ORIGINAL ARTICLE

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J. Chem. Metrol. 9:1 (2015) 1-15

journal of chemical metrology

A quantitative method for the measurement of hydrolyzed type-I collagen protein in dietary supplement syrup using HPLC-SEC-UV technique

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(Received January 16, 2015 Revised May 29 2015; Accepted June 3, 2015)

Abstract: Collagen is one of the most represented proteins in human body. Type-I collagen, also known as Peptan®, belongs to the collagen family. To measure hydrolyzed type-I collagen directly in supplement syrup, a HPLC-SEC-UV technique was applied as an analytical method. The method was validated considering the FDA (Food and Drug Administration) guidelines. Calibration curves were linear over the entire range from 200 to 1000 µg/mL and the regression coefficients (r) were above ≥ 0.99. Intra- and inter batch accuracies, determined as a deviation between nominal and measured values, ranged from 0.9 to 2.8% and from 0.5 to 1.7%, respectively. Type-I collagen showed extraction recoveries above 99%. The method was successfully applied to a sample of 25 mL supplement syrup, containing 10000 mg hydrolyzed type-I collagen. A final concentration of (10974.2 ± 172.9) mg in 25 mL was found.

Keywords: Collagen; Proteins; Quantitative Analysis; Method Validation; HPLC; Dietary Supplements.

1. Introduction

Collagen is the most abundant protein in the human body, which represents 30% of its dry weight [1-3] and has important beneficial impact on skin [4-6], connective tissues [7,8], tendons [9,10], bones [11,12] and cartilage [13,14]. Although there are more than 20 types of collagens, the types I, II, III, V and XI are the most available onces, representing 80 - 90% of the total collagens. [15,16]. Collagen, which is a water insoluble protein, belongs to the high molecular weight protein

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The article was published by Academy of Chemistry of Globe Publications
www.acgpubs.org/JCM © Published 06/ 06/2015
Online ISSN:1307-6183

group and has a fibrous structure. They could be found in various parts of the body, such as the type I in bones and the type II in cartilage. From the analytical point of view, its insoluble character, most of the time, generates problems for its analysis.

The insoluble collagens are usually transformed into water soluble proteins by enzymatic or acidic hydrolyses. The enzymatically hydrolyzed form of type I collagen is commercially available as Peptan®. The benefit of type I collagen is based on being a high source of all essential amino acids, excluding tryptophan. It is characterized by the abundance of glycine, proline and hydroxyproline which represent around 50% of the total amino-acid content [17]. The unique composition of the amino acids provides hydrolyzed type I collagen with nutri-functional properties, which cannot be found with other protein sources, and it is commercially available as a nutritional dietary suppliment.

Since the beginning of the 20th century, collagen has been studied extensively by a large number of research laboratories due to its above mentioned benefits. Therefore, there is an important requirement to develop reliable analytical methods for the quantitative determination of collagen proteins in nutritional dietary supplements, especially for manufacturer's in-house quality control laboratories.

Unfortunately, there are limited methodologies in the literature for direct quantification of type-I collagen in different sample types. The available techniques are mainly based on laser scanning microscope [18] and ELISA [19]. Hence, this study presents a fast and simple method for quantitive determination of hydrolyzed type I collagen in dietary supplement syrup, using HPLC-SEC-UV technique, which is based on size exclusion chromatography.

The method was validated considering the bioanalytical method validation guidance for industry of FDA (Food and Drug Administration) and was subsequently utilized for the measurement of the type-I collagen content in dietary supplement syrup that containing 10g of hydrolyzed fish type I collagen (Peptan®) in 25 ml syrup portion.

2. Experimental

2.1. Chemicals and materials

Type-I collagen standard, which is a hydrolyzed collagen extracted from fish (Peptan F 2000 LD), was obtained in extracted form from *Eliksir ilaç, gıda, sağlık, kozmetik ürünleri ve danışmanlık hizmetleri tic. ltd. şti.* (Istanbul, Turkey). Disodium hydrogen phosphate (BioXtra ≥ 99,0%) was purchased from Sigma Aldrich (St Louis, MO, USA). Methanol was obtained from Merck KGaA (Darmstadt, Germany). Sodium dihydrogen phosphate mono-hydrate was purchased from Appli-Chem GmbH (Darmstadt, Germany). Supplement syrup (500 mL), of which each 25 mL contains 10 g of hydrolyzed fish collagen (type-I), was obtained from *Eliksir ilaç, gıda, sağlık, kozmetik ürünleri ve danışmanlık hizmetleri tic. ltd. şti.* (Istanbul, Turkey).

2.2. Instrumentation

A HPLC system from Hitachi LaChrom Elite series was used for the chromatographic separation, applying a flow rate of 0.35 mL/min on an Agilent BioSEC 300 Á column (300 mm length × 4.6 mm i.d., 3.0 µm). An isocratic gradient with a mobile phase consisting of 150 mM phosphate buffer, pH= 7.0, was used for the chromatographic separations. The column temperature was maintained at room temperature. The auto-sampler syringe and the injection valve were successively washed with methanol/water (70/30; v/v) to reduce the carry-over contaminations. The injected sample volume into the system was 5 µL. A diode array detector of Elite LaChrom, L-2455 series, from Hitachi was utilized for detection of collagen, which was performed at a wavelength of 214 nm. Karl Fisher analyses were performed with a C30 coulometric titrator from Mettler Toledo and qNMR analysis with 600 MHz NMR from Varian.

2.3. Preparation procedure of calibration standard and QC sample solutions

A 1000 µg/mL concentrated standard stock solution (SSS) was prepared by dissolving 1 mg of type-I collagen in 1 mL distilled water. Using this stock solution, the calibration standard concentrations ranging from 200.0 µg/mL to 1000.0 µg/mL were prepared by dilution with distilled water. The prepared calibration standards were 200, 300, 400, 500, 600, 800 and 1000 µg/mL, respectively. A 1000 µg/mL of QC stock solution (QCSS) was prepared by dissolving 1 mg of type-I collagen in 1 mL distilled water, from which three QC solutions, 300, 500 and 800 µg/mL, were obtained by dilution with distilled water. All calibration standards and QC concentration levels were prepared prior to analysis according to the above described procedure.

2.4. Preparation procedure of syrup sample

Prior to sampling the container with syrup of type-I, the collagen was shaken strongly for a proper homogenization of the viscose syrup. After homogenization process, 10 µL of highly concentrated sample was diluted 1000 times with distilled water, from which 5 µL was injected into the HPLC-SEC-UV system and analyzed at a wavelength of 214 nm.

3. Results and Discussion

3.1. Method validation

The method validation in terms of linearity, repeatability, reproducibility and % recovery was carried out at three spiking levels, namely 300 (QC low), 500 (QC medium) and 800 µg/mL (QC high). Implementing diode array detection the selectivity/specificity of the method was reached using pure standard (hydrolyzed collagen extracted from fish). A single peak of standard with the highest intensity was observed at wavelength of 214 nm.

3.1.1. Linearity

Each of the seven standard concentrations was analyzed in triplicate using HPLC-SEC-UV. Calibration curves were created considering the integration of the peak areas of type-I collagen, which were determined to be linear over the entire range measured from 200 to 1000 µg/mL. As can be noticed, the regression coefficients (r) were above ≥0.99 (Table 1). The calibration curve parameters obtained in 3 days were suitable for the quantification of type-I collagen and supported the data obtained during the intra- and inter-day validation tests. In compliance with the FDA guidance [20], none of the calibration samples deviated by more than 15 % from the nominal value.

Table 1. Calibration curve and linearity

Analyte	Calibration Range (µg/mL)	Calibration equation (n=7)		
		Slope mean ± SD	Intercept mean ± SD	r mean ± SD
Type-I Collagen	200.0 – 1000.0	49576 ± 213	-619012 ± 49372	1.000 ± 4.590E-05

3.1.2. Repeatability and reproducibility

Repeatability (precision, expressed as coefficient of variation, %CV) and accuracy (expressed as percent error, %Bias) were calculated at three QC concentrations (300.0, 500.0 and 800.0 µg/mL). Five replicates of each QC point were analyzed to determine the intra-day accuracy and precision and four replicates for the rest of two days to determine the inter-day accuracy and precision (in total nine replicates). For instance, the obtained inter-run precision and accuracy data for QCs ranged between 0.9 to 2.8 % (%BIAS) (Table 2) are average values. Those values met the acceptance criteria indicating that the applied method was accurate and precise.

Table 2. The data of accuracy and precision

Run ID	Curve No	Nominal concentration (µg/mL) of type-I collagen					
		300.0	%Bias	500.0	%Bias	800.0	%Bias
		Type-I collagen measured concentration (µg/mL) and bias (%)					
Run-1	1	300.0	0.0	507.5	1.5	815.1	1.9
		303.6	1.2	515.2	3.0	820.4	2.5
		302.9	1.0	507.7	1.5	828.3	3.5
		303.6	1.2	515.2	3.0	820.4	2.5
		302.9	1.0	507.7	1.5	828.3	3.5
Mean Concentration		302.6		510.7		822.5	
Intra-Run Accuracy (%BIAS)		0.9		2.1		2.8	
%RSD		1.498		4.163		5.714	
Intra-Run Precision (%CV)		0.5		0.8		0.7	
N		5		5		5	
Run-2	2	299.4	-0.2	504.1	0.8	800.6	0.1
		296.3	-1.2	505.4	1.1	798.0	-0.2
Run-3	3	301.6	0.5	499.2	-0.2	802.9	0.4
		303.2	1.1	502.9	0.6	807.4	0.9
Mean Concentration		301.5		507.2		813.5	
Inter-Run Accuracy (%BIAS)		0.5		1.4		1.7	
%RSD		2.5		5.3		11.7	
Inter-Run Precision (%CV)		0.8		1.0		1.4	
N		9		9		9	

3.1.3. Recovery

In this method, the matrix was a neat solvent (normalized matrix), rather than any kind of biological matrices such as plasma, urine etc. The recovery assessment was performed by using the spikes before and after approach for type-I collagen at three QC concentrations such as 300.0 (low), 500.0 (medium) and 800.0 (high) µg/mL. The obtained recovery data for type-I collagen was ranged from 99.2 to 99.9 % (Table 3).

Table 3. The recovery data of type-I collagen

	Analyte peak areas in samples spiked before extraction (µg/mL)			Analyte peak areas in samples spiked after extraction (µg/mL)		
	300.0	500.0	800.0	300.0	500.0	800.0
	14384745	23807858	39077024	14378957	24117269	39086321
	14050769	23658768	38348906	14335755	24066746	39042457
	14241087	24196092	39616215	14008172	24080580	39363317
Mean	14225534	23887573	39014048	14240961	24088198	39164032
Recovery %	99.9	99.2	99.6			
n	3	3	3	3	3	3

3.1.4. Determination of LOD and LOQ

The limit of detection (LOD) of the detector was found to be 6.0 µg/mL, determined by noise value, which was three times higher than the signal to background noise (S/N) ratio. The limit of quantification (LOQ) was determined as 20 µg/mL, which is 10 times higher than the noise.

3.2. Purity assessment

As no traceable reference material of type-I collagen is available, a purity assessment study was performed within TÜBİTAK-UME. Applying mass balance methodology, while water content of the standard was determined by Karl Fisher coulometric titration, the impueites were determined by HPLC-SEC-UV and NMR.

3.2.1. Karl Fisher analysis

The Karl Fisher analysis was performed according to the internal operating procedure at 160 °C. Three parallel analyses were conducted for water content determination with Karl Fisher. The results obtained from these analyses are shown in Table 4.

Table 4. The data of Karl Fisher analysis

Analyze Number	Sample Weight (g)	Measured Water Content Value (%)
Analyze -1	0.05112 g	9.43
Analyze -2	0.05232 g	9.34
Analyze -3	0.05056 g	9.77
Mean	0.0513	9.51

Water impurity determined by Karl Fisher titration was assigned as IMP_{KF}.

3.2.2. HPLC-SEC-UV analysis

For HPLC-SEC-UV analyis, 5 mg of type-I collagen was disolved in 1 mL distilled water. The prepared sample, having high collagen I concentration was injected in triplicate into the HPLC-SEC-UV with an injection volume of 5 µL and analysed at wavelength 214 nm. The overlayed chromatograms of the resulting three analyses are illustrated in Figure 1.

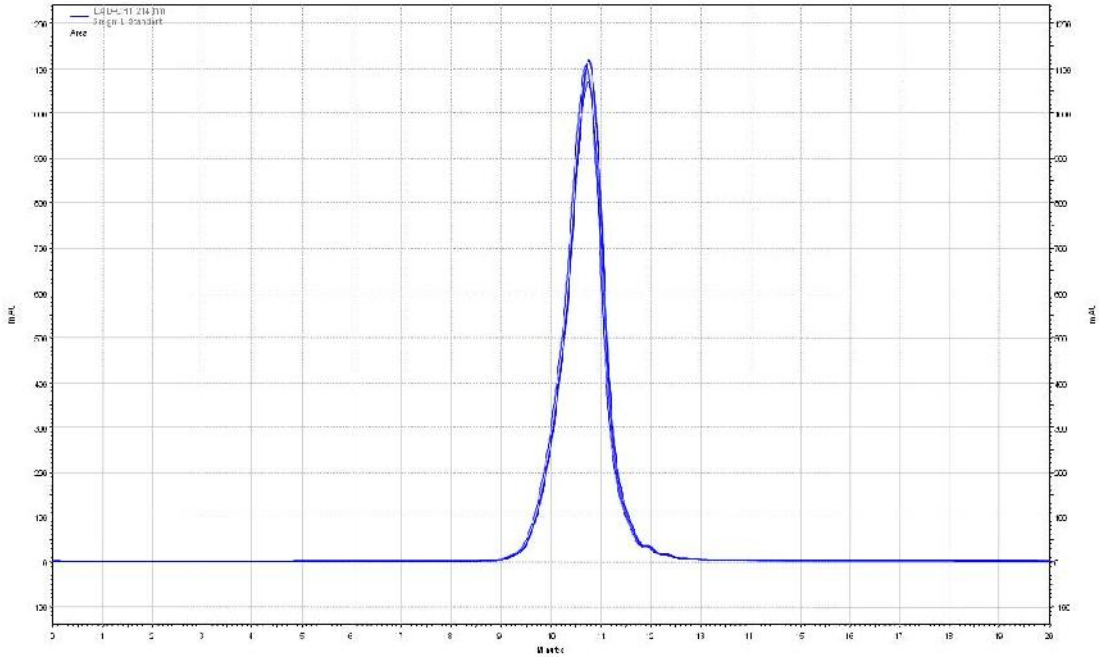

Figure-1: Overlayed chromatograms of resulting three analyses.

Impurity results obtained from the HPLC-SEC-UV analysis are summarized in Table 5.

Table 5. The data of HPLC-SEC-UV analysis

Analyze Number	Impurity Percentage (%)
Analyze -1	0.22
Analyze -2	0.15
Analyze -3	0.05
Mean	0.14

The structurally related impurities determined by HPLC are assigned as IMP_{HPLC}.

3.2.3. NMR analysis

As the resulting peaks were too broad, the purity estimation with NMR was not performed. A representative NMR spectrum of type-I collagen is shown in Figure 2.

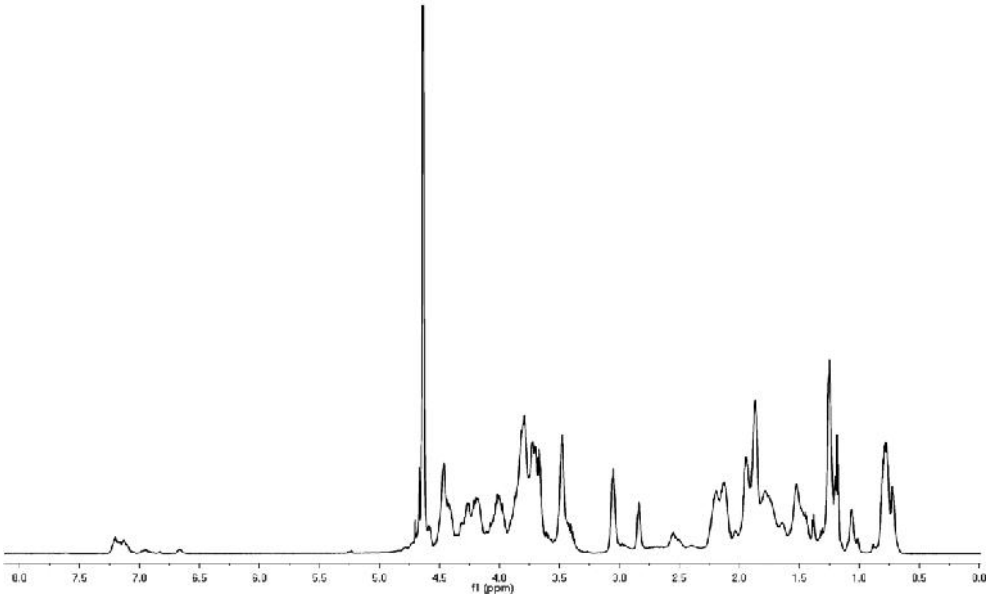

Figure-2: A representative spectrum of resulting NMR analysis.

Due to the high percentage of moisture in the material, inorganic impurities were not determined. Applying equation-1 the purity of type-I collagen was estimated to be 90.35%

$$Purity(\%) = 100 - (IMP_{HPLC} + IMP_{KF}) \qquad \text{Equation-1}$$

$$Purity(\%) = 100 - (0.14 + 9.51)$$

3.3. Estimation of uncertainty

Uncertainty is the parameter associated with the result of a measurement that characterizes the dispersion of the values that could be attributed to the measurement. The uncertainty of the method was evaluated according to EURACHEM/CITAC Guide CG 4 (3th edition) "Quantifying Uncertainty in Analytical Measurement" [21, 22]. The uncertainty sources for the current method are defined as follows:

i. uncertainty of standard stock solution
ii. uncertainty of calibration graph
iii. uncertainty of sample preparation
iv. uncertainty of repeatability
v. uncertainty of recovery

3.3.1. Uncertainty of standard stock solution

The model equation for the calculation of the concentration of standard stock solution is presented by equation-2.

$$C_{SSS} = \frac{m\ x\ Px1000}{V} = ...«g/mL \qquad \text{Equation-2}$$

P:purity (%)

m:mass (µg)

V: volume(mL)

1000: Conversion factor from mg to µg

In order to calculate the uncertainty of the standard stock solution, the uncertainty contribution for each parameter has to be estimated and combined according to equation-3.

$$\frac{u(C_{SSS})}{C_{SSS}} = \sqrt{(\frac{u(P)}{P})^2 + (\frac{u(m)}{m})^2 + (\frac{u(V)}{V})^2} \qquad \text{Equation-3}$$

$u(C_{SSS})$: Uncertainty of concentration of standard stock solution *(mg/mL)*

u(P): Uncertainty of purity

u(m): Uncertainty of weighing

u(V): Uncertainty of volumetric measurement

C_{SSS}: concentration of standard stock solution (mg/mL)

3.3.1.1. Uncertainty of purity of type-I collagen

The purity assessment of type-I collagen consists of Karl Fisher titrimetry and HPLC-SEC-UV analyses, thus, uncertainties of Karl Fisher (*u(KF)*) and HPLC-SEC-UV (*u(HPLC)*) analyses have to be calculated and combined according to equation-4 to obtain the uncertainty of purity, *u(P).*

$$u(P) = \sqrt{u(KF)^2 + u(HPLC)^2} \qquad \text{Equation-4}$$

Uncertainty of Karl Fisher analysis:

Karl Fisher analysis was performed using Mettler Toledo C30 columetric KF Titrator. According to the standard operating procedure, the sample was weighed and then placed into the automated KF oven. The results, i.e. percentage wetness, were obtained after a certain period of heating. This experiment was repeated three times. The uncertainty of Karl Fisher analysis, consisting of weighing of sample and repeatability of determination, was calculated using equation-5.

$$u(KF) = \sqrt{u(m_{sample})^2 + u(R)^2} \qquad \text{Equation-5}$$

u(KF): Uncertainty of Karl Fisher analysis

u(m_{sample}): Uncertainty of weighing of sample

u(R): Uncertainty of repeatability

The uncertainty of weighing of sample is calculated by equation-6.

$$u(m_{sample}) = \sqrt{2u(m_{calibration})^2} \qquad \text{Equation-6}$$

u($m_{calibration}$) is the uncertainty of calibration of the balance, obtained from the calibration certificate of the balance, which is 4.6710^{-4}. Since the balance is used to measure tare and sample, it was used two times for one sample measurement and the uncertainty value of calibration was multiplied by 2.

The uncertainty of repeatability was calculated by equation-7. n is the number of replicate, which is 3 in this experiment. SD, the standard deviation of the analysis, is 0.0256.

$$u(R) = \frac{SD}{\sqrt{n}} \qquad \text{Equation-7}$$

The wetness and *u(KF)* was obtained as 9,51% and 0,13%, respectively.

Uncertainty of HPLC-SEC-UV analysis:

For HPLC-SEC-UV analyis, 5 mg of type-I collagen was disolved in 1 mL distilled water, which is measured by pipette. The sample having high concentration was then injected in triplicate into the HPLC-SEC-UV with an injection volume of 5 µL and analysed at 214 nm. The purity was assessed by equation-8. The uncertainty source of HPLC-SEC-UV analysis is only repeatibility of measurement. $u(R)_{HPLC}$ was calculated by using equation-7 and found to be 0.0493.

$$IMP_{HPLC}(\%) = \frac{PA_n}{PA_T} x100 \qquad \text{Equation-8}$$

PA_n*: Peak area of impurity*

PA_T*: Total peak area of chromatogram*

Finally the uncertainty of purity, *u(P)*, was calculated as 0.837 % and *P* value was found to be 90.35 %.

$$u(HPLC) = \sqrt{u(m_{sample})^2 + u(V_{sample})^2 + u(R)^2} \qquad \text{Equation-9}$$

The uncertainty of sample weighing is determined using equation-6 and the uncertainty of the volumetric pipetting of the sample is determined using equation-10.

$$u(V_{sample}) = \sqrt{u(V_{temp})^2 + u(V_{calibration)})^2} \qquad \text{Equation-10}$$

$u(V_{sample})$*:Uncertainty of volumetric measurement of sample*

$u(V_{temp})$*:Uncertainty of temperature*

$u(V_{calibration})$*:Uncertainty of calibration of pipette*

$u(Vt_{emp})$ is calculated using equation-11

$$u\,(V_{temp}\,) = \frac{\Delta\, TVQ}{\sqrt{3}} \qquad \text{Equation-11}$$

$u(V_{temp})$*: Standard measurement uncertainty of the temperature effect*

V: Measured volume (mL)

Q: Average coefficient of volume expansion of the solvent (water) (1/˚C)

ΔT: Laboratory temperature variation (˚C)

√3: Rectangular distribution coefficient

Laboratory temperature variation was 3 ˚C, the expansion coefficient of the water was 2.110^{-4} 1/˚C and the measured volume was 1 mL. $u(V_{temp})$ was calculated as $3.64\ 10^{-4}$ mL. $u(V_{calibration})$ was 0.825, $u(V_{sample})$ is 0.825, $u(R)$ was calculated by using equation-6 and found to be 0.0493. $u(m_{sample})$ was $9.34\ 10^{-5}$ and $u(HPLC)$ was 0.827. Finally the uncertainty of purity, $u(P)$, was calculated as 0.837 and P value was found to be 90.35.

3.3.1.2. Uncertainty of standard weighing

The uncertainty of weighting of standard was calculated by equation-12.

$$u(m_{s\tan dard}) = \sqrt{2u(m_{calibration})^2} \qquad \text{Equation-12}$$

$u(m_{calibration})$ is the uncertainty of the calibration of the balance and was obtained from its calibration certificate as 4.6710^{-4}. Since the balance was used to measure tare and sample, it was used two times for one sample measurement and the balance uncertainty contribution for calibration was therefore multiplied by 2. $u(m_{standard})$ is $9.35\ 10^{-5}$ mg for a measured mass of 1 mg.

3.3.1.3. Uncertainty of volumetric measurement of standard

The volumetric measurement of stock solution preparation is equal to sample preparation for HPLC purity assessment, therefore $u(V_{sample})$ is 0.825 µL and measured volume is 1000 µL. Consequently, the uncertainty of stock solution was calculated based on equation-3

$$\frac{u(C_{SSS})}{1000} = \sqrt{(\frac{0.837}{90.35})^2 + (\frac{9.3510^{-5}}{1})^2 + (\frac{0.825}{1})^2}$$

C_{SSS} is 1000 µg/mL and $u(C_{SSS})$ is 9.298 µg/mL.

3.3.2. Uncertainty of calibration graph

The uncertainty of the calibration graph was calculated using equation-13, where $\boldsymbol{B_1}$ is the slope of the calibration curve, which is 49839.21, $\boldsymbol{p}$ is the total number of measurements to determine $C_{o,}$ in this case 3, $\boldsymbol{n}$ is the number of measurements for the calibration, 21, $\boldsymbol{C_o}$ is the determined concentration of the sample obtained using the calibration graph and is 813.47 and $\boldsymbol{C_{average}}$ is the mean value of the ordinate values (concentration levels) in regression line and it is 542.86. The value $\boldsymbol{S}$ is the residual standard deviation which is a function of calibration curve and it is 178716.55. This value was calculated automatically using the Curve.exe Software or Excel.

The function $\boldsymbol{Sxx}$, which is a sub function of equation-13, was calculated by equation-14, which was found to be 1430635. The value $\mathbf{C_i}$ represents the individual ordinate values. $u(C_0)$ is 1.367 µg/mL, C_0 is 831.47 µg/mL.

$$u(c_0) = \frac{S}{B_1}\sqrt{\frac{1}{p} + \frac{1}{n} + \frac{(c_0 - \bar{c})^2}{S_{xx}}} \quad \text{Equation-13}$$

$$S_{xx} = \sum_{i=1}^{n} (c_i - \bar{c})^2 \quad \text{Equation-14}$$

3.3.3. Uncertainty of sample preparation

For sample preparation, first, a solution was prepared by dissolving 10 µL of sample in 990 µL of distilled water. Then, 100 µL from this first solution was dissolved in 900 µL of distilled water. Volumetric dispensing was performed by pipette, applied four times across all sample preparation steps. Uncertainty was calculated by using equations-10 and -11 for each use of pipette. $u(V_{P1})$ is 0.25 µL, $u(V_{P2})$ is 0.90 µL, $u(V_{P3})$ is 0.36 µL, $u(V_{P4})$ is 0.89 µL.

3.3.4. Uncertainty of repeatability

The uncertainty of repeatability was determined using equation-15, where **n** is the number of replicates and **SD** is the standard deviation of replicates. The mean concentration of nine QC-high replicates (r) was found to be 813.5 mg/mL (n=9), with standard deviation of 11.7 (SD) mg/mL resulting in a u(r) of 3.9.

$$u(r) = \frac{SD}{\sqrt{n}} \quad \text{Equation-15}$$

3.3.5. Uncertainty budget

The final combined standard measurement uncertainty for the HPLC-SEC-UV method is calculated using equation-16. The summarised uncertainty budget is presented in Table 6.

In order to obtain the expanded uncertainty of measurement $U(C_{T\text{-}IC})$, the combined standard measurement uncertainty, $u(C_{T\text{-}IC})$, was multiplied by 2, which is the coverage factor for a 94.5% confidence level. The expanded and relative uncertainties are given in Table 6.

Table 6. The uncertainty budget of the method

Uncertainty sources	***x*-value**	***u(x)***	***u(x)/x***
Uncertainty of Standard Stock solution (µg/mL)	1000.00	9.30	0.00930
Uncertainty of Calibration Graph (µg/mL)	813.47	1.36672	0.00168
Uncertainty of pipette volume-1 in sample preparation (µL)	10.00	0.25003	0.02500
Uncertainty of pipette volume-2 in sample preparation (µL)	990.00	0.90016	0.00091
Uncertainty of pipette volume-3 in sample preparation (µL)	100.00	0.35686	0.00357
Uncertainty of pipette volume-4 in sample preparation (µL)	900.00	0.88757	0.00099
Uncertainty of Repeatability	813.5	3.9	0.00479
Uncertainty of Recovery	1.00	0.01068	0.01067
			0.02942
$C_{T\text{-}IC}$ *(mg/mL)*	438.97		
$u(C_{T\text{-}IC})$ *(mg/mL)*		12.92	
$U(C_{T\text{-}IC})$ *(mg/mL)*		25.83	
Relative uncertainty (%)		5.88	

$$\frac{u(C_{T-IC})}{C_{T-IC}} = \sqrt{(\frac{u(C_{SSS})}{C_{SSS}})^2 + (\frac{u(C_0)}{C_0})^2 + (\frac{u(V_{P1})}{V_{P1}})^2 + (\frac{u(V_{P2})}{V_{P2}})^2 + (\frac{u(V_{P3})}{V_{P3}})^2 + (\frac{u(V_{P4})}{V_{P4}})^2 + (\frac{u(r)}{r})^2} \quad \text{Equation-16}$$

$u(C_{T\text{-}IC})$: Combined standard measurement uncertainty of Type-I Collagen

$C_{T\text{-}IC}$: Concentration of Type-I Collagen

$u(C_{SSS})$: Combined standard measurement uncertainty of standard stock solution

C_{SSS}: Concentration of standard stock solution

$u(C_0)$: Combined standard measurement uncertainty of calibration graph

C_0: Determined concentration of the sample by using calibration graph

$u(V_{P1})$: Combined standard measurement uncertainty of pipette in sample preparation for first measurement of volume

V_{P1}: Volume of first measurement in sample preparation

$u(V_{P2})$: Combined standard measurement uncertainty of pipette in sample preparation for second measurement of volume

V_{P2}: Volume of second measurement in sample preparation

$u(V_{P3})$: Combined standard measurement uncertainty of pipette in sample preparation for third measurement of volume

V_{P3}: Volume of third measurement in sample preparation

$u(V_{P4})$: Combined standard measurement uncertainty of pipette in sample preparation for forth measurement of volume

V_{P4}: Volume of forth measurement in sample preparation

$u(r)$: Standard measurement uncertainty of repeatability

r: concentration of sample that repeatability experiments run

$u(R_m)$: Combined standard measurement uncertainty of recovery

R_m: mean recovery

3.4. Method application

The validated HPLC-SEC-UV method was applied for quantitative determination of hydrolyzed type-I collagen protein in dietary supplement syrup, in 25 mL, 10000 mg (1 dose) of Peptan®. A final concentration of 10974.2 mg in 25 mL was found using our method. The measurement result may be reported with its expanded uncertainty value as follows: 438.97 ± 25.83 mg/mL at k=2 and a 95% confidence level.

4. Conclusion

The reliable quantitation of proteins still represents one of the most difficult analytical challenges. With this study, a reliable quantitative method using HPLC-SEC-UV technique was developed for the first time to determine quantitatively the type- I collagen in supplement syrup. The method was developed according to FDA guidelines for Bioanalytical Method Validation. All uncertainty data was evaluated in accordance with EURACHEM uncertainty guides. The obtained results, using this method, indicated that this methodological approach could serve as a candidate reference measurement procedure for determination of type- I collagen in supplement syrup. Considering the obtained results, in conjunction with suitable reference materials, it can provide traceability of the currently used commercial assays.

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