METHOD FOR DETERMINATION OF AMINO ACID CONTENT IN PROTEIN PRODUCTS FOR MEDICAL USE

Gabriela MACOVESCU*, Ciprian CHELARU, Dana GURĂU

INCDTP - Division: Leather and Footwear Research Institute, 93 Ion Minulescu St., Bucharest, Romania, email: icpi@icpi.ro

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ABSTRACT. The paper presents a method for determination of amino acid content in collagen biomaterials for medical use produced in the Collagen Department of INCDTP - Division ICPI and its validation. The method has three stages: the first consists in hydrolysing the collagen biomaterial sample down to amino acids; the second step refers to derivatization of amino acids with N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) which is a silylation reagent; derivatized amino acids are detected using a mass spectrometer after gas chromatography. The method was validated to establish performance parameters and to check compliance with the intended purpose.

KEY WORDS: collagen, amino acids, biomaterials

METODĂ PENTRU DETERMINAREA CONŢINUTULUI DE AMINOACIZI DIN PRODUSELE PROTEICE PENTRU UZ MEDICAL

REZUMAT. Se prezintă o metodă de determinare a conţinutului de aminoacizi din biomaterialele colagenice pentru uz medical produse în Departamentul Colagen al INCDTP – Sucursala ICPI şi validarea ei. Metoda are trei etape: prima constă în hidroliza probei de biomaterial colagenic până la stadiul de aminoacizi; a doua etapă se referă la derivatizarea aminoacizilor cu N, O-bis (trimetilsilil) trifluoracetamidă (BSTFA) care este un reactiv de sililare; aminoacizii derivatizaţi sunt detectaţi pe un spectrometru de masă după gaz cromatografie. Metoda a fost validată pentru a se stabili parametrii de performanţă şi pentru verificarea conformării cu scopul propus.

CUVINTE CHEIE: colagen, aminoacizi, biomateriale

PROCÉDÉ DE DÉTERMINATION DE LA TENEUR EN ACIDES AMINÉS DANS DES PRODUITS PROTÉIQUES À USAGE MÉDICAL

RÉSUMÉ. On présente une méthode de détermination de la teneur en acides aminés de biomatériaux de collagène à usage médical fabriqués dans le Département Collagène de INCDTP-ICPI et sa validation. La méthode comporte trois étapes : la première consiste en l'hydrolyse de l'échantillon de biomatériau de collagène au stade des acides aminés ; la deuxième étape se rapporte à la dérivatisation d'acides aminés avec du N, O-bis (triméthylsilyl) trifluoroacétamide (BSTFA) qui est un réactif de silylation ; les acides aminés dérivés sont détectés sur un spectromètre de masse après chromatographie en phase gazeuse. La méthode a été validée pour établir les paramètres de performance et vérifier la conformité avec l'objectif prévu.

MOTS CLÉS : collagène, acides aminés, biomatériaux

** Correspondence to: Gabriela MACOVESCU, INCDTP - Division: Leather and Footwear Research Institute, 93 Ion Minulescu St., Bucharest, Romania, email: icpi@icpi.ro*

INTRODUCTION

Collagen is the main structural protein in soft, lax, semirigid and rigid conjunctive tissues (skin, bones, tendons, basal membranes, etc.), that primarily provides structural integrity to tissues, but also plays an important role in determining the cell phenotype and in cell adhesion.

Due to its excellent biocompatibility and biodegradability, well-defined structure, biological characteristics and to the way it interacts with the body, collagen is always one of the most widely used biomaterials. Extracted as aqueous solution or gel, type I fibrillar collagen may be moulded into various forms: medical devices, artificial implants, drug delivery systems and scaffolds for tissue regeneration, with an important role in today's medicine [1-5].

As a natural protein, collagen cannot heal by itself the infected tissue because bacteria may use it as a substrate. In severe wound infections, systemic drug delivery may lead to an insufficient drug concentration in the infected area or to side effects associated with the drug and/or to systemic toxicity. This deficiency was successfully solved by local drug delivery, developing drug delivery systems with collagen as a substrate and an antibiotic/antiseptic as a drug for infection control [2, 4, 6, 7].

The primary structure of collagen is determined by the sequence of the 20 amino acids that "build" first α-helix polypeptide chains (secondary structure) by peptide bonds, and then the tertiary and quaternary macromolecular superstructure is formed by means of α-helixes.

Amino acids are particularly important for the human body. They are basic structural elements of proteins, compounds with a remarkable biological importance. Amino acids may be obtained from proteins by acid, basic or enzymatic hydrolysis of peptide bonds.

The following amino acids are included in the collagen composition: glycine (1/3), alanine, proline, hydroxyproline (1/3) and other amino acids (1/3). Amino acids are found in a triple helix conformation: two polypeptide chains are identical, and the third slightly differs in the amino acid composition [8-10].

In the polypeptide chain the amino acids form peptide bonds by coupling the carboxyl group to an amino group; once bound in the protein chain, the amino acid "turns" into a "residual" amino acid, and the carbon, nitrogen, hydrogen and oxygen atoms involved in the bonds form the "skeleton" of the protein. As a result, it is necessary to know the amino acid composition of the products for medical purposes when establishing links between the affected tissues and the products used in the treatments.

As a result, a study was conducted on methods of analysis of amino acid content in protein products, biological fluids, foodstuffs and fodders, in order to develop a qualitative and quantitative method for the identification of amino acids in collagen-based medical materials, for better knowledge of product structure, reproducibility of batches and for improving their quality.

The proposed method was validated in order to establish performance parameters and to check compliance to the intended purpose by determining: detection limit, quantification limit, selectiveness, sensitivity, robustness, accuracy and reliability of the method.

The method of amino acid analysis in collagen-based materials for medical use was verified in order to ensure reproducibility and is supported by determinations carried out on samples from the Collagen Department of ICPI.

MATERIALS AND METHOD

Method Principle

Determination of amino acids from collagen materials for medical use is carried out based on the following three basic stages:

a) Hydrolysis of collagen materials for medical use, with 6M hydrochloric acid, for 24 hours at 1000C;

b) The resulting amino acids are evaporated to dryness to remove moisture and resuspended with acetonitrile to derivatize with N, O-bis (trimethylsilyl) trifluoracetamide (BSTFA) which is a silylation reagent;

c) Derivatized amino acids are detected using a mass spectrometer after gas chromatography.

Reagents

Only reagents of known analytical grade and distilled water or demineralized water or

equivalent purity water are used.

- L-alanine (Ala), L-glycine (Gly), L-proline (Pro), L-aspartic acid (Asp), L-glutamic acid (Glu), all these amino acid standards were purchased from Sigma-Aldrich;
- Derivatizing agent N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) purchased from Merck;
- Hydrochloric acid (HCl) 6 M was used as hydrolysis agent (HCl) 0,1 M;
- Acetonitrile, purchased from Merck.

Equipment

- NANOCOLOR VARIO C2 thermoblock for hydrolysis of collagen materials;
- TurboVap II equipment for concentration of samples using nitrogen gas;
- FOCUS GC Gas chromatograph coupled with DSQ II MS mass spectrometer, AutoSampler TriPlus, flame ionization detector, Split/Split less injector, XCalibur Thermo Scientific software, equipped with a non-polar capillary column;
- capillary column: TR 5MS: 5% phenyl – 95% dimethylpolysiloxane, length: 60 m, inner diameter: 0,32 mm, film thickness: 0,25 µm.

WORK METHOD

Sample Preparation

- About 5 mg solid samples were weighed with an accuracy of 0.01 mg and approximately 100 mg liquid samples were weighed with an accuracy of 0.01 mg;
- 1 ml of 6 M hydrochloric acid solution as hydrolysis agent was added, the tube was covered and placed in the aluminium thermoblock at 100°C±20°C for 24 hours for hydrolysis;
- Using a pipette, a volume of 100 µl of hydrolysed is introduced in a vial placed in TurboVap to remove moisture with nitrogen gas;
- The dried amino acid residues were dissolved in a volume of 100 µl of acetonitrile;
- They are derivatized with a volume of 100 µl of N, O-bis (trimethylsilyl) trifluoroacetamide;
- The sealed vial is subjected to ultrasound for 1 minute;
- The vial is placed in the thermoblock at 100°C+2°C for 30 min. to complete the derivatization reaction;
- The vial is placed in the gas chromatograph sample stand;
- 10 injections of 1 µl per sample are performed.

Standard Stock Solutions

Standard amino acid stock solutions were prepared by dissolving each amino acid in 0.1 M HCl down to a concentration of 5 µmol/ml. The solutions were stored at 4°C until analysis. The calibration standards at 4 different concentrations (0.625-5 µmol/ml were prepared using standard amino acid stock solutions.

Standard amino acid samples were processed by dissolving about 0.1 mg of each amino acid in 1 ml of 0.1 M hydrochloric acid. A 100 µl volume was dried under a constant nitrogen stream.

The remaining amino acid was dissolved in a volume of 100 µl acetonitrile and derivatised with a volume of 100 µl of N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) and analyzed by GC/MS.

RESULTS AND DISCUSSIONS

Since collagen materials for medical purposes in the form of sponges, gels, atomized or liquid hydrolysates are stabilized for good preservation over time, acid hydrolysis has been chosen until the matrix is destroyed and individual amino acids are released.

Hydrochloric acid has been chosen because it has been found to be the most widely used, with the advantage that it is not an oxidizing acid and can be removed quickly from the system.

Several variants of hydrolysis have been attempted, ranging from 2 hours to 24 hours, and it has been observed that a good separation of the amino acids from the samples requires a longer time, therefore a 24-hour hydrolysis at 100°C was opted for.

N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) (Figure 1) is a common silylation reagent that replaces acidic protons of amino acids (e.g. SH, OH, NH and COOH) with nonpolar trimethylsilane groups (TMS).

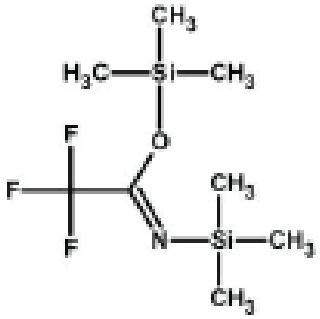
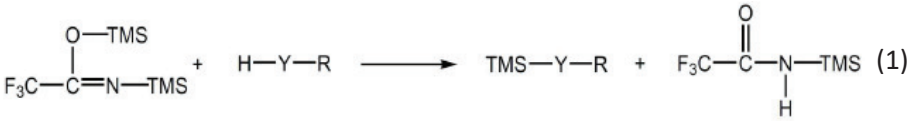


Figure 1. Chemical formula of N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA)

According to some studies, by replacing acidic protons with nonpolar TMS groups, polarity of an amino acid is reduced and its volatility is increased [11-17] which is an advantage in gas chromatographic analysis.



(1)

Equation (1) represents derivatization (silylation reaction) of amino acids in which TMS=Si(CH3)3, Y=O, S, NH, COO and R= alkyl or aryl radical.

The advantage of derivatization with TMS is that it requires a single step, while other derivatization methods usually have two or more reaction steps.

The major drawbacks of TMS derivatives are the sensitivity to moisture and the derivatization of Gly and Glu, which are affected by the polarity of the solvent [18-20]. For example, both the di-trimethylsilyl derivative and the trimethylsilyl derivatives of glycine are obtained after derivatization using acetonitrile or acetone as solvents [18].

Amino acids derivatized with TMS are typically analyzed using gas chromatography coupled with mass spectrometry (GC / MS) and identified according to a combination of retention times and mass spectra [21, 22].

GC / MS is a powerful tool for separating and identifying components in complex mixtures and plays a central role in amino acid analysis, presenting the advantages of improved resolution, sensitivity and quantification of smaller amounts in the sample as well as rapid analysis.

The literature reports contradictory results with regard to optimal amino acid derivatization conditions using BSTFA. These results may be related to several limitations that were observed in those studies, such as the limited number of iterations for each of the derivatization conditions [22-24], using either peak height or peak area without evaluating the calibration linearity, sensitivity, or the limit of detection (LOD), using either SCAN mode or SIM mode, or using relatively long GC temperature programs (e.g. 54 min [25], 62 min [26], and 90 min [27]).

The derivatized amino acids were analyzed with a FOCUS GC gas chromatograph coupled with a DSQ II MS mass spectrometer, TriPlus AutoSampler, flame ionization detector, Split/Split less injector, XCalibur Thermo Scientific software equipped with a nonpolar capillary column: TR 5MS: 5% phenyl – 95% dimethylpolysiloxane, length: 60 m, inner diameter: 0.32 mm, film thickness: 0.25 µm; ultra-high purity helium was used as carrier gas at a constant flow rate of 20 ml/min. The column transfer flux was 1.0 ml/min.

The column temperature was programmed to increase from 700°C to 1700°C at a rate of 100°C/min, and then was raised to 280°C at a rate of 30°C/min, the temperature at which the elution took place for 8 min. Total running time was 21mins 66sec.

The sample was injected at 280°C in split injection mode (ratio 1:20) using an injection volume of 1 µl.

For each sample, triple injections were performed and the results were averages of the three determinations. Acetonitrile was also used as a control solvent before each injection. Three solvent ampoules, namely acetonitrile, methanol, acetone, were used successively as cleaning solvents for the autosampler injection syringe.

Amino acids were detected with a mass spectrometer in automatic scanning mode (SCAN), and the individual identification was performed using the specific retention times of the reference materials and the spectra library of the device. In Selected Ion Monitoring Mode (SIM), identification was made based on specific fragments of maximum intensity.

The mass spectrometer operated in full scan mode (SCAN) at m/z of 50 to m/z of 650, with a scan time of 0.3 s and in the selected ion monitoring mode (SIM) with a scanning time of 0.2 s.

VALIDATION OF THE METHOD

The validation method and the analysis procedure of the amino acid content were performed according to validation guides for EURACHEM analytical methods.

The concentration range is the interval between the lower and upper concentration of the analyte in the assay for which it has been demonstrated that the procedure has an appropriate level of precision, accuracy and linearity.

Linearity is the ability of an analytical method to yield results proportional to the concentration of the analyte in the sample.

As a result, these parameters have been studied for the four amino acid standards which have an important share in collagen hydrolysates, namely:

- Glycine;
- Proline;
- Glutamic acid;
- Aspartic acid.

To evaluate the linearity and sensitivity of the signal in relation to the concentration, eight linear calibrations were generated for each amino acid.

The calibration curves of each amino acid were plotted in the 0.625-5 µmol/ml range, and the linearity range for which the correlation coefficient that characterizes the regression line R2 was obtained, was examined visually.

The mass spectrometer operated in automatic scanning mode (SCAN) and selected ion monitoring mode (SIM). The mass spectrometer operated in full scan mode (SCAN) at m/z of 50 to m/z of 500, with a scan time of 0.3 s and in Selected Ion Monitoring Mode (SIM) with a scan time of 0.2 s.

The performance parameters of the reference amino acid method, concentrations, limit of detection (LOD), limit of quantification (LOQ) and calibration curves were statistically calculated using Excel 2010 and are shown in Table 1. All statistical tests were performed at a confidence level of 95% and k = 2.

Table 1: Performance parameters of the amino acid determination method

Performance parameters / Amino acid	Correlation coefficient R^2	Limit of detection LOD **µmol/ml**	Limit of quantification LOQ **µmol/ml**	Retention time
Glycine SCAN	0,9999	0.004365	0.014549	8,98
Glycine SIM	0,9996	0.01817	0.060565	8,98
Proline SCAN	0,9998	0.010724	0.035745	11,40
Proline SIM	0,9999	0.002622	0.00874	11,40
Glutamic acid SCAN	0,9995	0.001592	0.005308	13,59
Glutamic acid SIM	0,9999	0.003978	0.013261	13,60
Aspartic acid SCAN	0,9999	0.005337	0.01779	13,37
Aspartic acid SIM	0,9998	0.096521	0.321737	13,33

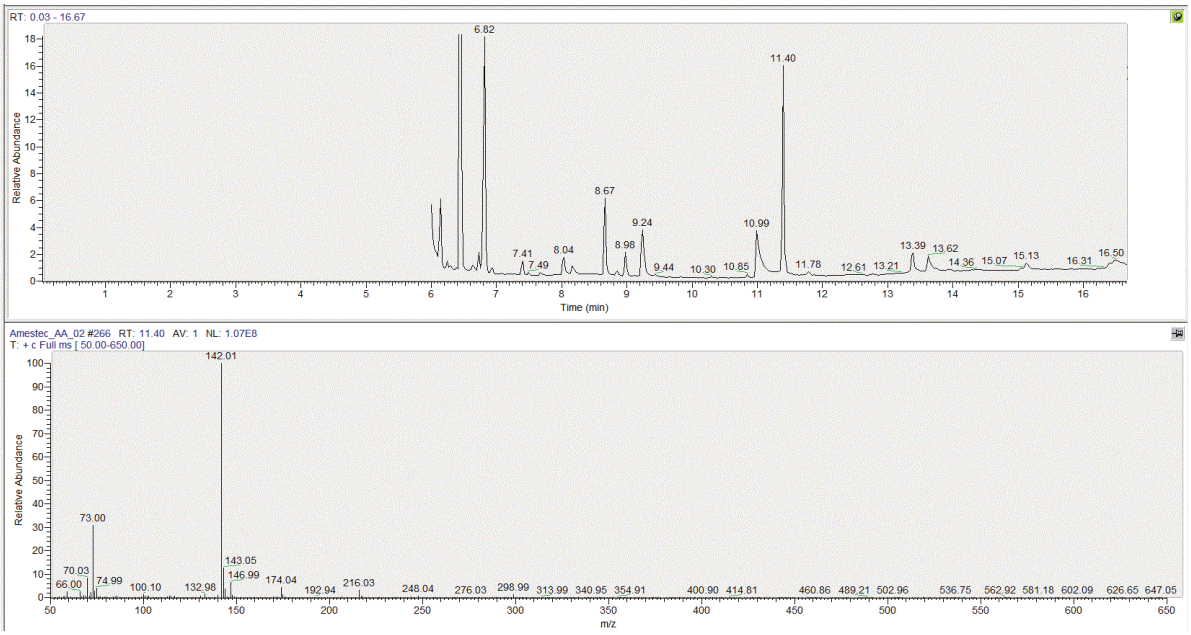


Figure 2. GC-MS chromatogram of standard amino acids in the mixture

ANALYSES OF BIOMATERIALS FOR MEDICAL USE

Seven samples of collagen materials for medical use were studied and physically and chemically characterized, the results are presented in the table below:

Table 2: Characterization of collagen products for medical use

Characteristics / Product	Dry substance, %	Ash*, %	Total nitrogen*, %	Appearance
Pancol L1	85,79	1,64	15,78	White spongy foil
Pancol L2	87,43	1,96	15,19	White spongy foil
Gevicol G1	87,57	2,72	16,45	Violet spongy foil
Gevicol G2	86,52	1,46	14,80	Violet spongy foil
Gel Zetta Skin 5	2,10	0,95	17,62	Transparent gel
Collagen hydrolysate HO 8 (Liquid)	28,76	0,56	17,62	Yellow liquid
Collagen hydrolysate HO8 (Atomized)	95,64	0,45	17,70	Yellowish powder

*values are recalculated free of volatile matter

The samples were hydrolyzed with 6M hydrochloric acid at 100°C±2°C for 24 hours, and the aliquot of the hydrolyzed sample was dried with nitrogen gas.

The derivatization was performed with N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA), and subsequently the derivatized amino acids were analyzed with a FOCUS GC gas chromatograph coupled with a DSQ II MS mass spectrometer, TriPlus AutoSampler, flame ionization detector, Split/Split less injector, XCalibur Thermo Scientific software, equipped with a non-polar capillary column.

Samples of collagen materials for medical use, hydrolysed, derivatized and analyzed by the mass spectrometric gas chromatographic method are shown in Figures 3-14, and the amino acid composition in Tables 3-9.

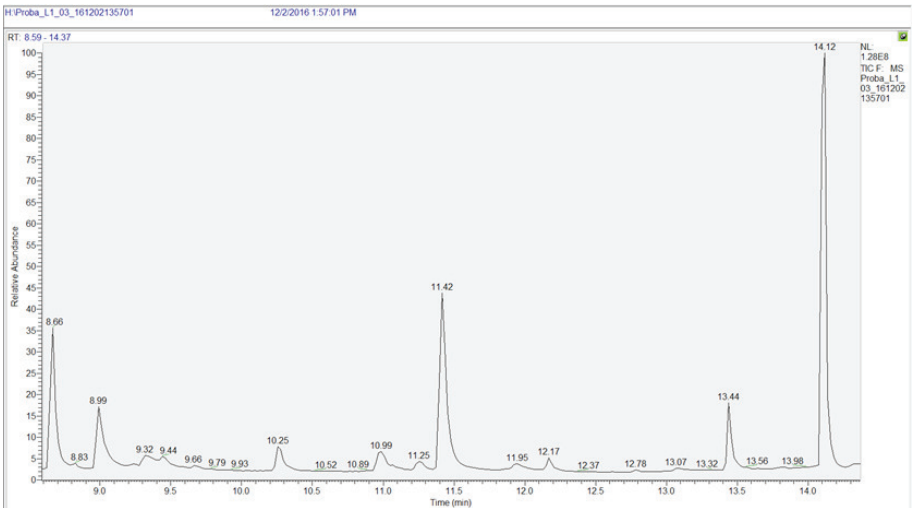


Figure 3. GC-MS profile of amino acids in Pancol L1

The amino acid composition of the Pancol L1 sample and the retention times obtained are shown in Table 3.

Table 3: Amino acid composition of Pancol L1 sample

SAMPLE L1_pancol				
No.	Retention time	Compound	Majority fragment	%
1	8.66	Alanine	116	13.20
2	8.99	Glycine	102	8.40
3	10.25	Valine	144	3.16
4	10.99	Leucine	158	3.85
5	11.25	Isoleucine	158	1.21
6	11.42	Proline	142	22.94
7	11.95	Serine	204	0.92
8	12.17	Threonine	218	1.15
9	13.44	Hydroxyproline	230	5.33
10	14.12	Tartaric acid	147	39.85

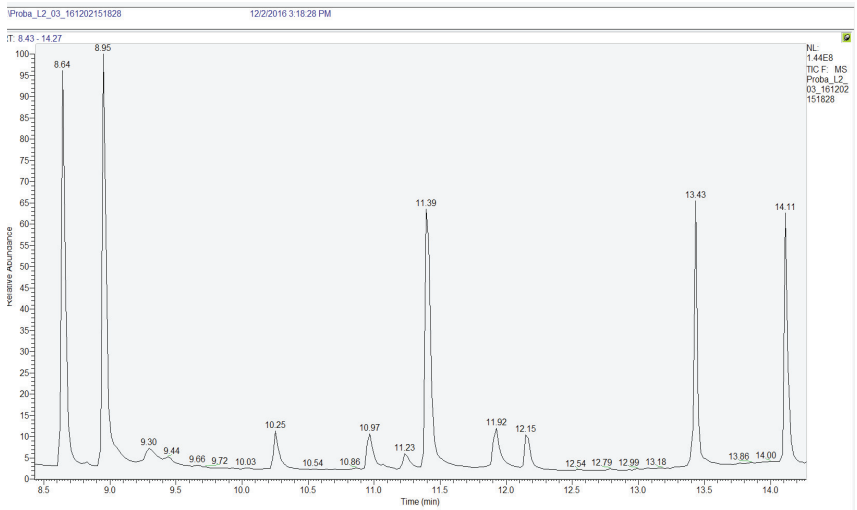


Figure 4. GC-MS profile of amino acids in Pancol L2

Table 4: Amino acid composition of Pancol L2 sample

SAMPLE L2_pancol				
No.	Retention time	Compound	Majority fragment	%
1	8.64	Alanine	116	20.79
2	8.95	Glycine	102	24.56
3	10.25	Valine	144	2.36
4	10.97	Leucine	158	3.14
5	11.23	Isoleucine	158	1.26
6	11.39	Proline	142	18.72
7	11.92	Serine	204	2.89
8	12.15	Threonine	218	2.19
9	13.43	Hydroxyproline	230	11.47
10	14.11	Tartaric acid	147	12.62

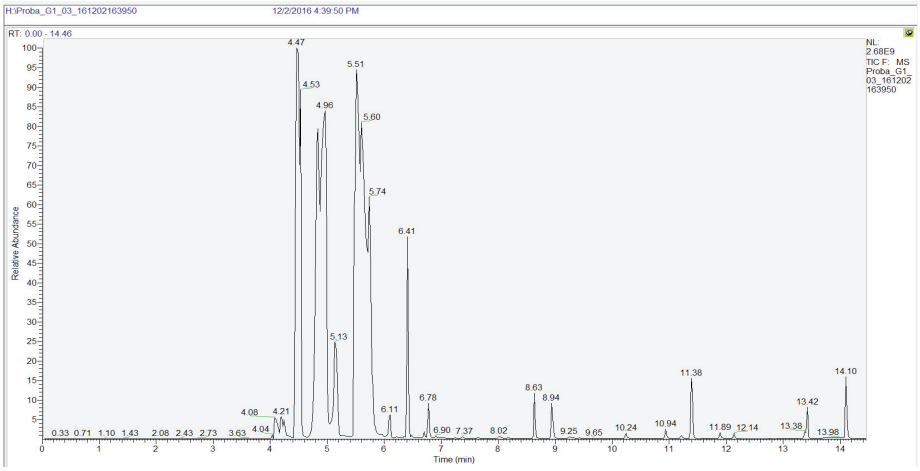


Figure 5. GC-MS chromatogram of Gevicol G1 sample

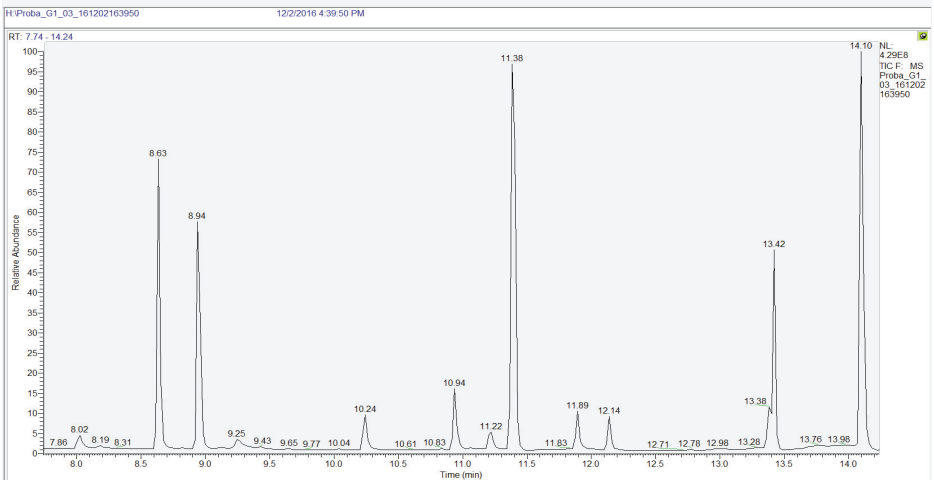


Figure 6. GC-MS profile of amino acids in Gevicol G1

Table 5: Amino acid composition of Gevicol G1 sample

SAMPLE G1_gevicol				5.83 mg
No.	Retention time	Compound	Majority fragment	%
1	8.02	Lactic acid	147	1.11
2	8.63	Alanine	116	13.70
3	8.94	Glycine	102	13.69
4	10.24	Valine	144	2.35
5	10.94	Leucine	158	3.54
6	11.22	Isoleucine	158	1.27
7	11.38	Proline	142	27.33
8	11.89	Serine	204	2.19
9	12.14	Threonine	218	1.74
10	13.38	Acid Aspartic	232	2.37
11	13.42	Hydroxyproline	230	8.09
12	14.1	Tartaric acid	147	22.61

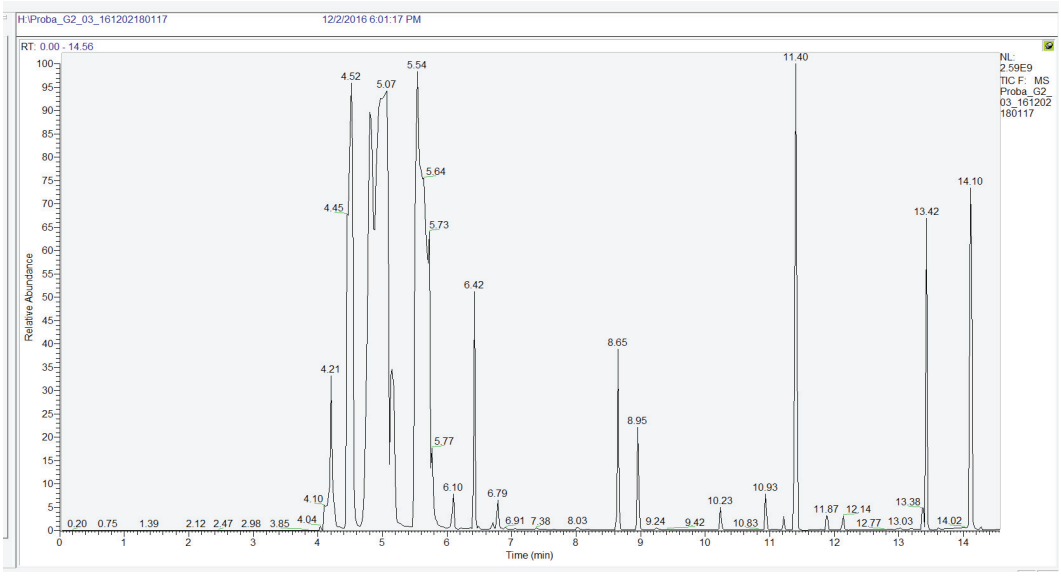


Figure 7. GC-MS chromatogram of Gevicol G 2 sample

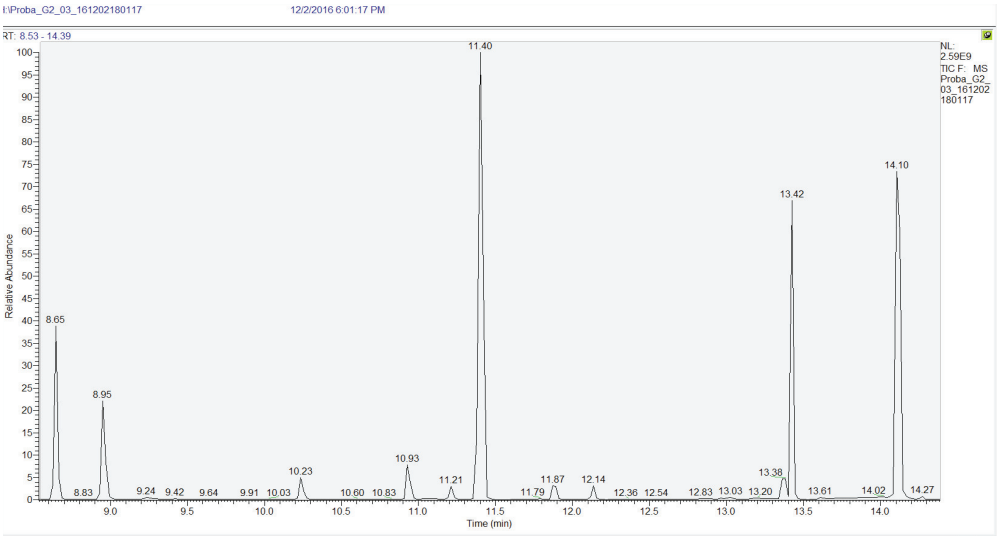


Figure 8. GC-MS profile of amino acids in Gevicol G2

Table 6: Amino acid composition of Gevicol G2 sample

SAMPLE G2_gevicol				5.13 mg
No.	Retention time	Compound	Majority fragment	%
1	8.65	Alanine	116	9.41
2	8.95	Glycine	102	6.50
3	10.23	Valine	144	1.39
4	10.93	Leucine	158	2.23
5	11.21	Isoleucine	158	0.72
6	11.4	Proline	142	34.69
7	11.87	Serine	204	1.16
8	12.14	Threonine	218	0.76
9	13.36	Aspartic acid	232	1.75
10	13.42	Hydroxyproline	230	14.20
11	14.1	Tartaric acid	147	27.18

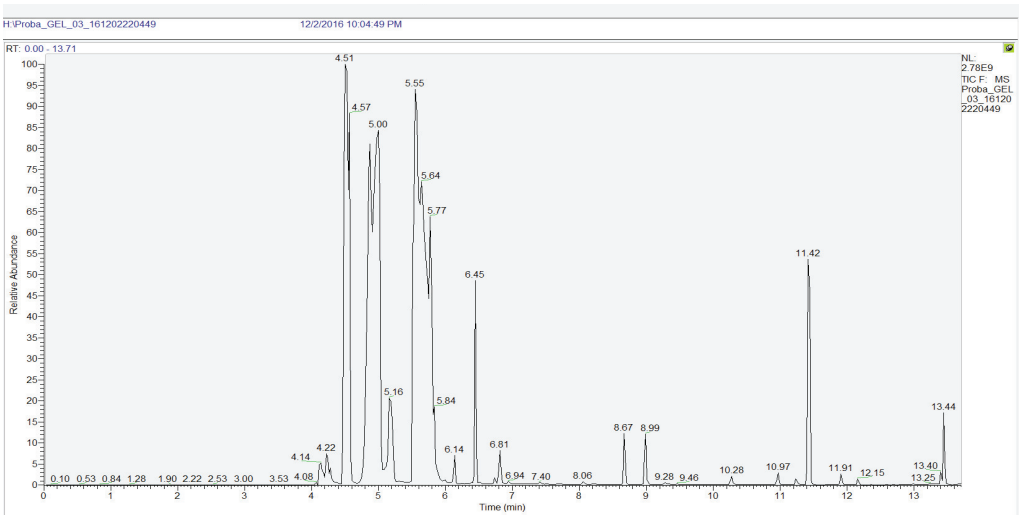


Figure 9. GC-MS chromatogram of Gel zeta sample

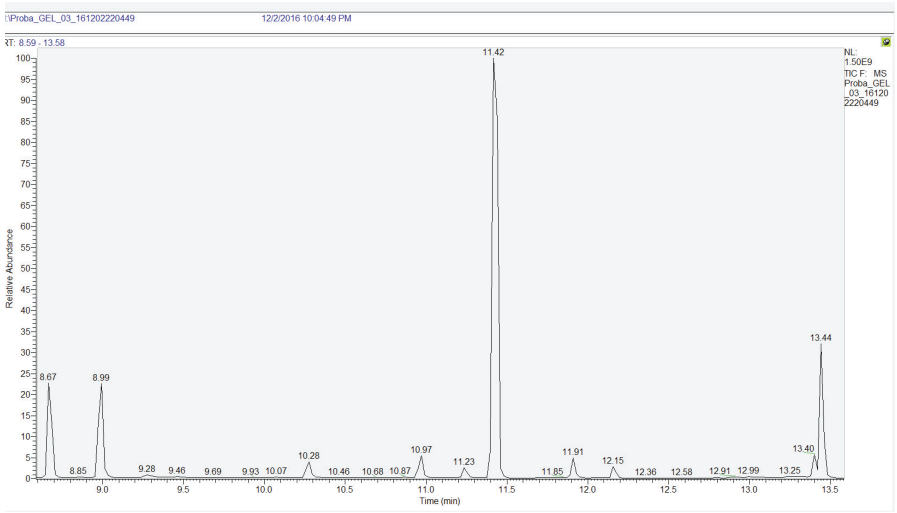


Figure 10. GC-MS profile of amino acids in Gel zeta

Table 7: Amino acid composition of Gel zeta sample

SAMPLE GEL_zeta				
No.	Retention time	Compound	Majority fragment	%
1	8.67	Alanine	116	10.13
2	8.99	Glycine	102	10.65
3	10.28	Valine	144	1.91
4	10.97	Leucine	158	2.22
5	11.23	Isoleucine	158	1.01
6	11.42	Proline	142	57.25
7	11.91	Serine	204	1.67
8	12.15	Threonine	218	1.07
9	13.4	Acid Aspartic	232	1.67
10	13.44	Hydroxyproline	230	11.19
11	14.12	Tartaric acid	147	0.43
12	14.32	Phenylalanine	218	0.32
13	15.77	Lysine	174	0.49

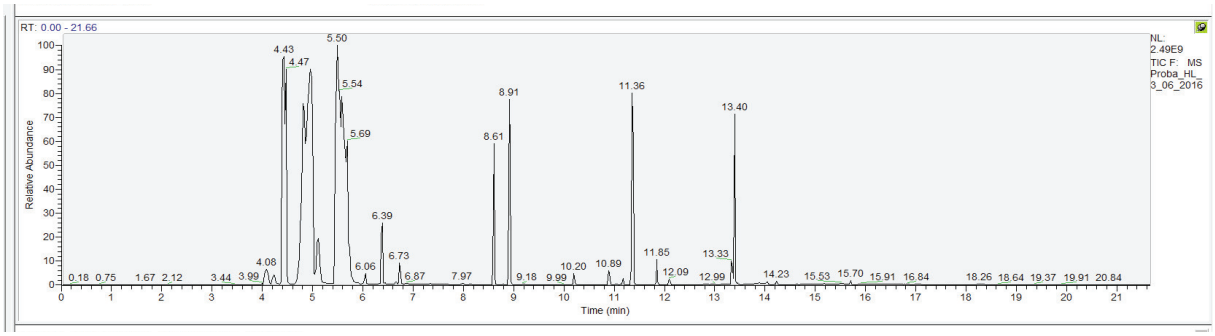


Figure 11. GC-MS chromatogram of liquid HO8 sample

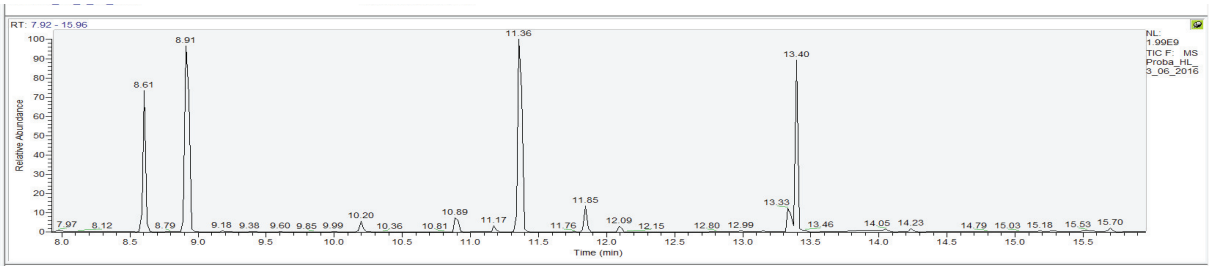


Figure 12. GC-MS profile of amino acids in liquid HO8

Table 8: Amino acid composition of liquid HO8 sample

Sample: Liquid hydrolysate HO8				
No.	Compound	Majority fragment	Retention time	%
1	Alanine	116	8.61	14.36
2	Glycine	102	8.91	28.65
3	Valine	144	10.2	1.30
4	Leucine	158	10.89	2.26
5	Isoleucine	158	11.17	0.66
6	Proline	142	11.36	30.04
7	Serine	204	11.85	2.63
8	Threonine	218	12.09	0.82
9	Aspartic acid	232	13.33	2.96
10	Hydroxyproline	230	13.4	14.98
11	Glutamic acid	156	14.05	0.51
12	Phenylalanine	218	14.23	0.38
13	Lysine	174	15.7	0.47

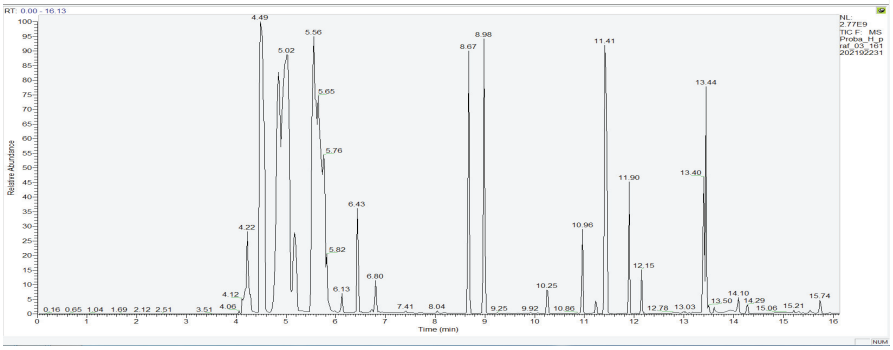


Figure 13. GC-MS chromatogram of atomized HO8 sample

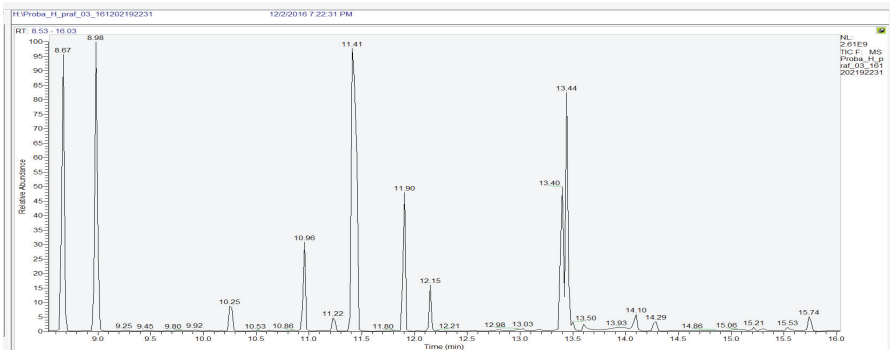


Figure 14. GC-MS profile of amino acids in atomized HO8

Table 9: Amino acid composition of atomized HO8 sample

SAMPLE HO8 atomized hydrolysate				
No.	Retention time	Compound	Majority fragment	%
1	8.67	Alanine	116	14.96
2	8.98	Glycine	102	16.92
3	10.25	Valine	144	1.90
4	10.87	Glycerol	147	0.03
5	10.96	Leucine	158	4.18
6	11.22	Isoleucine	158	0.87
7	11.41	Proline	142	29.67
8	11.9	Serine	204	6.98
9	12.15	Threonine	218	1.95
10	13.4	Acid Aspartic	232	8.98
11	13.44	Hydroxyproline	230	11.02
12	14.1	Tartaric acid	147	0.96
13	14.29	Phenylalanine	218	0.66
14	15.74	Lysine	174	0.91

CONCLUSIONS

Validation of the method included assessment of independent acid hydrolysis procedures, amino acid derivatization and GC/MS analysis.

To fully evaluate the performance of these procedures, a standard amino acid mixture allowed the GC/MS method to be verified, including derivatization and effects of acid hydrolysis on amino acids.

The validated analytical method for determining the amino acid content of collagen biomaterials for medical purposes fulfills all the conditions necessary for use and application for the intended purposes.

It was shown that this method can be used both for collagen hydrolysates and gels as well as for spongy collagen matrices because it is repeatable and reproducible.

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