, in repeatability conditions, in order to evaluate exactness and precision.

Repeatability (Precision)

The precision of the method was evaluated based on repeatability conditions according to SR ISO 3496 / 1997 pt.10.1 [9], with a dependence \( r_{\text{max}} = 0.0131 + 0.0322 \times \text{Mean} \), which is expected to fall within a probability interval (usually 95%), \( r_{\text{max}} \) is the maximum permissible absolute difference between two independent results obtained with

Table 4

| Sample no. | Concentration, x (mg/mL) | Absorbance response, y (u.a., n = 3 repetitions) | STDEV | STDEV² |
|------------|--------------------------|---------------------------------|-------|-------|
| 1          | 0.5                      | 0.113                           | 0.094 | 0.118 |
|            |                          | 0.1                             | 0.101 | 0.085 |
|            |                          | 0.1                             | 0.1   |       |
| 6          | 3.5                      | 0.718                           | 0.666 | 0.633 |
|            |                          | 0.5                             | 0.857 |       |

For freedom degrees n-1 = 4, F=15.98, PG=11.272203, PG=F

Table 5

| HYDROXYPROLINE CONCENTRATION ON THE STANDARD CURVE AND ON THE COLLAGEN SAMPLE OF 4G WEIGHT |
|--------------------------------------------------------------------------------------------|
| No. of repetitions | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---------------------|---|---|---|---|---|---|---|
| Hyp. c (µg/mL) concentration, calibration curve | 0.033 | 0.029 | 0.066 | 0.066 | 0.054 | 0.083 | 0.082 |
| Hyp. % concentration, Collagen for m = 4.00 g sample and V = 4 mL hydrolysate with dilution factor Eₐ = 6.25 | 0.005 | 0.004 | 0.010 | 0.010 | 0.008 | 0.013 | 0.013 |
| No. of repetitions | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| Hyp. c (µg/mL) concentration, calibration curve | 0.039 | 0.031 | 0.072 | 0.093 | 0.057 | 0.037 | 0.086 |
| Hyp. % concentration, Collagen for m = 4.00 g sample and V = 4 mL hydrolysate with dilution factor Eₐ = 6.25 | 0.011 | 0.005 | 0.011 | 0.014 | 0.009 | 0.006 | 0.009 |
| No. of repetitions | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
| Hyp. c (µg/mL) concentration, calibration curve | 0.035 | 0.035 | 0.07 | 0.066 | 0.059 | 0.059 | 0.078 |
| Hyp. % concentration, Collagen for m = 4.00 g sample and V = 4 mL hydrolysate with dilution factor Eₐ = 6.25 | 0.013 | 0.010 | 0.003 | 0.010 | 0.009 | 0.006 | 0.012 |

Statistical results for Hyp. %, from sample of collagen hydrolysate from fish for m=4.00 g sample

| Mean value | 0.000286 |
| STDEV | 0.000068 |
| LOD collagen = 3*STDEV | 0.01 |
| LOQ collagen = 10*STDEV | 0.03 |
| LOD = 3*LOQ | 0.01 |

Table 6

ANALYSIS OF COMPLIANCE WITH PERFORMANCE CRITERIA FOR RECOVERY

| Mean absorbances obtained for witness samples |
| For sample set I | 0.034 u.a. < 0.040 u.a. |
| For sample set II | 0.038 u.a. < 0.040 u.a. |
| For sample set III | 0.036 u.a. < 0.040 u.a. |
| LOD = 3*SD | 0.05 µg/mL (0.01% m/m) |
| LOQ = 10*SD | 0.2 µg/mL (0.03% m/m) |
| Recovery = 90-120% | 105.7% |
| RSD = max 20% | RSD = 17.1% |
the same method in the same laboratory by the same operator using the same apparatus and \( X_{\text{mean}} \) is the average of the two results of the content of the hydroxyproline. Repeatability conditions are verified based on results obtained with the same method, identical samples, in the same laboratory, by the same operator, using the same equipment. The following parameters are used: \( r = \) repeatability limit, \( S_{\text{r}} = \) repeatability standard deviation, \( x_{ij} = \) determined value for each experiment, \( X_{\text{mean}} = \) mean value of experiments. Three sets of samples, each analyzed in 6 repetitions, were taken into study, in the same laboratory, by different analysts, using the same equipment, in different days, separated by a long interval of time. Results are presented in table 7. The following was noted: for set I - \( r_{\text{max}} = 0.026\% \), for a mean value of 0.388\% Hyp. and \( |x_1 - x_2| = 0.023 < r_{\text{max}} \); for set II - \( r_{\text{max}} = 0.018\% \), for a mean value of 0.163\% Hyp. and \( |x_1 - x_2| = 0.010 < r_{\text{max}} \), and for set III (standard solution) - \( r_{\text{max}} = 0.014\% \), for a mean value of 0.032\% Hyp. (LOQ) and \( |x_1 - x_2| = 0.014\% = r_{\text{max}} \).

Results are within the established performance criteria because the condition for absolute difference is met:

\[
\Delta = \frac{|x_1 - x_2|}{n-1} \leq r_{\text{max}}.
\]

(\( \Delta \) is the absolute difference between the double samples of the routine determinations is and \( r_{\text{max}} \) is the calculated repeatability limit - RSD (repeatability) is usually refers to the standard deviation of simultaneous duplicates or replicates, \( S_{\text{r}} \)).

### Accuracy (Exactitude), Bias and Precision

Accuracy is a systematic error expressed as the difference between the average value for a high number of repeated determinations and the real value according to equation (6) [30]. Table 8 presents the results which prove the compliance with performance criteria for accuracy.

Exactness measures closeness between results of measurements and the accepted real value and is determined through recovery experiments from samples with known added concentration standard [30]. Bias characterizes the systematic error of an analytical procedure and is equal to the mean deviation (positive or negative) of analytical results compared to the real value (known or attributed) according to equation (7).

\[
\text{Accuracy} = \frac{X_{\text{mean}} - \text{Reference value}}{\text{Reference value}} \times 100
\]

(6)

\[
\text{Bias}\% = 100 \frac{X_{\text{mean}} - \text{Reference value}}{\text{Reference value}} \times 100
\]

(7)

Fidelity conveys the closeness between a result of a test and the accepted reference value and it is usually expressed as a measure of error [30]. Precision refers to reaching an agreement between independent testing results, obtained on the basis of predetermined stipulated conditions. The measure of precision is typically expressed through imprecision terms and is evaluated as the standard deviation of the test’s results. It is the random error. Lower precision is determined by a higher standard deviation. The fidelity of the method has been tested using the certified reference material (TET003RM from FAPAS) [31], for a short period of time, and the accuracy, precision and fidelity were evaluated in the specified conditions. The certified reference value is 0.805\% Hyp. ± 0.028 \%, and the data obtained is presented in table 9. Data for the evaluation of method precision presented in table 10 confirm the performance characteristics which comply with the legal and laboratory-imposed requirements.

### Table 7

| Double samples | Hyp. standard matrix |
|----------------|----------------------|
| Type of sample | Sample set I | Sample II | Sample set III |
| Parameter      | Hyp. (%)  | Hyp. (%)  | Hyp. (%)  |
| R 1            | 0.388     | x1 = Min 0.134 | 0.039     |
| R 2            | x2 = Max 0.173 | 0.164     | 0.039     |
| R 3            | 0.384     | x2 = Max 0.258 | 0.051     |
| R 4            | 0.389     | 0.166     | 0.051     |
| R 5            | x2 = Max 0.396 | 0.161     | 0.043     |
| R 6            | 0.390     | 0.160     | 0.033     |
| Mean of R1, R6 results | 0.385     | 0.163     | 0.022     |

### Table 8

| Parameter | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|-----------|---|---|---|---|---|---|---|---|
| % Hyp.    | 0.761 | 0.738 | 0.785 | 0.766 | 0.734 | 0.798 | 0.811 | 0.808 |
| Accuracy % | \( \Delta_m = \frac{|X_{\text{mean}} - \text{Reference value}|}{\text{Reference value}} \times 100 \) | \( \Delta_m = 0.071 < 0.08 \) |

### Table 9

| Parameter | 9 | 10 | 11 | 12 | 13 | 14 | 15 | Mean value |
|-----------|---|----|----|----|----|----|----|------------|
| % Hyp.    | 0.810 | 0.828 | 0.828 | 0.822 | 0.835 | 0.844 | 0.834 | 0.809 ± 0.067 (f.d.=2) |
| Accuracy % | \( \Delta_m = \frac{|X_{\text{mean}} - \text{Reference value}|}{\text{Reference value}} \times 100 \) | \( \Delta_m = 0.05 < 0.08 \) |
### Table 9

| Sample type | Certified reference material MRC |
|-------------|----------------------------------|
| Certified value % Hyp. | 0.305 |
| Mean accuracy = certified value mean, % Hyp. | 0.004 |
| Bias (exactness error) | 0.5 |
| SR. % Hyp. | 0.0372 = 0.019 |
| RSD % (CVR %) | 4.6 |
| Fidelity, % | 59.3 |

- **SR.** = standard deviation for n participants.
- **RSD** % = relative standard deviation according to equation (8):

\[
RDS = \frac{SD}{X_{mean} \times 100} \tag{8}
\]

- **CVR**% is the coefficient of variation for n participants and is declared in percent by Eurachem Guide definition [26].

### Table 10

| Fidelity, MRC – FAPAS TET 003 RM, repeated analysis under reproducibility conditions | Determining accuracy, bias, and fidelity of the method |
|---------------------------------|------------------------------------------------------|
| 0.0372 (LOQ) | 0.2 | 0.4 |
| \( \Delta m = |C_{mean} - C_{theoretic}| \) \( \% (m/m) \) Hyp. | 0.004 |

\[
\Delta m = |C_{mean} - C_{theoretic}| \tag{9}
\]

- **Extended uncertainty (k=2, for level of confidence 95%) for 0.4% (m/m) Hyp.**

\[
C_{MRMC} = \pm 0.038 \% (m/m) \) Hyp., \quad U_{c2\%} = 9.7% \tag{10}
\]

\[
U_{MRMC} = \sqrt{u_{MRMC}^2 + u_{CMRMC}^2} \tag{11}
\]

\[
U_{MRMC} = 2\times u_{\Delta m} \tag{12}
\]

- **U** is the uncertainty associated to the mean measured value and the certified value. Because the obtained value is outside the linearity domain, 1+1 sample dilution was performed (dilution factor f.d. = 2) so as to fit on the calibration curve. In case of a certified reference material, averaged content deviation, determined experimentally, and the certified value must be situated within a limit of ± 10%. Fidelity (in percentages) must be within the 90-110% interval. Recovery data are acceptable if they are within a limit of ± 10% from the target value. From the data presented in table 11, for evaluation of method performance, the obtained value is compared to \( U_{C} \) and it can be observed that there is no significant difference between measurement result and certified value because \( \Delta m \leq U_{C} \) [31]. Table 12 presents the centralized values of the budget of uncertainties for measurement.

**Uncertainty**

The calculation of combined and extended uncertainty for \( \Delta m \) (the absolute difference between the measurement average and the theoretic certified value, \( c_{mean} \), is the measurement average value and \( c_{theoretic} \), is the theoretic certified value, \( -X_{mean} \) is the mean value of measurement.

- \( \Delta m \) is the absolute difference between the measurement average and the theoretic certified value, \( c_{mean} \), is the measurement average value and \( c_{theoretic} \), is the theoretic certified value, \( -X_{mean} \) is the mean value of measurement.

**Fig.2 Ishikawa diagram for the determination of the sources of uncertainty**
Table 11

RESULTS OBTAINED FOR EVALUATING UNCERTAINTY

| Date regarding uncertainty | Obtained values  |
|---------------------------|------------------|
| \( \Delta m = l \Delta m_{\text{unc}} \) | \( \Delta m = 1.0805 - 0.801 = 0.04 \% \) Hyp. |
| Uncertainty associated with measurement result \( u_m = \Delta m \) | \( u_m = 0.037 \% \) Hyp |
| Uncertainty associated with certified value \( u_{m,\text{cert}} \) | \( u_{m,\text{cert}} = 0.028 \% \) Hyp (MRC solution standardization certificate) |
| Combined uncertainty associated to the mean measured value and the certified value \( u_{\Delta} = \sqrt{u_{m}^2 + u_{\text{uncert}}^2} \) | \( u_{\Delta} = \sqrt{0.037^2 + 0.014^2} = 0.04 \% \) Hyp |
| Extended uncertainty \( U_{\Delta} = 2 \times u_{\Delta} \) | \( U_{\Delta} = 2 \times 0.04 = 0.08 \% \) Hyp |

Fidelity (as percentage) 99.3\%, within the 90\% - 110\% interval

Table 12

CENTRALIZED VALUES OF THE BUDGET OF UNCERTAINTIES FOR MEASUREMENT

| Parameter | \( X \) values | Uncertainty \( u(x) \) | Relative standard uncertainty \( u(x)/x \) |
|-----------|----------------|---------------------|----------------------------------|
| Sample mass, g | 4 | 0.0016 | 0.0016/4 = 0.0004 |
| Standard calibration solution | 5 \( \mu \)g/mL Hyp. | 0.06 \( \mu \)g/L | 0.06/5 = 0.012 |
| Spectrophotometer, for \( \lambda = 558 \) nm and an absorbance = 0.300 u.a. corresponding to \( c = 1.833 \mu \)g/mL Hyp. | 0.02 - 0.01 u.a., corresponding to \( c = 0.02 \mu \)g/mL Hyp. read on the calibration curve | 0.02 \( \mu \)g/mL | 0.02 - 1.833 = 0.10 (\( U_{\text{uncert}} \)) |
| Drawing of the calibration curve (standard deviation \( S_0 \)) | \( X_{\text{med}} = 1.833 \mu \)g/mL | 0.012 | 0.012 - 1.833 = 0.006 |

Table 13

QUANTIFICATION OF UNCERTAINTY COMPONENTS OF THE METHOD AT 0.400 \% HYP

Combined uncertainty/method \( u_c = (0.033)^2 + (0.122)^2 = 0.126 \mu \)g/mL

Extended uncertainty \( U_{\Delta} = 2 \times 0.04 = 0.08 \% \) Hyp

Percentage uncertainty \( U_{\%} = 0.252 \mu \)g/mL \times 100/2.56 \mu \)g/mL = 9.84 \%

\( U_{\max} = 0.0195 + 0.0529 \times X_{\text{med}} \) [23]

\( U_{\max} = 0.062 \% \) Hyp

7.74\% from the mean obtained by 0.801\% Hyp

Table 14

DETERMINING VALUE INTERVALS FOR HYDROXYPROLINE CONTENT (\( \mu \)g/ML), NEEDED TO DRAW A SHEWHART CHART ACCORDING TO SR ISO 3496:1997 [23]

| Sample no. | 1 | 2 | 3 | 4 | 5 | 6 |
|------------|---|---|---|---|---|---|
| Hydroxyproline \( \mu \)g/mL | 4.167 | 4.566 | 4.359 | 4.087 | 3.906 | 4.067 |
| Sample no. | 7 | 8 | 9 | 10 | 11 | 12 |
| Hydroxyproline \( \mu \)g/mL | 3.982 | 3.615 | 4.063 | 4.250 | 4.262 | 4.393 |

Mean value \( S_2 \) 2SD 3SD Mean value = 2SD Mean value = 3SD

4.166 \mu \)g/mL

Target value = 0.651 \%

0.274 0.548 0.822 3.618 - 4.714 3.344 - 4.988

Quantification of uncertainty components of the method at 0.400 \% Hyp. is presented in table 13. It can be noticed that the calculated percentage uncertainty \( U_{\%} > U_{\max} \%

Stability and control chart (internal reproducibility)

Stability refers to stability over time of the analyzed substance from the matrix. The control chart is a visual surveillance instrument of the analytical measurement

The map of control for Hyp.

Fig.3 Graphical representation of the results on the control map
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