Experimental Observations About Improving the Properties of Collagen Extracts for Applications in Agriculture

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This study highlights the fact that some properties of collagen extracts obtained by thermo-chemical and enzymatic processes from leather by-products may be modified and corrected during concentration or filtration operations in order to achieve performance specific to applications in agriculture (seed, soil and plant treatment in various phases of vegetation), as an alternative to treatment using synthetic substances. Concentration of collagen hydrolysates at atmospheric pressure leads to polydispersions with high molecular weights favourable to structuring collagen films, that will gradually release organic nitrogen, for plant nutrition in various vegetation stages. Concentration under vacuum results in polydispersions with low molecular weights, richer in free amino acids and oligopeptides that can penetrate cell membranes. Also, filtration under vacuum using low-porosity membranes (0.45-0.80 μm) determines deagglomeration, selection and concentration of particles with sizes ranging from 1 to 10 nm and from 10 to 100 nm, characteristic to amino acids and dipeptides, into filtrates.

Keywords: collagen extracts, polydispersion, amino acids, agricultural applications

Collagen is a fibrous protein that is the essential component of connective tissue, distributed in various forms in almost all living organisms. Over the years, 27 types of collagen have been found and analysed [1], but the most intensely studied is type I collagen for which the most diverse applications have been identified.

In recent years, collagen applications have evolved with the development of processing and characterisation techniques, intensely studying applications of collagen-based materials in bioeconomy and various niche industrial fields. Collagen-based materials obtained in various forms, from gelatins and collagen hydrolysates, to complex 2D/3D structures, membranes, hydrogels, aerogels have been largely created for applications in the medical field. Due to its special characteristics, such as biodegradability and low antigenicity [2], collagen has been directed towards tissue engineering [3, 4], implants [5-8], burn treatment, new drug delivery systems [9-11]. Recent studies show results of creating 3D collagen architectures such as high density gels for tissue engineering, starting from concentrated gelatin solutions, by renaturation of triple helix at 28-32 degrees Celsius for opaque gels or at temperatures below 25 degrees Celsius for transparent gels [12]. Ultrasonication applied in the nucleation phase was found to increase the self-assembling rate of collagen [13]. Other studies have examined the possibilities of improving collagen-based gel properties and inducing new functionalities by co-gelling with other types of proteins (from blood, milk, soy, cereal) [14], or by additivating with various vegetable extracts (e.g. papain used in dentistry to remove affected dentine and remineralization) [15]. Particularly interesting research studies in ophthalmology have examined the possibility of creating 3D biodegradable porous collagen matrices as implants for the primary treatment of open angle glaucoma [16]. There are studies on collagen gels for applications in the quick treatment of chronic pressure ulcers (located in the heel) developed in adult and elderly patients diagnosed with diabetes [17].

An important research direction in terms of collagen-based biomaterials is related to the types of collagen crosslinkers and their dosing. For instance, it was found that different gel properties may be obtained depending on different crosslinker doses, by self-assembling collagen molecules simultaneously with crosslinking with N-hydroxysuccinimide derivative of adipic acid, in the development of fish skin collagen gels (catfish) [18]. Moreover, experiments for wound treatment applications were conducted by reinforcing the fish scale 3D collagen matrix with a combination of curcumin linked to graphene nanofibers [19]. Studies on 3D collagen sponges modified with 2,3-dialdehyde cellulose as crosslinker resulted in increased denaturation temperature, improved viscoelastic behaviour and mechanical properties, which constitutes a potential for neural tissue regeneration [20].

From another perspective there is research aimed at direct assembling of collagen and smooth muscle cells into a cylindrical 3D geometry using a casting technique in order to obtain tissue rigid enough to withstand handling [21]. In order to obtain collagen architectures with increased resistance and adherence, mixtures of fish collagen and polycaprolactone were developed by electrospinning technique, allowing the development of composite nanofibrous scaffolds with applicability in organ or tissue engineering, as well as in other areas [22]. 3D printing of calcium phosphate-collagen composites was used to make osteo-conductive implants with improved performance compared to traditional techniques, in order to replace synthetic bone grafts [23].

However, collagen has proven its efficacy in other types of applications as well. Many studies were dedicated to obtaining food gelatins, structuring food membranes and to using collagen as food supplement, such as recent studies on combining alginate nanoparticles and calcium chelating to correct calcium deficiencies [24].

Due to intrinsic or acquired functionalities, collagen-based materials are found in many niche areas. Some

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studies have analysed the use of collagen to precipitate wine polyphenols [25] or to develop 2D structures for foil wrapping [26]. Collagen extracts are used in cosmetics, in nail polish and make-up, in the preparation of various cleansing and care products such as shampoo, creams, hair curling products [27, 28]. Biogas development from collagen hydrolysate extracted from tanned leather by-products and neutralized with KOH-H_3PO_4 was studied and it was found to be a more feasible variant than aerobic storage of lime fleshings, because microorganism growth is quicker [29]. An important application of collagen extracts with low molecular weight is obtaining surfactants, either for the cosmetics industry, if the resource is safe in terms of purity degree, or for light construction materials, if hydrolysates come from uncertain sources [30-32]. It was also proved that the use of collagen extracts as additives for aminoplast adhesives in a proportion of approximately 5% considerably limits formaldehyde emissions from thermally processed adhesive films [33, 34].

There are studies on using the organic nitrogen from collagen in soil remediation [35] and fertilization of agricultural crops [36]. Studies have been conducted on: obtaining collagen hydrolysates from leather by-products to fertilize horticultural crops [37, 38]; treatment of cereal seeds to stimulate germination and reduce amounts of insecticide and fungicide [39], or even to fertilize wheat and rice crops [40]; the use of collagen extracts from fish skins to increase rapeseed production by stimulating growth and reducing silique dehiscence [41]; the use of collagen hydrolysate from bovine leather in the foliar treatment of grapevine to attenuate effects induced by iron deficiency particularly due to calcareous soils [42]. Each of the mentioned applications requires certain chemical and physical properties specific to collagen-based materials, which cannot be achieved through the simple extraction of collagen, and needs additional processing to correct some properties or induce functionalities.

This study deals with the effects induced by thermal concentration and membrane filtration of collagen extracts developed for applications in agriculture. In this regard, collagen hydrolysates were obtained from semi-processed bovine hide. Hydrolysates were subsequently processed in order to develop film-forming properties and to be refined. The purpose of concentration is to facilitate formation of collagen films that allow slow release of proteins with low molecular weight for plant nutrition and protection in various stages of vegetation [35, 41, 42]. The purpose of refinement is to enrich collagen polydispersions into small proteinaceous fragments with immediate availability for penetration of plant cell membranes and growth stimulation [36, 38, 39].

Experimental part

Materials

Leather by-products having the following characteristics: maximum 55 % volatile matter, maximum 7% total ash, minimum 14% total nitrogen, pH value of aqueous extract 3.5-5.0.

Chemical materials: calcium hydroxide from Utchim Ramnicu Valcea, Romania, having the following characteristics: 0.5% volatile matter, 60% calcium oxide, sulfuric acid, concentration of 96%, from S. C. Chimopar S.A. Romania, Alcalase 2.4 L from Novozymes, Denmark, with activity of 2.4 enzymatic units/g, optimal enzymatic activity at temperature = 60 °C and *p*H range = 7-9; acetic acid, concentration of 99 %.

Cellulose acetate filter from Sartorius Stedim Biotech GmbH, with pore size: 1.20; 0.80; 0.45μm.

Analytical methods

STAS 8574-92, Finished hides and fur finished hides; SR ISO 5397-96, Finished leathers. Total nitrogen determination; SR EN ISO 4045-02, Finished leathers – Chemical analyses - pH determination; SR EN ISO 13903-05, Animal feeding stuffs. Determination of amino acids content; Sorensen method to determine aminic nitrogen; STAS 8602-90, Finished leathers. Chrome oxide determination.

IR spectral analysis of collagen hydrolysates by FT/IR-4200 (Jasco) with ATR device equipped with diamond reflection crystal, in spectral range: 7800÷350 cm^{-1}.

Dynamic Light Scattering (DLS) investigation was performed using the ZetaSizer device Nano ZS (Malvern, UK) to determine particle size and distribution.

Collagen hydrolysates were obtained from tanned leather by-products, by chemical-enzymatic compact processing, at atmospheric pressure and temperature of 65-80°C, for 6 h.

Two hydrolysate concentration models were performed: a) at atmospheric pressure, under static conditions, by hot air current heating; b) under vacuum, by heating under dynamic conditions.

To enrich collagen hydrolysate into small-sized compounds, vacuum filtration was carried out, using cellulosic membranes with different porosities: 1.2; 0.8; 0.45 μm.

Results and discussion

Table 1 presents physico-chemical characteristics of collagen hydrolysates before and after thermal concentration at atmospheric pressure and under vacuum.

No.	Characteristics, MU	HCN4	HCN4C	HCN5	HCN5C	HCN6	HCN6CV
1	Dry substance, %	10.66	43.86	8.85	53.36	9.47	30.25
2	Total ash, %	4.22	3.19	4.86	2.90	1.58	5.45
3	Total nitrogen, %	16.14	17.05	16.84	17.07	16.58	16.43
4	Protein substance, %	90.71	95.85	94.58	95.95	93.14	92.33
5	Amino nitrogen, %	2.27	0.45	1.19	0.29	1.59	1.16
6	pH	7.24	8.34	9.87	8.37	8.33	8.21

Table 1
PHYSICO-CHEMICAL CHARACTERISTICS OF COLLAGEN HYDROLYSATE BEFORE AND AFTER CONCENTRATION

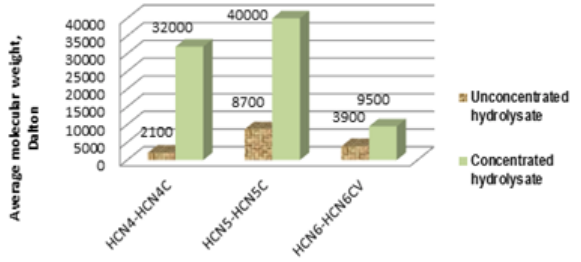
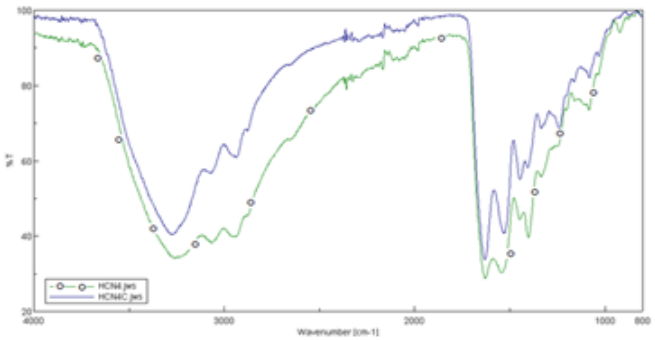


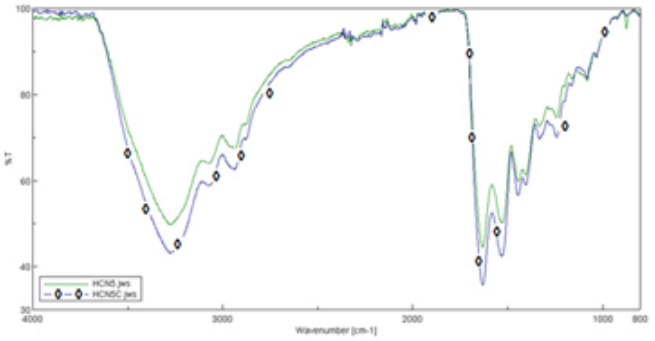
Fig. 1. Average molecular weights of the collagen hydrolysate

During concentration of collagen hydrolysates by thermal effect, water elimination results in association of small peptides and increased average molecular weight.

Collagen hydrolysates extracted from leather by-products, HCN4, HCN5, HCN6, contain amino nitrogen > 1 %. According to the Sörensen method, amino nitrogen > 1 % indicates average molecular weights < 13 000 Daltons, figure 1.



(a)



(b)

Fig. 2. FT/IR-ATR spectral analysis of collagen hydrolysate before and after atmospheric concentration : (a) HCN4- HCN 4C; (b) HCN 5- HCN5C

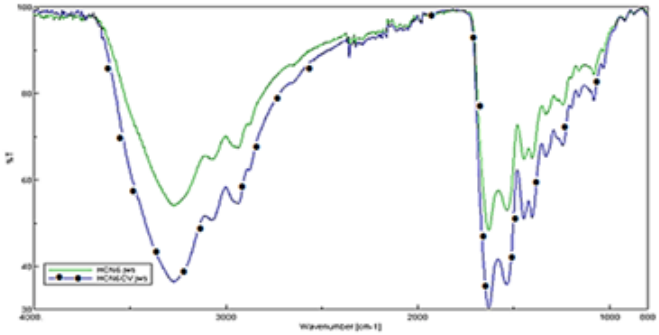


Fig. 3. FT/IR-ATR spectral analysis of collagen hydrolysate before and after vacuum concentration

After concentration at atmospheric pressure, at relatively high temperature there is a significant decrease of amino nitrogen content, which shows that, simultaneously with water elimination, there is an association of free amino acid and oligopeptides with polypeptide chains and a significant increase of the average molecular weight for HCN4C, HCN5C above 30 000 Daltons.

The decreased amino nitrogen content and the increased average molecular weight to values specific to gelatins are favourable to structuring collagen films from collagen hydrolysates extracted from leather by-products.

In the vacuum concentration variant, this phenomenon is much attenuated, due to the lower working temperature. Under these conditions, the average molecular weights of the concentrated HCN6CV hydrolysate is approximately 4 times lower compared to the average molecular weight of hydrolysates concentrated at atmospheric pressure.

This concentration variant is favourable to the preservation of a content of free amino acids in collagen hydrolysates that is able to produce plant growth stimulating effects shortly after application.

IR spectroscopy showed that after concentration there are no major changes at structural level that might affect the bio-stimulating potential of collagen polydispersions on plant growth.

Figures 2 and 3 comparatively show FT/IR-ATR spectra for unconcentrated collagen hydrolysates HCN4, HCN5, HCN6 and their counterparts concentrated by heating, at atmospheric pressure, HCN4C, HCN5C, and under vacuum, HCN6CV.

Wave numbers and spectral attributions of peptide fragments from raw and concentrated collagen hydrolysates [43, 44, 45], experimentally obtained from semi-processed bovine leather, are given in table 2.

Wave numbers of vibrations are found to be less affected in the atmospheric concentration variant and much less in the vacuum concentration variant.

Wavelength shifts either indicate a structural rearrangement of existing proteins in the raw hydrolysate, or are the expression of new protein fragments with different structural characteristics, all the more so if wave numbers without correspondent in the unconcentrated-concentrated pairs of samples are highlighted. For hydrolysate HCN4, before concentration, a high number of vibrations is recorded at the following wavelengths: 3262.65 cm^{-1} , 3064.98 cm^{-1}, 1542.46 cm^{-1}, 1200.16 cm^{-1}, 920.529 cm^{-1}, 847.247 cm^{-1}, without correspondent in HCN4C, after atmospheric concentration, but where there are vibrations at new wavelengths: 3276.15 cm^{-1}, 3072.70 cm^{-1}, 2877.92 cm^{-1}, 1529.92 cm^{-1}, 1240.65 cm^{-1}, 1162.55 cm^{-1}. In the case of vacuum concentrated hydrolysate, the phenomenon is very limited: 3067.88 cm^{-1}, 2649.40 cm^{-1} in HCN6, compared to 3077.52 cm^{-1}, 1631.17 cm^{-1} in HCN6CV.

The profile of spectral wave numbers, associated with the differentiated modification of amino nitrogen content, indicates the possibility of processing collagen hydrolysate for concentration, depending on the desired goal. If collagen hydrolysates are intended for plant nutrition, atmospheric concentration may be used to male polydispersions with higher molecular weights, favourable to structuring collagen films, which will gradually release organic nitrogen for plant nutrition in various stages of vegetation. If collagen hydrolysates are intended for germination stimulation or plant growth, vacuum concentration may be used, resulting in polydispersions with lower molecular weights, richer in free amino acids and dipeptides that are able to penetrate cell membranes.

For a rich content in free amino acids and small peptides, collagen hydrolysates were filtered under vacuum through cellulosic membranes with porosities of 1.20 μm, HCN7.1; 0.80 μm, HCN7.2; 0.45 μm, HCN7.3.

Table 3 presents physical-chemical characteristics of collagen hydrolysates, before and after selective filtration.

Amino nitrogen content of collagen extracts is in strict correlation with the average molecular weight, an important property of collagen extracts.

Previous research [41, 46] showed (by HPLC analysis) that collagen hydrolysates with average molecular weights up to 15000 Daltons contain free amino acids (histidine, alanine, glutamic acid, arginine, glycine, leucine, isoleucine, methionine, aspartic acid, valine, proline) and oligopeptides (phenylalanine/leucine, isoleucine/lysine), that provide bioactive properties.

In this study, the presence of compounds with low molecular weights, such as free amino acids and oligopeptides, was highlighted by particle size measurements by Dynamic Light Scattering, using a ZetasizerNano ZS device, Malvern. For collagen hydrolysates obtained from semi-processed bovine leather, histograms in figure 4 show the presence of several types

Table 2
SPECTRAL FT/IR-ATR CHARACTERISTICS OF COLLAGEN HYDROLYSATE BEFORE AND AFTER CONCENTRATION

No.	Frequency range of samples, cm^{-1}						Characteristic frequency range, cm^{-1}	Assignments
	HCN4	HCN4C	HCN5	HCN5C	HCN6	HCN6CV		
1		3276.15	3274.22	3276.15	3272.29		3200-3600	ν_{OH}, in alcohol, phenol, carboxylic acid
2	3262.65							
3		3072.70		3077.52		3077.52	2380-3333	δ_{NH} in salts of amino acids
4	3064.98		3067.88		3067.88			
5	2939.63	2938.67	2936.74	2938.67	2938.67	2939.63	2850-3000	ν_{CH}, ν_{CH2}, in aliphatic chain
6		2877.92	2879.85	2879.85		2879.85		
7					2649.40			
8	1629.24	1631.17	1630.20	1631.17	1630.20	1631.17	1610-1660	δ_{NH}, band I-free amino acid
9		1529.92	1528.00	1530.89	1534.75	1535.71	1485-1550	δ_{NH}, band II-amino acid
10	1542.46							
11	1448.93	1446.04	1444.11	1445.07	1447.00	1447.96	1425-1475	δ_{CH2},in amino acid (proline)
12	1401.68	1405.54	1404.57	1404.57	1404.57	1403.61	1400-1465	ν_{CN}, in amino acid
13	1335.15	1332.26	1334.19	1332.26	1333.22	1332.26	1315-1350	δ_{CH}, in amino acid (aspartic and glutamic acids)
							1264-1450	
14		1240.65	1241.62	1240.65	1244.51	1244.51	1230-1250	ν_{CN}, δ_{NH} in amino acid
							1235-1270	ν_{CO}, ν_{CC}, in tyrosine
							1181-1420	δ_{COH} or ν_{CO}, in serine
15	1200.16				1201.12	1200.16	1120-1253	ν_{CO}, in amino acid(aspartic and glutamic acids)
16	1158.69	1162.55	1160.62	1162.55	1160.62	1161.59		
17	1082.52	1081.56	1082.52	1082.52	1082.52	1082.52	1075-1150	ν_{CO}, in amino acid (threonine)
							724-1174	$\gamma_{r\ CH2}$ in amino acid
18	1034.31	1032.98	1032.98	1031.42	1032.98	1033.34	724-1174	$\gamma_{r\ CH2}$ in amino acid
19				992.458				
20	920.529				920.529	921.493		
21	876.174	874.246	874.246	874.246	876.174	875.210		
22	847.247							

No.	Characteristics, MU	HCN7	HCN7.1	HCN7.2	HCN7.3
1	Dry substance, %	9.49	9.56	9.81	10.09
2	Total ash, %	6.74	5.81	5.65	5.54
3	Total nitrogen, %	16.54	16.72	16.74	16.75
4	Protein substance, %	92.94	94.00	94.04	94.14
5	Amino nitrogen, %	0.57	0.74	0.76	0.89
6	Average molecular weights, kDa	26.00	20.20	19.50	16.00
7	pH	8.54	8.52	8.49	8.47

Table 3
PHYSICO-CHEMICAL CHARACTERISTICS OF COLLAGEN HYDROLYSATE BEFORE AND AFTER FILTRATION

No.	Sample	Average particle size, nm	PdI
1	HCN7	1032	0.707
2	HCN7.1	619	0.531
3	HCN7.2	454	0.445
4	HCN7.3	431	0.428

Table 4
POLYDISPERSITY IN COLLAGEN HYDROLYSATE

of compounds with various distributions of particle sizes. Particles with sizes above 100 nm are dominant, which coincides with the prevailing presence of polypeptides, reflected in the value of average molecular weight, in correlation with amino nitrogen content, but also coincides with the presence of particle agglomerates. Compared to the dominant range in the studied raw hydrolysate, differences are induced by the selectivity of membranes used in filtration, in correlation with their porosity.

Table 4 shows the average particle size and polydispersity (Pdl) of raw collagen hydrolysates and of those refined by selective filtration.

It is noticed that reflected light intensity measurements for collagen hydrolysates HCN7 and HCN7.1 indicate types of particles with a high share in the range of 100-1000 nm, while the presence of very small particles (1-10 nm) is not visible due to agglomeration, and the presence of large particles (1000-10000 nm) is very discreet. After filtration using 30-50% lower porosity membranes, the presence of particles with sizes in the ranges of 1-10 nm and 10-100 nm is recorded in filtrates, which reflects both deagglomeration of particles due to vacuum, and selection and concentration of small particles in the range of amino acids and oligopeptides. Also, a wider distribution of particle size is recorded in all size ranges, which confirms that there was a high concentration of particle agglomerates in the raw hydrolysate, including sizes > 10000 nm, which under the vacuum effect have deagglomerated and released particles have penetrated the membrane pores, enriching the filtrate in particles not visible in the raw hydrolysate.

Figure 5 shows the variation of collagen hydrolysate polydispersity depending on the porosity of membranes used in selective filtration compared to raw collagen hydrolysate polydispersity.

Polydispersity of collagen hydrolysate refined by cellulosic membranes shows a decrease concomitantly with a decrease in selective membrane porosity, as a result of retaining particles larger than the membrane pores and changing the polydispersity profile.

Figure 6 comparatively presents FT/IR-ATR spectra for the collagen hydrolysate before and after refining by selective membranes with various porosities.

Wave numbers and spectral attributions in raw collagen hydrolysates and those refined by membrane filtration, extracted from semi-processed bovine leather, are presented in table 5.

Filtration by cellulosic membranes with pore sizes of 1.2μm produces minor shifts in wavelengths in the filtered hydrolysate. Hydrolysates filtered using 0.80μm and 0.45 μm porosity membranes show marked differences compared to the unfiltered hydrolysate, but slightly close to each other. These changes are associated with the emergence of new compounds as a result of breaking apart from agglomerates, under vacuum conditions.

Results of IR spectral analysis are in accordance with particle size determinations by DLS, that reflect a completely different profile of particle size and distribution in the unfiltered hydrolysate compared to the one filtered by membranes with porosity < 1.20 μm.

Test results indicate that, under the technical conditions of the experimental model, refined hydrolysates may be obtained, with a spectrum of compounds controllable depending on the porosity of selective membranes.

Both concentration and filtration using selective membranes facilitate the development of various collagen polydispersions, richer either in free amino acids and oligopeptides, bioactive compounds for applications in stimulation and specific nutrition of plants, or in polypeptides with high molecular weights, in order to develop films or more complex spatial structures, with applications in industry, as well as in agriculture or other branches of bioeconomy.

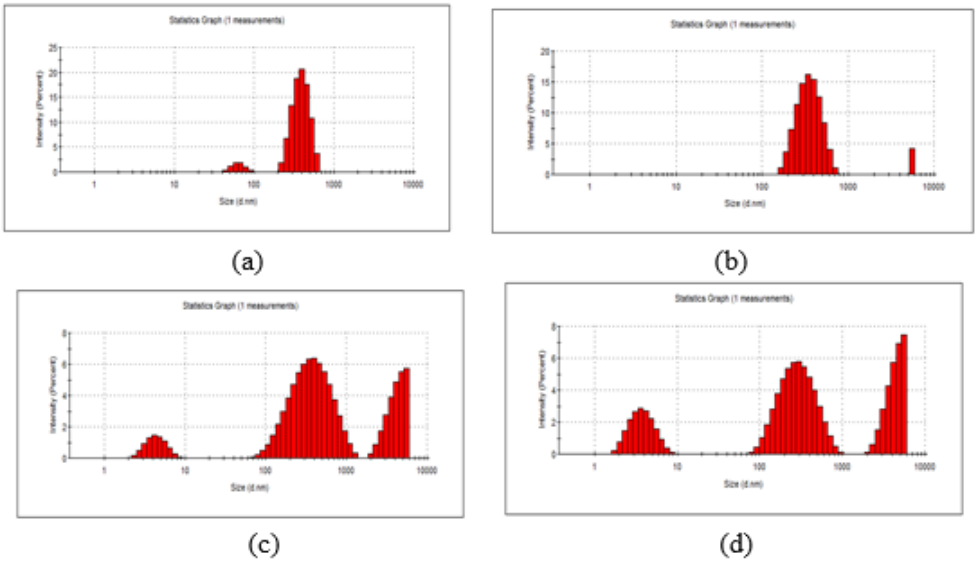


Fig. 4. Particle size in collagen hydrolysate: (a) HCN7; (b) HCN7.1; (c) HCN7.2; (d) HCN7.3

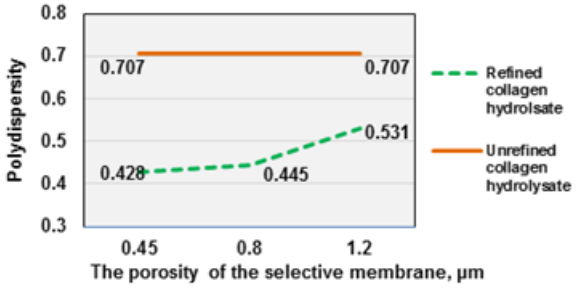


Fig. 5. Polydispersity variation in refined collagen hydrolysate

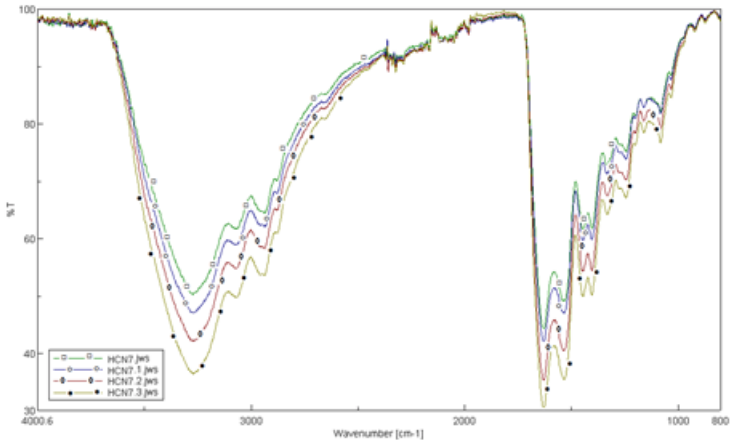


Fig. 6. FT/IR-ATR spectral analysis of collagen hydrolysate before and after refining by cellulosic membranes

Table 5
SPECTRAL FT/IR-ATR CHARACTERISTICS OF COLLAGEN HYDROLYSATE BEFORE AND AFTER REFINING

No.	Frequency range of samples, cm^{-1}				Characteristic frequency range, cm^{-1}	Assignments
	HCN7	HCN7.1	HCN7.2	HCN7.3		
1	3274.22	3273.26	3274.22	3272.29	3200-3600	ν_{OH}, in alcohol, phenol, carboxylic acid
4	3076.55				2380-3333	**δ_{NH} in salts of amino acids**
5		3067.88	3070.77	3065.95		**ν_{CH}, ν_{CH2}, in aliphatic chain**
8			2938.67	2938.67	2850-3000	
9	2937.70	2937.70		2937.70		
10	2879.85	2879.85	2878.89	2879.85		
11		2661.93		2658.08		
14	1630.20	1630.20	1631.17	1631.17	1610-1660	**δ_{NH}, band I-free amino acid**
18	1533.78	1534.75	1535.71	1533.78		
19	1447.96	1447.00	1447.00	1446.04	1425-1475	δ_{CH2},in amino acid (proline)
22	1403.61	1404.57	1404.57	1404.57	1400-1465	ν_{CN}, in amino acid
24	1335.15	1332.26	1332.26	1332.26	1315-1350 1264-1450	δ_{CH}, in amino acid (aspartic and glutamic acids)
26	1243.55	1244.51	1244.51	1244.51	1230-1250 1235-1270 1181-1420	ν_{CN}, δ_{NH} in amino acid ν_{CO}, ν_{CC}, in tyrosine δ_{COH} or ν_{CO}, in serine
28	1201.12	1202.09	1201.12	1201.12	1120-1253	ν_{CO}, in amino acid (aspartic and glutamic acids)
30	1160.62	1159.66	1160.62	1160.62	724-1174	$\gamma_{r\ CH2}$, in amino acid
32	1082.52	1081.56	1081.56	1081.56		
36	1033.34	1034.31	1032.38	1032.38		
40	920.529	923.422	922.458	921.493		
41	876.174	874.246	873.282	875.210		

Conclusions

Thermal concentration at atmospheric pressure may improve film-forming properties of collagen hydrolysates extracted from leather by-products, as a result of increased average molecular weight.

Vacuum thermal processing leads to concentration of collagen hydrolysates while maintaining an amino nitrogen content higher than 1%, which corresponds to the presence of free amino acids in polydispersion.

Collagen hydrolysates may be concentrated at atmospheric pressure or under vacuum without undergoing major structural changes or affecting the bio-stimulating and nutritional potential of collagen polydispersions in plant crops.

Selective cellulosic membrane filtration of collagen hydrolysates extracted from leather by-products leads to enrichment of polydispersions with very small particles, in the ranges of 1-10 nm and 10-100 nm, associated to the presence of free amino acids and small oligopeptides, with bio-stimulating potential in plant crops.

Acknowledgement: This work was supported by the grants of Romanian National Authority for Scientific Research and Innovation, CCCDI – UEFISCDI, project number: PN-III-P3-3.5-EUK-2016-0029 (contract no. 93/2017); PN 16.34.01.07 (contract no. 26N/2016); BIOFOL_CER (contract no. 55/2016).

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Manuscript received: 7.03.2017