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Characteristics of Collagen Preparations from Leather Wastes by the High Pressure Liquid Chromatography Method

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Abstract
The raw trimming waste from the leather industry is considered potential hazardous waste as a consequence of the chrome tanned leather process. On the other hand, leather waste contains a large amount of precious protein – collagen, which has many uses. Nowadays, collagen preparations obtained from leather waste are available on the market. This paper presents a procedure for the determination of amino acids in five collagen preparations of animal origin. Recent improvements in HPLC-based methods for analysing amino acids have made it feasible to analyse different sample types accurately. In this study the 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatization procedure was applied. The amino acid analysis indicated the presence of 18 amino acids (Asp, Ser, Glu, His, Gly, Arg, Thr, Ala, Pro, Cys, Tyr, Val, Lys, Met, Ile, Leu, Phe and Hyp) in the collagen samples. Glycine, alanine, proline and hydroxyproline were the most abundant amino acid, whereas the lowest contents corresponded to serine, tyrosine, valine and izoleucine. The analysis proposed can be used with confidence in collagen quality control to guarantee appropriate amino acid content.

Key words: *amino acid analysis, collagen, leather wastes, HPLC.*

Introduction

The solid waste produced in the leather industry represents huge amounts on a worldwide basis. For every ton of raw material processed, 50 kg of raw trimmings are generated [1]. Globally, on average 6.82 mn tons of raw hides and skins are processed into leather [2]. At 5%, which is about 340 thousand tons of raw trimming wastes are generated annually, which is potentially hazardous because of the chromium it contains. Also, the leather waste disposed in landfills can cause leachate problems [3]. This is why in several countries it is characterised and treated as a chemical waste [4]. It is imperative to develop a simple method for the utilszation of this waste to provide a practically feasible and economically viable solution. In some countries (China, Spain), raw trimming wastes are used as raw material for the production of various products, such as glue, collagen peptides, industrial gelatin, feed and fertilisers [1].

Collagen protein is a biopolymer which has various advantages. It has wide raw material resources and is renewable, degradable and biocompatible. Thus, it is extensively used in making health care products, food [5], cosmetics [6], biomedical materials [7], animal feed and agriculture [8]. A characteristic feature of collagen molecules is their structure, which is a triple right-handed helix formed by three left-handed α polypeptide chains. Until 2010, 29 types of this protein were identified, differing mainly in the conformation of the α chains and their mutual combination in the triple helix [9]. Despite the identification of many types of collagen proteins, only a few of them are used in the construction of biomaterials, among which are the fibrous collagen types I, II, III, V and IX. The most common collagen protein is type I collagen, which is a protein with strong and thick fibers, with high thermal stability and it is characterised by high extensibility and strength [10, 11].

Nowadays, collagen preparations obtained from different source are available on the market, whose potential applications depend on the content and quality of the ingredients that they contain. It is widely accepted that the most important characteristics of a collagen preparation designed for cosmetic and medical purposes are its molecular mass distribution and the amino acid content of the collagen fragments. Suitable values of these characteristics allow good compatibility with the other components of the final product as well as its desired softening effect on the skin because of hydration and good penetration ability [12, 13].

The first method used to determine the individual amino acids was paper chromatography [14]. Gas chromatography methods were subsequently developed [15], based on the separation of free amino acids using ion-exchange columns, followed by post-column derivatisation with ninhydrin, which were introduced a long time ago [16]. In the past 20 years, however, there has been a rapid increase in the use of HPLC systems with analytical detectors [17]. Several years ago a new method for amino acid analysis using the novel reagent AQC (6-aminoquinolyl-N-hydroxysuccinimidylcarbamate) was introduced Notable advantages of this method include rapid, quantitative and linear reactions, highly stable substituted urea adducts, good reaction yields and highly sensitive detection. Moreover, the derivatisation procedure takes approximately one minute, and the amino acid derivatives, including secondary amino acids, are quite stable. It is currently one of the most modern methods for the determination of amino acids [18, 19].

Therefore, the objective of this work was to identify the amino acids in collagen preparations obtained from leather waste. To achieve this goal, e precolumn derivatisation with a new reagent – AQC,

Table 1. *Characteristics of collagen preparations.*

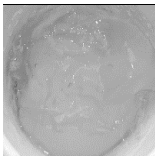
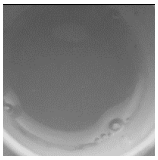
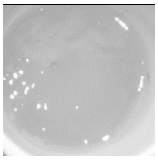
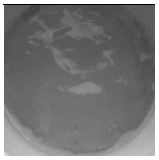
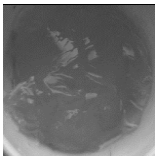
Parameter	Collagen preparations				
	C2	C3	C3W	C4	C5
Appearance					
Colour	Gold	Grey	Pale yellow	Beige	Yellow
Odour	Very weak	Very weak	Weak	Weak	Very weak
pH, –	3.70	3.70	3.70	3.80	3.80
TA, %	1.23	1.69	1.62	1.33	1.57
DR, %	3.49	2.85	2.78	3.81	3.28
Physical state	Gel	Gel	Viscous liquid	Gel	Gel
Trade Names and forms	Soluble Collagen	Soluble Collagen	Soluble Collagen	Soluble Collagen with Elastin	Soluble Collagen with Elastin

Table 2. *Selected gradient program for the elution of 17 amino acids.*

Time, min	Eluent A, %	Eluent B, %
0.0	100	0
0.5	98	2
15.0	93	7
19.0	70	30
32.0	67	33
33.0	67	33
34.0	0	100
37.0	0	100
38.0	100	0
64.0	100	0

HPLC analysis and spectrophotometric analysis (for hydroxyproline, Hyp) were applied. This method provides an important advance in amino acid analysis compared with other techniques reported. The amino acid profile of collagen may provide useful and basic information for the cosmetic and medical industries.

Materials and methods

Chemicals

Sulfuric acid, monohydric citric acid, sodium hydroxide, 60% perchloric acid, propanol, isopropanol and sodium acetate were obtained from Chempur (Poland). Chloramine-T trihydrate (N-chloro-4-toluenesulfonamide, sodium salt), 4-dimethylaminobenzaldehyde and L-hydroxyproline as a standard were obtained from Fisher Scientific (UK). Reagent solutions for spectroscopy analysis were prepared according to literature [20]. Acetonitrile (HPLC super gradient grade) and methanol (HPLC super gradient grade) were obtained from J.T. Baker (Holland). The Waters AccQ•Tag Chemistry Package was obtained from Waters (Milford, MA, USA). The chemistry package consists of a Waters AccQ•Fluor Reagent Kit (Waters AccQ•Fluor Borate Buffer, Waters AccQ•Fluor Reagent Powder, Waters AccQ•Fluor Reagent Diluent), Waters AccQ•TagAmino Acid Analysing Column (Nova-Pak™ C18, 4 μm, 150 × 3.9 mm), Waters AccQ•Tag Eluent A Concentrate (acetate–phosphate aqueous buffer) and Waters Amino Acid Hydrolysate Standard. Each ampoule (1 cm^3) of Amino Acid Standard contains a 2.5 mM mixture of the 17 hydrolysate amino acids (aspartic acid ((Asp)), serine ((Ser)), glutamic acid ((Glu)), glycin ((Gly)), histidine ((His)), arginine ((Arg)), threonine ((Thr)), alanine ((Ala)), proline ((Pro)), cysteine ((Cys)), tyrosine ((Tyr)), valine ((Val)), methionine ((Met)), lysine ((Lys)), isoleucine ((Ile)), leucine ((Leu)), and phenylalanine ((Phe)), with the exception of cysteine (1.25 mM). Reagent solutions for HPLC analysis were prepared according to the Waters AccQ•Tag Chemistry Package Instruction Manual (Waters, Milford, MA, USA) [21].

Apparatus

Amino acid analysis was carried out using a Shimadzu Prominence-i LC-2030C 3D system equipped with an LC-2030 pump, autosampler injector, column oven and LC-2030/2040 PDA detector (Shimadzu, Kyoto, Japan). The HPLC system was controlled using the software Lab Solutions (Shimadzu, Kyoto, Japan). All samples were filtered using 0.45 μm membrane filters: Acrodisc Syringe Filter 13 mm, GHP (Waters, Milford, MA, USA). An ultrasonic cleaner – Sonic-2 (30-80 °C) (Polsonic, Warsaw, Poland) was used as a heating block. The hydroxyproline concentration was determined using spectrophotometric analysis. The absorbance was measured at 557 nm on a UV-9200 – UV/VIS spectrophotometer (RayLeigh, Pekin, China). The pH was measured with a CP-411 pH meter (Elmetron, Zabrze, Poland).

Samples' characteristics and preparations

A collagen preparation for the purpose of cosmetics was purchased from a company on the Polish market. This company specialise in producing fibrous proteins of animal origin in a natural and hydrolysed form. Collagen preparations were obtained by extraction of protein from calf connective tissue, available as a concentrated dispersion. The collagen samples tested had the following properties: total ash (TA): 1.23-1.69%, dry residue (DR): 2.78-3.81%, and molecular weight: approximately 360 Daltons for all samples tested. Also, two collagens had an addition of elastin (C4, C5). Samples' characteristics are presented in ***Table 1***. Physico-chemical tests of C2-C5 preparations were performed according to the BN-85 6149-03 standard [22].

All collagen preparations were subjected to acid hydrolysis at 105 °C for 16 hours [1]. The procedure of hydrolysis was followed except that sulfuric acid was used instead of hydrochloric acid. After hydrolysis of the collagen preparations (C2-C5) the amino acids were determined by the HPLC method, described below. The concentration of hydroxyproline was determined by using L-hydroxyproline as the standard, according to literature [20].

Derivatization procedure

Ten microliters of a filtered hydrolysated sample or calibration standard were

transferred to a 1.5 cm^3 vial, 70 µL of borate buffer added, and the solution was briefly vortexed. Then 20 µL of reconstituted AccQ. Fluor reagent (3 mg/cm^3 in acetonitrile) was added and the mixture immediately vortexed for several seconds. The vial was closed and left to stand for one minute at room temperature. It was then heated in a heating block at 55 °C for 10 min. Heating converts a minor by-product of tyrosine to a major mono-derivatized compound. Derivatives were stable at room temperature for up to 1 week. A 10 µL injection of the derivatized standard contained 50 pmol of each amino acid derivative (except cysteine, at 25 pmol).

Chromatographic conditions

AccQ•Tag amino acid column Nova-Pak C18, 4 µm (150 × 3.9 mm) from Waters was used. The column was thermostated at 37°C, where the flow rate was 1.0 cm^3/min. and 10 µL the injection volume (concentration of amino acids 5-200 pmol). A gradient mobile phase was used for chromatography. The mobile phase consisted of eluent A (prepared from Waters AccQ•Tag Eluent A concentrate, adding 200 cm^3 of the concentrate to 1 L of distilled water, eluent B (60% acetonitrile). Satisfactory separation of the amino acids was achieved using the gradient profile in ***Table 2***. Before beginning the gradient, the column was equilibrated in 100% Eluent A for 10 min at 1 cm^3/min. Detection was carried out with a UV detector at 254 nm.

Results and discussion

In this study, precolumn derivatisation with HPLC analysis (for 17 amino acid) and spectrophotometric analysis (for hydroxyproline) were used to identify the amino acid composition in collagen preparations. The elution profile of the amino acid standard obtained on the Prominence-i system (Shimadzu) and Nova-Pak C18 column are shown in ***Figure 1***.

This chromatogram clearly shows the baseline separation of all derivatised amino acids in 65 minutes. The following 17 hydrolysate amino acids can be identified: (2) Aspartic acid, (3) Serine, (4) Glutamic acid (5) Histidine (6) Glycine, (7) NH_3 – ammonium, (8) Arginine, (9) Threonine, (10) Alanine, (11) Proline, (12) Cysteine (13) Tyrosine, (14) Valine, (15) Lysine, (16) Metionine, (17) Isoleucine, (18) Leucine & (19) Phenylalanine.

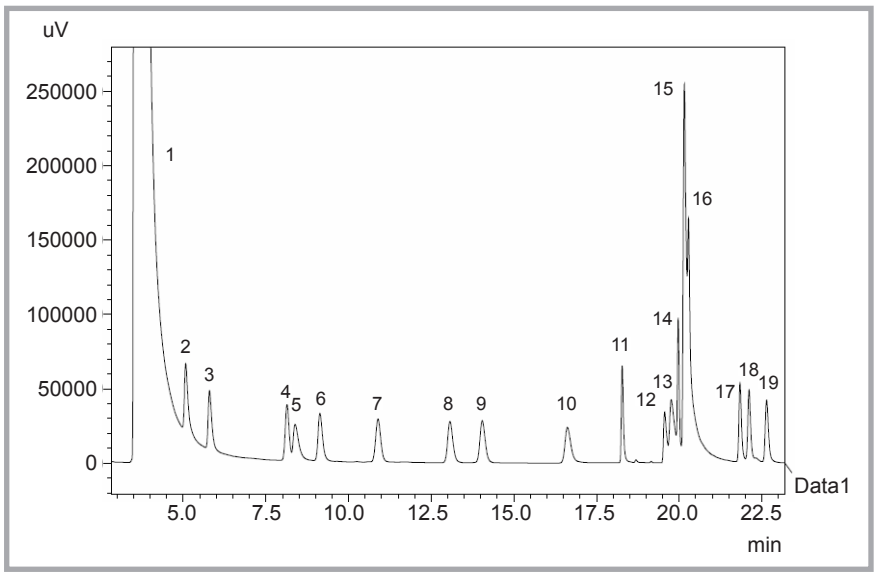


Figure 1. *Chromatogram of the AQC-derivatised amino acid standard with UV detection. Peaks: (1) AMQ, (2) Asp, (3) Ser, (4) Glu, (5) His, (6) Gly, (7) NH_3, (8) Arg, (9) Thr, (10) Ala, (11) Pro, (12)Cys, (13) Tyr, (14) Val, (15) Lys, (16) Met, (17) Ileu, (18) Leu, (19) Phe.*

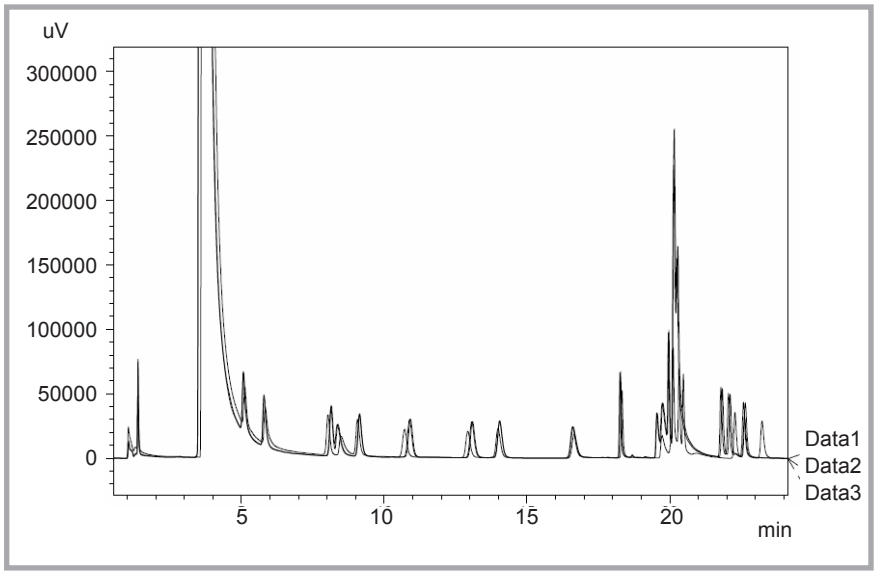


Figure 2. *Overlay of 3 replicate runs of the AQC-derivatized amino acid standard (Data 1, Data 2, Data 3) measured with UV detection.*

AMQ, as a by-product of hydrolysis, producing a large peak – 1 that is easily resolved chromatographically. Other hydrolysis products do not interfere with the analysis.

Validation of the method was performed by precision evaluation. The precision of the method is expressed as the relative standard deviation (RSD) of the retention time and peak area for three consecutive assays performed on the same day [23]. The overlay of three chromatograms of the standard in ***Figure 2*** shows the satisfactory reproducibility and robustness of the method.

The values of relative standard deviation of the retention time were in the range from 0.37 to 0.67. The values of relative standard deviation of peak area were in the range from 0.19 to 1.23. RSD values obtained in our work are in the range reported by other authors using AQC with UV detection [23, 24]. Retention time stability is in the range of < 0.5% RSD and peak area precision < 2.5% RSD [24]. Only the results of the RSD of the retention time for Glu (0.65) and His (0.67) slightly exceed a value of 0.5. Nevertheless, it can be stated that the results obtained confirmed the reproducibility and robustness of the method.

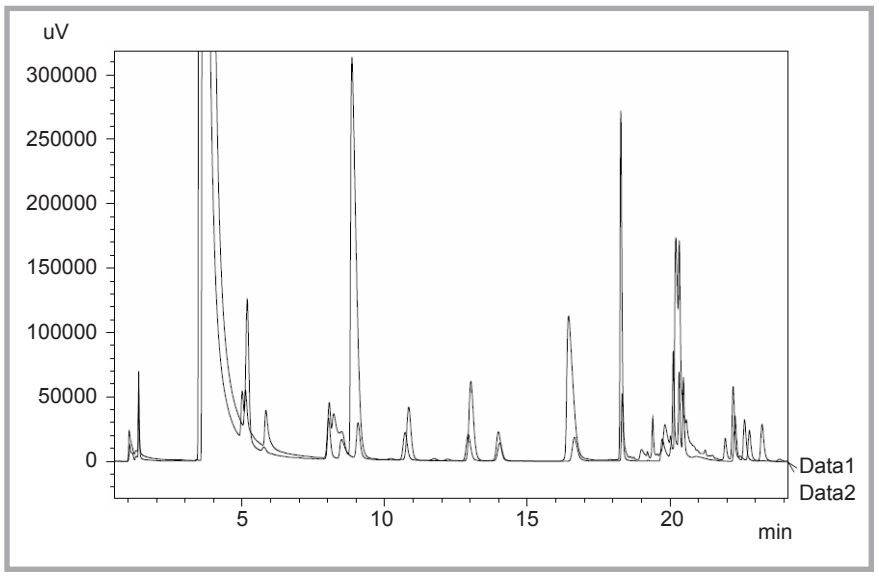


Figure 3. *Overlay of two chromatograms measured with UV detection: Data 1 – AQC-derivatised amino acid standard, and Data 2 – AQC-derivatised hydrolysed collagen sample (Collagen C5).*

Figure 3 shows superimposed chromatograms of C5 collagen and standard samples. Each amino acid was identified by comparing the retention time with the standard sample. The amino acid profile of collagen samples revealed about 24 peaks, most of which for the collagen sample coincide with the peaks for the standard. Comparing the standard and sample peak profiles, the following 17 amino acids were identified: Asp, Ser, Glu, His, Gly, Arg, Thr, Ala, Pro, Cys, Tyr, Val, Lys, Met, Ile, Leu, Phe. The hydroxyproline concentration was determined using spectrophotometric analysis. Analysing ***Figure 3***, it clearly shows that detection by PDA is completely sufficient for amino acid concentrations, and there is no need for a fluorescence detector in this case.

The amino acid contents (pmol/μL) of the samples analysed are reported in ***Table 3***, showing the arithmetic mean of three replicates of amino acid concentrations and the standard deviation calculated. All collagen samples analysed came from calf connective tissue, which means the amino acid profile has to be similar in every sample tested. The samples analysed showed high values of amino acids but differed in amino acid concentrations. Serine (5.09-8.40 pmol/μL), tyrosine (14.51-84.45 pmol/μL), valine (27.63-77.81 pmol/μL) and isoleucine (7.55-40.16 pmol/μL) showed the lowest contents, while Glycine (517.01-1339.46 pmol/μL), alanine (166.12-588.99 pmol/μL), proline (213.79-489.07 pmol/μL) and hydroxyproline (181.19-379.16 pmol/μL) were the most abundant amino acids in the samples tested. It should be mentioned that the Gly-Pro-Hyp sequence occurs very frequently in the structure of I-IV collagen [25]. For this reason, the amino acids mentioned occur in the largest amount in collagen, which is confirmed by the results obtained. Moreover, the content of glycine in samples C2, C3, C3W, C4 and C5 equaled 29.63%, 29.41%, 30.32%, 33.12% and 32.59%,, respectively. These results are also consistent with the literature, which states that glycine comprises about 30% of all amino acids constituting collagen [25]. This confirms the correctness of the tests carried out.

The greatest differences were observed for collagen C5. In this case, higher concentrations of almost all amino acids, except Glu, His, Val & Liz, were observed in comparison with other samples. Collagen C2 was characterised by the lowest concentration of almost all the amino acids identified. In this case, three amino

Table 3. *Concentration of amino acid in hydrolysed collagen measured with UV detection.* ***Note:*** ** nd – not detected, ** SD – standard deviation.*

Amino acid	Mean of amino acid concentration, pmol/μL									
	C2	SD**	C3	SD**	C3W	SD**	C4	SD**	C5	SD**
Asp	167.59	2.07	214.25	4.15	180.08	2.07	162.21	4.15	251.95	6.22
Ser	nd*	0.03	5.09	0.24	6.44	0.47	5.73	0.24	8.40	0.71
Glu	95.31	2.23	158.96	4.57	93.23	4.57	86.02	4.57	102.15	2.28
His	72.36	2.38	116.28	2.38	161.66	4.76	175.04	2.38	135.08	4.76
Gly	517.01	45.20	863.03	22.6	814.73	22.61	1037.28	22.61	1339.46	45.21
Arg	102.94	6.73	175.28	2.24	158.46	2.24	145.14	4.48	224.42	6.73
Thr	23.10	4.56	38.85	2.28	34.35	4.56	31.25	4.56	48.66	4.56
Ala	166.12	0.22	297.08	2.18	248.46	4.37	502.58	4.37	588.99	4.37
Pro	213.79	0.23	295.16	2.29	289.53	4.59	371.23	31.69	489.07	22.96
Cys	20.58	2.34	37.38	2.37	15.15	4.74	64.05	23.70	73.42	23.70
Tyr	nd*	–	14.51	0.32	16,63	0.64	50.95	6.44	84.45	3.22
Val	nd*	–	77.81	0.33	55.53	1.57	24.87	3.27	27.63	1.63
Liz	88.91	0.01	176.66	1.72	123.49	2.62	72.99	3.44	77.54	1.72
Met	23.24	0.76	46.18	3.50	32.28	3.53	19.08	7.07	70.85	4.51
Ile	7.55	0.93	15.89	2.28	9.79	1.85	35.54	1.85	40.16	4.28
Leu	30.75	0.45	52.90	0.45	47.48	0.45	111.84	2.27	112.89	2.27
Phe	13.67	4.18	24.56	2.09	20.83	2.09	55.18	2.09	61.94	2.09
Hyp	201.94	0.46	324.26	0.69	379.16	0.23	181.19	2.29	372.45	2.29
Total	1744.86		2934.13		2687.28		3132.17		4109.51	

acids (Ser, Tyr and Val) were not identified, most likely because of the low, undetectable concentration in the formulation tested. Differences detected in the concentration of amino acid in the samples tested could suggest different properties of the collagen extracted. A similar observation was made by Dimova et al. [12], whoanalysed amino acid profiles in collagen preparation (KKI). The collagen tested for the purposes of cosmetics was prepared from calfskin of healthy animals at the Institute of Organic Chemistry, BAS. Amino acid analysis of KKI was carried out after hydrolysis in 6N HCI at 105 °C for 16 hours. The data show the characteristics for collagen: a high content of glycine, alanine & proline, the presence of hydroxyproline, as well as the absence of cysteine [12]. A similar high value of glycine, alanine and proline was also obtained for cow skin by Ward and Courts [26]. Thus, it can be concluded that the collagen preparations tested (C2-C5) were characterised by a typical amino acid composition for calfskin. However, the content of each amino acid in these preparations was different.

Conclusions

1. Collagen preparations tested (C2-C5) were characterised by a typical amino acid composition for calfskin. High values of glycine, alanine, proline and hydroxyproline were noted in all samples.
2. The method developed shows the quite fast and simultaneous determination of 17 AQC derivatised amino acids.
3. The analysis proposed can be used with confidence in collagen quality control to guarantee appropriate amino acid content. The highest value of glycine, proline, alanine and hydroxyproline in the C5 preparation shows that it is the preparation with the most advantageous amino acid composition.

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