Anal Bioanal Chem (2015) 407:1981–1987
DOI 10.1007/s00216-014-8443-y

RESEARCH PAPER

Development and validation of a generic method for quantification of collagen in food supplement tablets using liquid chromatography coupled with time-of-flight mass spectrometry

Bilgin Vatansever · Merve Oztug Senal · Muslum Akgoz · Ahmet C. Goren

Received: 20 November 2014 / Revised: 18 December 2014 / Accepted: 19 December 2014 / Published online: 14 January 2015

Abstract A generic method for the quantification of type II collagen in protein-based dietary supplements is described. This quantitative analysis was conducted using liquid chromatography–electrospray ionization–time-of-flight mass spectrometry (LC–ESI–TOF MS). Compared to classical methods with the use of isotope-labeled standards, our method includes, for the first time, the quantification of hydroxyproline using histidine as an internal standard. Separation of the analytes was performed on a Phenomenex Synergi 4 μm Fusion-RP 80 Å column (150 × 2.0 mm, 4.0 μm) with a mobile phase made of 10 mM ammonium formate in water (A) and 10 mM ammonium formate in methanol (B). The assay was fully validated according to FDA guidelines, and the method exhibited sufficient specificity, accuracy, and precision. Intra- and inter-batch accuracy, determined as a deviation between nominal and measured values, ranged from −4.8 to 9.1% and from 0.9 to 6.4 %, respectively. All analytes (hydroxyproline and histidine) at three concentration levels showed extraction recoveries from 89 to 98 %. The method was successfully applied to protein-based dietary supplements of the pharmaceutical industry.

Keywords Collagen · Quantitative analysis · Method validation · Protein · LC–ESI–TOF MS · Pharmaceutical applications

Electronic supplementary material The online version of this article (doi:10.1007/s00216-014-8443-y) contains supplementary material, which is available to authorized users.

B. Vatansever (✉) · M. O. Senal · M. Akgoz · A. C. Goren
Chemistry Group Laboratories, TUBITAK UME, PBox 54, 41470 Gebze, Kocaeli, Turkey
e-mail: bilgin.vatansever@tubitak.gov.tr

Introduction

Collagen is one of the most widely distributed proteins in the mammalian world and makes up approximately 30 % of the proteins within the body [1–3]. Collagen is found in most tissues and mutations of collagen can lead to various diseases at the tissue level. Due to this reason, collagen is intimately involved in many important disease processes including atherosclerosis [4–6], arthritis [7–9], cirrhosis [10–12], and tumor invasion [13–15]. The superfamily of this protein now includes more than 20 collagen types, but 80–90 % of the collagen is present in the body as the most prominent types as I, II, III, V, and XI [16, 17]. For example, type I and type III collagen improves skin texture and firmness [18–20]. Fortunately nowadays, lost body collagen stores can be effectively replaced through the intake of appropriate dietary supplements in the right amount of regulatory requirements. Non-controlled dosages of collagen supplements can lead sometimes to undesired side effects. Because of this fact, there is a need to develop a robust and reliable analytical method for its quantitative determination in collagen dietary supplements. Hydroxyproline (HYP) is a major component of collagen, where it serves to stabilize the helical structure. Because HYP is largely restricted to collagen, the measurement of HYP provides a direct measure of collagen content [21–23]. Hence, the present work was undertaken to develop a generic method for quantification of collagen in food supplement tablets using liquid chromatography coupled with high-resolution time-of-flight mass spectrometry. The method was validated according to the bioanalytical method validation guidance for industry of FDA and was subsequently used for the measurement of the collagen content in dietary supplement tablets containing 40 mg of undenaturated type II

collagen, 200 mg methylsulfonylmethane (MSM), and 300 mg *Boswellia serrata* extract.

Experimental

Chemicals and materials

Type II collagen standard (66.7 %, from chicken) was obtained from Gongan Lijing Biochemical Co., Ltd. (Hubei, mainland China). An amino acid mixture was purchased from Zivak Technologies (Istanbul, Turkey). Methanol, hydrochloric acid (37 %), isooctane, and chloroform were obtained from Merck KGaA (Darmstadt, Germany). Propyl chloroformate (PCF, 98 %), ammonium formate (98 %), and pyridine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glycine was obtained from Molekula GmbH (München, Germany) and propanol from Riedel-de Haën (Seelze, Germany). ESI-L Low Concentration Tuning Mix (100 mL) of Agilent Technologies was purchased from Supelco (Turkey). Supplement tablets that contain 40 mg of undenatured type II collagen were obtained from MDC İlaç ve Sağlık Hizmetleri A.Ş. (Istanbul, Turkey).

Preparation procedure of standard and QC sample solutions

The standard and quality control (QC) samples were hydrolyzed in acidic condition. Considering the purity of 66.7 %, prior to hydrolysis, 120 mg of each standard and QC samples (i.e., equivalent to 80 mg of 100 % of each standard and QC samples) was weighed separately in glass flasks and clogged tight with septum. Both prepared QC and standard samples were deoxygenated simultaneously for 45 min under a nitrogen stream. The same deoxygenating process was applied to 40 mL of 6 M HCl solution. After the deoxygenating process, 20 mL of deoxygenated 6 M HCl was added carefully via syringe onto each standard and QC sample. The resulting mixture solutions of standard and QC samples were incubated at 110 °C for 24 h. After incubation, the hydrolyzed standard and QC sample solutions were transferred into Falcon tubes. Both solutions were centrifuged at +4 °C and 6000 rpm for 15 min. The resulting supernatant was filtrated using filters with a pore size of 45 μm (CHROMAFIL® PET-45/25). Subsequently, the clear supernatant was aliquoted in 1.5-mL Eppendorf tubes at −20 °C until analysis. Each aliquot contained 500 μL of solution.

Preparation procedure of supplement tablets

After the pulverizing of a supplement tablet, the same hydrolysis preparation procedure as of the standard and QC samples was applied for the preparation of the supplement tablets.

Preparation of standard stock and QC stock solutions

The filtrated supernatant of the standard sample solution was aliquoted in eight parallel 1.5-mL Eppendorf tubes wherein each tube contained 500 μL of standard sample solution. In the supernatant, presenting 6 M HCl was evaporated up to dryness under vacuum at 50 °C. After that, all aliquots were reconstituted by adding 250 μL of distilled water. After the reconstitution process, all aliquots were combined in one 2.0-mL Eppendorf tube. The same preparation procedure was applied for the QC stock solution. The final concentration of the resulting standard stock (SSS) and QC stock (QCS) solutions of type II collagen was 8 mg mL^{-1}.

Preparation of the internal standard working solution

The amino acid standard mixture obtained from Zivak Technologies was used as internal standard stock solution for the analysis. The standard mixture contained the following 35 amino acids: glutamine (GLN), arginine (ARG), serine (SER), citrulline (CIT), 1-methylhistidine (1-MHIS), 3-methylhistidine (3-MHIS), glycine (GLY), glycine-proline dipeptide (GPR), sarcosine (SAR), alanine (ALA), gamma-aminobutyric acid (GABA), betaaminoisobutyric acid (BAIB), alpha-aminobutyric acid (ABA), proline (PRO), methionine (MET), ornithine (ORN), valine (VAL), aspartic acid (ASP), histidine (HIS), glutamic acid (GLU), lysine (LYS), tryptophan (TRP), leucine (LEU), isoleucine (ILE), phenylalanine (PHE), aminopimelic acid (APA), cystathionine (CTH), cystine (C-C), tyrosine (TYR), alpha-aminoadipic acid (AAA), threonine (THR), asparagine (ASN), delta-hydroxylysine (HLY), prolinehydroxyproline dipeptide (PHP), and thiaproline (TPR). Internal standard stock solution (ISS) was aliquoted in six parallel 1.5-mL Eppendorf tubes wherein each tube consisted of 500 μL of ISS. After that, all aliquots were evaporated up to dryness under vacuum at 50 °C and finally reconstituted by adding 200 μL of distilled water. All the six parallel aliquots containing 200 μL of ISS were derivatized. The used reagents and derivatization steps are shown in Table 1.

Three layers were formed after step 5. A volume of 200 μL was removed from the resulting upper layer of the six parallel aliquots and evaporated up to dryness under vacuum at 50 °C. All six aliquots were reconstituted by adding 100 μL of methanol/water (2:1 *v*/*v*) mixture. After reconstitution, all six aliquots were combined in one Eppendorf tube in order to achieve an internal standard working solution (ISWS) with a total volume of 600 μL.

Preparation of the calibration curve

The SSS was diluted in distilled water to give a final concentration of 6.0 mg mL^{-1} (upper limit of quantification, ULOQ).

Table 1 Derivatization steps and used reagents

Used reagents (UR)	Derivatization steps of 200 μL ISS aliquot
UR1: 1000 ppm glycine solution	Step 1: addition of 50 μL of UR1, vortex
UR2: propanol:pyridine (7:1 *v*/*v*) solution	Step 2: addition of 200 μL of UR2, vortex
UR3: isooctane:PCF (5:1 *v*/*v*) solution	Step 3: addition of 200 μL of UR3, vortex[a]
UR4: chloroform:isooctane:PCF	Step 4: addition of 100 μL of UR4, vortex[a]
(24:16:1 *v*/*v*/*v*) solution	Step 5: addition of 200 μL of UR5, vortex
UR5: 5 % HCl solution	

[a] Period of 1 min after vortex

Serial dilutions of ULOQ were performed to obtain aqueous working solutions for calibration standards and QCs with end volumes of 200 μL. The prepared calibration standard concentration levels were 0.25 (lower limit of quantification, LLOQ), 0.50, 1.0, 2.0, 3.0, 4.0, and 6.0 mg mL^{-1}. The QCs were prepared in a similar manner to give final concentrations of 0.75, 2.50, and 5.0 mg mL^{-1}. All the samples including calibration standards, QCs, and tablet samples were derivated as well. The performed derivatization process is described in Table 2.

A volume of 200 μL was removed from the resulting upper layer of the aliquots and evaporated up to dryness under vacuum at 50 °C. All aliquots were reconstituted by adding 200 μL of methanol/water (2:1 *v*/*v*) mixture and injected into the HPLC–high-resolution (HR) time-of-flight mass spectrometry (TOF MS) system.

Chromatography

A HPLC system from Hitachi LaChrom Elite series was used for the chromatographic separations. Chromatographic separations were performed at a flow rate of 0.25 mL min^{-1} on a Phenomenex Synergi 4 μm Fusion-RP 80 Å column (150×2.0 mm, 4.0 μm). A binary gradient with a mobile phase consisting of 10 mM ammonium formate in water (A) and 10 mM ammonium formate in methanol (B) was used for the LC separation. A linear gradient was applied starting from 0 min with 30 to 80 % eluent B in 30 min and back to the initial condition within 5 min followed by equilibration for 10 min. The column temperature was maintained at 40 °C using a column oven. The autosampler syringe and the injection valve were successively washed with methanol/water (70/30; *v*/*v*) to reduce the carryover. The sample volume injected into the system was 20 μL.

Table 2 Preparation of calibration curve, QCs, and tablet samples

Used reagents (UR)	Derivatization steps of 200-μL aliquots of calibration standards, QCs, and tablets
UR1: 1000 ppm glycine solution	Step 1: addition of 10 μL of ISWS, vortex
UR2: propanol:pyridine (7:1 *v*/*v*) solution	Step 2 addition of 50 μL of UR1, vortex
UR3: isooctane:PCF (5:1 *v*/*v*) solution	Step 3: addition of 200 μL of UR2, vortex
UR4: chloroform: isooctane:PCF	Step 4: addition of 200 μL of UR3, vortex[a]
(24:16:1 *v*/*v*/*v*) solution	Step 5: addition of 100 μL of UR4, vortex[a]
UR5: 5 % HCl solution	Step 6: addition of 200 μL of UR5, vortex

[a] Period of 1 min after vortex

Mass spectrometry

A micrOTOF-Q II mass spectrometer from Bruker Daltonics (Bremen, Germany) was utilized to carry out the quantitative determination and detection of HYP. Experiments were performed in positive electrospray ionization (ESI)–MS mode with the following parameters: capillary energy, −5000 V; end plate offset, −500 V; nebulizer gas, 3.0 bar; dry gas, 6.0 L min^{-1}; dry temperature, 150 °C; and scan range, 100–500 *m*/*z* with a scan rate of 5 Hz. Prior to measurements, the mass spectrometer was calibrated with ESI-L Low Concentration Tuning Mix of Agilent Technologies.

Results and discussion

Data processing

Data evaluation was performed with the DataAnalysis software package of Bruker Daltonics (Bremen, Germany). Histidine (HIS) was chosen as an internal standard. HIS indicated a unique mass of 369.142 *m*/*z* after derivatization with PCF while hydroxyproline showed a mass of 260.152 *m*/*z*. The peak areas of HYP and HIS were obtained from extracted ion chromatograms with isolation widths of ±0.1 and ±0.5 *m*/*z* units, respectively (see Electronic Supplementary Material Table S1). Both ions were applied smoothing a factor of 10 which was based upon a Gaussian function. Data evaluation and calculation of the results were accomplished with Microsoft Excel 2000. Figure 1 represents an extracted ion chromatogram (EIC) of one run. A representative EIC of HYP in Fig. 1b and an EIC of IS (HIS) in Fig. 1c are depicted. Because leucine (LEU) and isoleucine (ILE) give the same mass as HYP after derivatization with PCF, both amino acids appear on the EICs (Fig. 1b). Pure HYP was derivatized with PCF for the identification of the HYP peak. After derivatization, pure HYP was analyzed with the same LC–ESI–TOF MS parameters of the method. The retention time of the pure

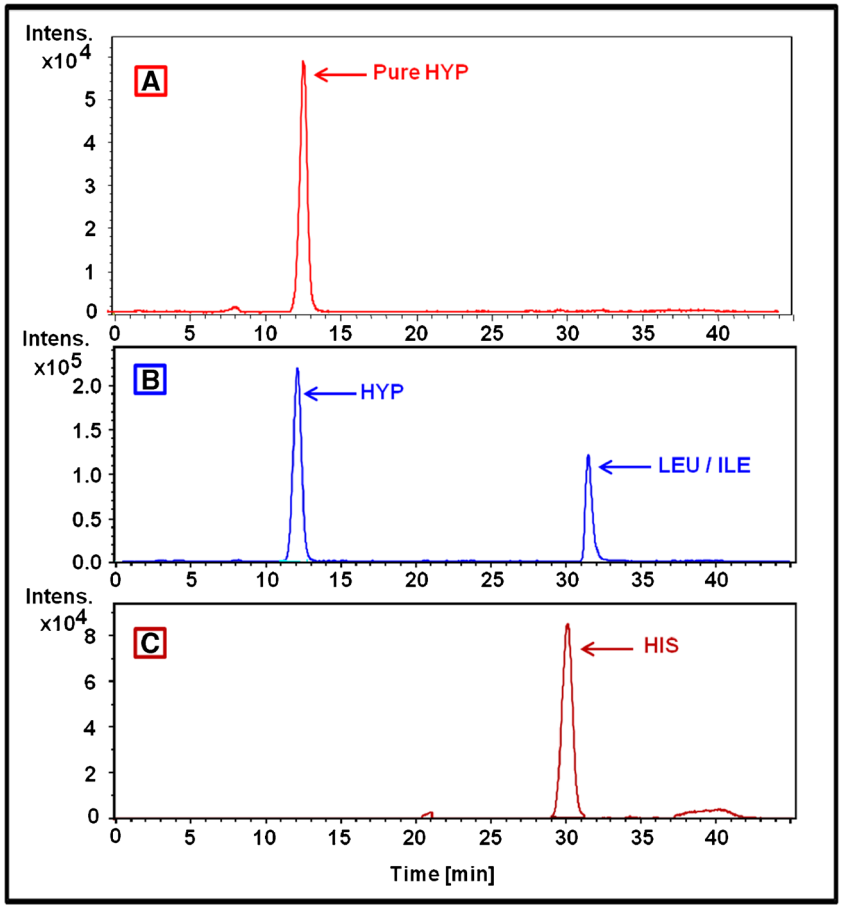

Fig. 1 Representative extracted ion chromatograms of pure HYP (**a**), HYP in standard mixture (**b**), and internal standard HIS (**c**)

HYP was identical to the HYP peak as depicted in Fig. 1a and b.

Method validation

The method validation in terms of linearity, repeatability and reproducibility, and percent recovery was carried out at three spiking levels, namely 0.75 (QC low), 2.5 (QC medium), and 5.0 mg mL^{-1} (QC high).

Linearity test

Two replicates of each of the seven standard concentration levels were analyzed via LC–HR ESI–TOF MS. Calibration curves were generated using the ratios of the peak areas of HYP and HIS (HYP/HIS) and were linear over the entire range measured from 0.25 to 6.0 mg mL^{-1}. As can be noticed, the regression coefficients (r) were above ≥0.99 (Table 3). The calibration curve parameters obtained on each of the 3 days were suitable for the quantification of HYP and supported the data obtained during the intra- and inter-day validation tests. In compliance with the FDA guidance [24], no calibration samples were deviated more than 15 % from the nominal value.

Repeatability and reproducibility

Precision (expressed as percent relative standard deviation, %CV) and accuracy (expressed as percent error, %Bias) were calculated at three QCs (concentrations of 0.75, 2.5, and 5.0 mg mL^{-1}). Five replicates of each QC point were analyzed every day to determine the intra-day accuracy and precision and four replicates for the rest of the 2 days to determine the inter-day accuracy and precision (in total, nine replicates). The acceptance criteria for the intra-run QC accuracies should be within the range ±15 % (%CV) and ≤15 % (%Bias) at the

Table 3 Calibration curve and linearity

Compound	Calibration range (mg mL^{-1})	Calibration equation (n=7)		
		Slope Mean±SD	Intercept Mean±SD	r Mean±SD
HYP	0.25–6.0	1.069±0.052	1.051±0.046	0.993±0.001

Table 4 The accuracy and precision data

		HYP nominal concentration (mg mL^{-1})					
Run ID	Curve no.	0.75	%Bias	2.5	%Bias	5.0	%Bias
		HYP measured concentration (mg mL^{-1}) and bias (%)					
Run 1	1	0.84	12.0	2.4	−4.0	5.0	0.0
		0.83	10.7	2.4	−4.0	4.9	−2.0
		0.81	8.0	2.4	−4.0	5.7	14.0
		0.82	9.3	2.3	−8.0	5.6	12.0
		0.79	5.3	2.4	−4.0	5.2	4.0
Mean concentration		0.82		2.4		5.3	
Intra-run accuracy (%Bias)		9.1		−4.8		5.6	
%RSD		0.019		0.045		0.356	
Intra-run precision (%CV)		2.3		1.9		6.7	
n		5		5		5	
Run 2	2	0.83		2.74		5.49	
		0.81		2.75		5.38	
Run 3	3	0.68		2.74		4.55	
		0.77		2.73		4.40	
Mean concentration		0.80		2.5		5.1	
Inter-run accuracy (%Bias)		6.4		0.9		2.7	
%RSD		0.055		0.179		0.469	
Inter-run precision (%CV)		6.9		7.2		9.2	
n		9		9		9	

concentration levels. The obtained inter-run precision and accuracy data for QCs ranged between 0.9 and 6.4 % (%Bias) and between 6.9 and 9.2 % (%CV), respectively (Table 4). Those values met the acceptance criteria, indicating that the present method was accurate and precise.

Recovery test

Instead of any kind of biological matrix such as plasma, urine, etc., in this method, the matrix was a neat solvent (normalized matrix). The recovery assessment was done by using the spike before and spike after approach for both analytes (HYP) and internal standard (HIS) at three QC concentration levels (0.75 (low), 2.5 (medium), and 5.0 (high)mg mL^{-1}). The obtained recovery data for HYP ranged between 89 and 93 % and for HIS between 94 and 98 %, respectively (Table 5).

Carryover test

In the current method, methanol/water (70/30; *v*/*v*) solution was used to wash the syringe and injection port multiple times before and after each injection. Under these washing conditions, the signal (area under the peak) observed on the retention time of HYP was below 20 % as compared to the one found at the LLOQ after the injection of three blank samples.

Stability test

The autosampler stability of HYP was evaluated at room temperature over 48 h using five replicates of QC samples at 0.75 and 5.0 mg mL^{-1}. The measured concentrations of HYP in these QCs were comparable to the nominal values, with accuracy ranging from 6.0 to 9.1 % (Table 6), indicating that HYP was stable also on the batch-top for at least 48 h at room temperature. The freeze–thaw stability of QCs at 0.75 and 5.0 mg mL^{-1} was assessed after three cycles over 3 days at −20 °C and analyzed together with one set of three QCs and regular QCs. The measured concentrations of HYP in these QCs were comparable to the nominal values, with accuracy

Table 5 The recovery data of analyte (HYP) and internal standard (HIS)

	Analyte peak areas in samples spiked before extraction (mg mL^{-1})			Analyte peak areas in samples spiked after extraction (mg mL^{-1})		
	0.75	2.5	5.0	0.75	2.5	5.0
	4,549,361	12,112,334	20,470,088	3,912,541	11,263,159	27,669,861
	4,857,233	11,679,521	20,220,327	4,441,608	11,899,587	27,369,959
	3,909,525	11,301,692	31,653,750	6,486,338	16,252,753	22,700,176
Mean	4,438,706	11,697,849	24,114,722	4,946,829	13,138,500	25,913,332
%Recovery	90	89	93			
n	3	3	3	3	3	3
	Internal standard peak areas in samples spiked before extraction (mg mL^{-1})			Internal standard peak areas in samples spiked after extraction (mg mL^{-1})		
	4,848,435	4,574,618	3,696,695	4,266,683	4,485,306	4,456,007
	5,228,839	4,409,190	3,728,201	5,040,577	4,564,291	4,740,684
	4,326,206	4,337,780	5,071,464	5,324,982	4,544,714	4,093,228
Mean	4,801,160	4,440,529	4,165,453	4,877,414	4,531,437	4,429,973
%Recovery	98	98	94			
n	3	3	3	3	3	3

ranging from −5.3 to −10.1 % (Table 6). The long-term stability data of Eureka Lab Division [25] was considered as reference for the long-term stability of HYP at −20 °C. According to the reference of Eureka Lab Division, HYP is stable for at least 36 months at −20 °C [25].

Uncertainty evaluation of the method

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 (third edition) titled "Quantifying Uncertainty in Analytical Measurement" [26, 27]. The determined uncertainty sources for the current method are the following defined parameters: (1) uncertainty of the weighing of the starting sample, (2) uncertainty of the stock solution, (3) uncertainty of the calibration curve, and (4) uncertainty of repeatability. The relative measurement uncertainty of the method was 9.87 % (Table 7).

Application to a dietary supplement tablet

The present method was applied as a quality control checking process for the pharmaceutical industry in order to quantify type II collagen in dietary supplement tablets. Therefore, dietary supplement tablets containing 40 mg of type II collagen were chosen for this study. Four tablets were selected at random from one production batch. Two parallel samples from each supplement tablet were prepared according to the present method and then analyzed using liquid chromatography coupled with time-of-flight mass spectrometry. The amounts of type II collagen in the dietary supplement tablets after back calculation are shown in Table 8. Under consideration of the relative measurement uncertainty (9.93 %) of the developed and validated method, the final mean result of type II collagen in tablets was found to be 42.78±4.25 mg.

Table 6 Autosampler and freeze–thaw stability of HYP

	48-h autosampler stability			
	QC low 0.75 mg mL^{-1}	Bias%	QC high 5.0 mg mL^{-1}	Bias%
	0.84	12.0	5.03	0.6
	0.83	10.7	4.93	−1.4
	0.81	8.0	5.67	13.4
	0.82	9.3	5.64	12.8
	0.79	5.3	5.24	4.8
Mean conc.	0.82		5.30	
Accuracy (%Bias)	9.1		6.0	
%RSD	0.02		0.34	
Precision (%CV)	2.4		6.4	
n	5		5	
	3-cycle freeze and thaw stability			
	0.68	−9.3	4.53	−9.4
	0.68	−9.3	4.55	−9.0
	0.77	2.7	4.40	−12.0
Mean conc.	0.71		4.49	
Accuracy (%Bias)	−5.3		−10.1	
%RSD	0.052		0.081	
Precision (%CV)	7.3		1.8	
n	3		3	

Table 7 Results from uncertainty evaluation of the method

Uncertainty sources	x^a	$u(x)$	$u(x)\,x^{-1}$
Weighing of the starting sample (mg)	120.88	0.2767	0.0023
Stock solution (mg mL^{-1})	8.0	0.0411	0.0051
Calibration curve (C_0) (mg mL^{-1})	2.53	0.0996	0.0394
Repeatability	1	0.0296	0.0296
Combined standard measurement uncertainty (U_C)		0.26	
Expanded measurement uncertainty (U_{exp})		0.51	
Relative measurement uncertainty (%)		9.93	

[a] Value

Conclusion

There is an increase in various diseases that are treated by taking a protein-based dietary supplement. Parallel to the drastically rising production of these protein-based supplements within the pharmaceutical industry, the need to develop different analytical methods for the quality control check of finished products has increased. In this study, we have demonstrated a new approach for the quantitative analysis of type II collagen

Table 8 Amounts of type II collagen in four supplement tablets

	Type II collagen (mg)
Tablet 1	35.48
	35.52
Tablet 2	53.96
	55.74
Tablet 3	35.51
	34.06
Tablet 4	46.24
	45.69
Mean	42.78
SD	8.85
n	8

in dietary supplement drugs using the LC–ESI–TOF MS methodology. This methodology was reliable and showed highly reproducible chromatographic and statistical results in terms of precision and accuracy during the validation. We are sure that our robust method can be useful for the pharmaceutical industry to control their collagen-containing end products.

References

1. Ricard-Blum S (2011) The collagen family. Cold Spring Harb Perspect Biol 3(1):a004978
2. Morrison G, Fraser DD (2009) Collagen-related biomarkers in severe sepsis: a big stretch? Crit Care 13(3):154
3. Hu W, Li H, Wang C, Gou S, Fu L (2012) Characterization of collagen fibers by means of texture analysis of second harmonic generation images using orientation-dependent gray level co-occurrence matrix method. J Biomed Opt 17(2):026007
4. Shekhonin BV, Domogatsky SP, Muzykantov VR, Idelson GL, Rukosuev VS (1985) Distribution of type I, III, IV and V collagen in normal and atherosclerotic human arterial wall: immunomorphological characteristics. Coll Relat Res 5(4):355–368
5. Rekhter MD (1999) Collagen synthesis in atherosclerosis: too much and not enough. Cardiovasc Res 41(2):376–384
6. Rekhter MD, Zhang K, Narayanan AS, Phan S, Schork MA, Gordon D (1993) Type I collagen gene expression in human atherosclerosis. Localization to specific plaque regions. Am J Pathol 143(6):1634–1648
7. Bagchi D, Misner B, Bagchi M, Kothari SC, Downs BW, Fafard RD, Preuss HG (2002) Effects of orally administered undenatured type II collagen against arthritic inflammatory diseases: a mechanistic exploration. Int J Clin Pharmacol Res 22(3–4):101–110
8. Barnett ML, Kremer JM, St Clair EW, Clegg DO, Furst D, Weisman M, Fletcher MJ, Chasan-Taber S, Finger E, Morales A, Le CH, Trentham DE (1998) Treatment of rheumatoid arthritis with oral type II collagen. Results of a multicenter, double-blind, placebo-controlled trial. Arthritis Rheum 41(2):290–297
9. Mullazehi M, Wick MC, Klareskog L, van Vollenhoven R, Ronnelid J (2012) Anti-type II collagen antibodies are associated with early radiographic destruction in rheumatoid arthritis. Arthritis Res Ther 14(3):R100
10. McGee JO, Fallon A (1978) Hepatic cirrhosis—a collagen formative disease? J Clin Pathol Suppl 12:150–157
11. Murata K, Kudo M, Onuma F, Motoyama T (1984) Changes of collagen types at various stages of human liver cirrhosis. Hepato-Gastroenterology 31(4):158–161
12. Gay S, Fietzek PP, Remberger K, Eder M, Kuhn K (1975) Liver cirrhosis: immunofluorescence and biochemical studies demonstrate two types of collagen. Klin Wochenschr 53(5):205–208
13. Wang ZN, Xu HM (2000) Relationship between collagen IV expression and biological behavior of gastric cancer. World J Gastroenterol 6(3):438–439
14. Honma K, Miyata T, Ochiya T (2007) Type I collagen gene suppresses tumor growth and invasion of malignant human glioma cells. Cancer Cell Int 7:12
15. Provenzano PP, Eliceiri KW, Campbell JM, Inman DR, White JG, Keely PJ (2006) Collagen reorganization at the tumor-stromal interface facilitates local invasion. BMC Med 4(1):38
16. Myllyharju J, Kivirikko KI (2001) Collagens and collagen-related diseases. Ann Med 33(1):7–21
17. Aho S, Turakainen H, Onnela ML, Boedtker H (1993) Characterization of an intronless collagen gene family in the marine sponge Microciona prolifera. Proc Natl Acad Sci U S A 90(15):7288–7292
18. Feng Y, Zhao J, Gold MH (2008) Skin rejuvenation in Asian skin: the analysis of clinical effects and basic mechanisms of intense pulsed light. J Drugs Dermatol 7(3):273–279
19. Pacak CA, MacKay AA, Cowan DB (2014) An improved method for the preparation of type I collagen from skin. J Vis Exp 83:e51011
20. Postlethwaite AE, Wong WK, Clements P, Chatterjee S, Fessler BJ, Kang AH, Korn J, Mayes M, Merkel PA, Molitor JA, Moreland L, Rothfield N, Simms RW, Smith EA, Spiera R, Steen V, Warrington K, White B, Wigley F, Furst DE (2008) A multicenter, randomized, double-blind, placebo-controlled trial of oral type I collagen treatment in patients with diffuse cutaneous systemic sclerosis: I. Oral type I collagen does not improve skin in all patients, but may improve skin in late-phase disease. Arthritis Rheum 58(6):1810–1822
21. Samuel CS (2009) Determination of collagen content, concentration, and sub-types in kidney tissue. Methods Mol Biol 466:223–235
22. Kliment CR, Englert JM, Crum LP, Oury TD (2011) A novel method for accurate collagen and biochemical assessment of pulmonary tissue utilizing one animal. Int J Clin Exp Pathol 4(4):349–355
23. Colgrave ML, Allingham PG, Jones A (2008) Hydroxyproline quantification for the estimation of collagen in tissue using multiple reaction monitoring mass spectrometry. J Chromatogr A 1212(1–2):150–153
24. Guidance for industry: bioanalytical method validation US Department of Health and Human Services FaDA, Center for Drug Evaluation and Research, Rockville, MD (2001) Available from: http://www.fda.gov/CVM (2001)
25. Eureka LD (2011) Hydroxyproline in urine by UV/VIS-FAST
26. GUM (Guide to the expression of uncertainty in measurement) available free of charge from the Eurachem Website (2014): https://www.eurachem.org/index.php/publications/guides
27. EURACHEM / CITAC guide CG 4, third edition (2012); Quantifying uncertainty in analytical measurement. Available from https://www.eurachem.org/images/stories/Guides/pdf/QUAM2012_P1.pdf