COLLAGEN AND KERATIN HYDROLYSATES AS VALUABLE ADDITIVES FOR RENEWABLE NEW PRODUCTS IN CIRCULAR ECONOMY

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ABSTRACT. Collagen and keratin-based waste represents a valuable biomass resource with still insufficiently exploited potential. The leather industry and sheep breeding activity generate important protein waste based on collagen and keratin which at world level amounts to 5.6 million and 1 million tonnes, respectively, every year. The processing of protein-based waste through water or chemical-enzymatic hydrolysis, allows the refinement of the molecular weights and distribution with different potential functionalities for versatile new products with applications in industry (deliming agents, filling and finishing additives), agriculture, medicine, or other fields. The versatile properties as a function of hydrolysis process were shown by analyses of protein degradation (ATR-FTIR spectroscopy), molecular weight distribution (SDS-PAGE electrophoresis) and particle size distribution (DLS analyses). The specific molecular weight distribution of collagen and keratin hydrolysates after hydrolysis at 100°C and 130°C was highlighted, thus proving the potential of refining the compositions for different applications with ecological impact. The collagen hydrolysis at 100°C leads to fragmentation of large molecules into small molecules meanwhile the temperature of 130°C favors the increase of medium size population of peptides, in agreement with SDS-PAGE electrophoresis results. Instead, the small molecules of keratin were doubled by hydrolysis at 130°C, in good correlation with ATR-FTIR determination of AI/AA ratios.

KEY WORDS: protein hydrolysates, molecular distribution, collagen, keratin

UTILIZAREA HIDROLIZATELOR DE COLAGEN ȘI CHERATINĂ CA ADITIVI VALOROȘI PENTRU NOI PRODUSE REGENERABILE ÎN ECONOMIA CIRCULARĂ

REZUMAT. Deșeurile pe bază de colagen și cheratină reprezintă o resursă valoroasă de biomasă cu potențial încă insuficient exploatat. Industria de pielărie și activitatea de creștere a oilor generează importante deșeuri proteice pe bază de colagen și cheratină, care la nivel mondial se ridică la 5,6 milioane, respectiv 1 milion de tone în fiecare an. Prelucrarea deșeurilor pe bază de proteine prin hidroliză în apă sau chimico-enzimatică permite rafinarea greutăților moleculare și distribuția acestora, cu diferite potențiale funcționalități pentru noi produse versatile cu aplicații în industrie (agenți de decalcificare, aditivi de umplere și finisare), agricultură, medicină, sau alte domenii. Proprietățile versatile în funcție de procesul de hidroliză au fost demonstrate prin analize de identificare a degradării proteinei (spectroscopie ATR-FTIR), distribuție a greutății moleculare (electroforeza SDS-PAGE) și distribuție a dimensiunii particulelor (analize DLS). S-a evidențiat distribuția specifică a greutății moleculare a hidrolizatelor de colagen și cheratină după hidroliză la 100°C și 130°C, dovedind astfel potențialul de rafinare a compozițiilor pentru diferite aplicații cu impact ecologic. Hidroliza colagenului la 100°C duce la fragmentarea moleculelor mari în molecule mici, în timp ce temperatura de 130°C favorizează creșterea populației de dimensiuni medii de peptide, în acord cu rezultatele electroforezei SDS-PAGE. În schimb, ponderea moleculelor mici de cheratină s-a dublat prin hidroliză la 130°C, în bună corelație cu valoarea rapoartelor AI/AA determinate prin spectroscopie ATR-FTIR.

CUVINTE CHEIE: hidrolizate de proteine, distribuție moleculară, colagen, cheratină

LES HYDROLYSATS DE COLLAGÈNE ET DE KÉRATINE COMME ADDITIFS PRÉCIEUX POUR DE NOUVEAUX PRODUITS RENOUVELABLES DANS L'ÉCONOMIE CIRCULAIRE

RÉSUMÉ. Les déchets à base de collagène et de kératine représentent une ressource précieuse en biomasse dont le potentiel est encore insuffisamment exploité. L'industrie du cuir et l'activité d'élevage ovin génèrent d'importants déchets protéiques à base de collagène et de kératine qui s'élèvent respectivement à 5,6 millions et 1 million de tonnes par an au niveau mondial. Le traitement des déchets à base de protéines à l'aide de l'eau ou de l'hydrolyse chimique-enzymatique permet d'affiner les poids moléculaires et leur répartition avec différentes fonctionnalités potentielles pour de nouveaux produits polyvalents avec des applications dans l'industrie (agents de déchaulage, additifs de remplissage et de finition), l'agriculture, la médecine ou d'autres domaines. Les propriétés polyvalentes en fonction du processus d'hydrolyse ont été démontrées par des analyses de la dégradation des protéines (spectroscopie ATR-FTIR), de la distribution des poids moléculaires (électrophorèse SDS-PAGE) et de la distribution granulométrique (analyses DLS). La distribution du poids moléculaire spécifique des hydrolysats de collagène et de kératine après hydrolyse à 100°C et 130°C a été mise en évidence, prouvant ainsi le potentiel d'affinage des compositions pour différentes applications à impact écologique. L'hydrolyse du collagène à 100°C conduit à la fragmentation des grosses molécules en petites molécules tandis que la température de 130°C favorise l'augmentation de la population de taille moyenne de peptides, en accord avec les résultats de l'électrophorèse SDS-PAGE. Au lieu de cela, les petites molécules de kératine ont été doublées par hydrolyse à 130°C, en bonne corrélation avec la détermination ATR-FTIR des rapports AI/AA.

MOTS CLÉS : hydrolysats de protéines, distribution moléculaire, collagène, kératine

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INTRODUCTION

The leather industry is the oldest industry that reuses a waste, the animal skin. Although the ecological impact of processing 8 million tons of hides and skins annually in the world represents a reduction of 5 million tons of greenhouse gases (equivalent to the greenhouse gases produced by 1 million cars) [1], and the creation of products with added value, nevertheless, leather processing yield is 20-25 % and generates significant amounts of protein waste, which are mostly stored. The sheep breeding activity produces also low-quality wool which alongside with leather industry releases keratin-based waste amounting around 1 million tonnes annually [2].

Leather products are durable, processed with chemical materials containing heavy metals (basic salts of trivalent chromium), made from raw materials of petroleum origin (acrylic polymers, condensation syntans, phenol-formaldehyde resins, fatliquoring agents) or using energy-consuming chemicals (ammonium salts) and therefore, hardly biodegradable, which raises an important environmental problem regarding their storage, after the end of their life cycle.

The modern leather industry must respond to the principles of green chemistry [3], which must: i) prevent waste, ii) save materials, iii) use less dangerous materials, iv) start from benign chemical materials, v) be energy efficient, vi) use renewable raw materials, vii) reduce reaction intermediates, viii) use catalysis, ix) produce degradable materials, x) prevent and monitor pollution and, xi) prevent chemical accidents. The leather industry makes constant efforts to meet the requirements of green chemistry [4].

Organic waste in the European Union amounts to an estimated value of 138 million tons annually, of which almost 40% is stored [5], representing a serious environmental problem, which has boosted both research in the field and legislation in the direction of orienting the economy towards neutrality, circularity and products with a reduced carbon footprint. In this regard, intense research is being done on the replacement of non-biodegradable chemical materials or with fossil carbon content, with renewable materials, which help improve the biodegradability of leather, while maintaining the final quality and value of use, but with reduced environmental impact. The sustainability of leather processing is one of the most difficult problems of the leather industry, which must change a safe and versatile technological process (tanning with chromium III salts), which has dominated this industry for more than 150 years, with metal-free alternatives, without aldehydes, without bisphenols and to ensure biodegradability.

Superior valorization of collagen and keratin waste through separation, solubilization and refinement of the molecular weight allows the development of new materials with functionalization potential for applications in the leather industry (deliming agents, retanning, filling, finishing), agriculture, medicine or other fields [6].

The effects of the valorization of these by-products ensure the reduction in the number of chemical materials of petroleum origin and of the greenhouse gases emitted by the storage of this waste.

The work presents the possibility of making protein hydrolysates with different properties and functionalization potential through hydrolysis processes under different conditions, which allows obtaining versatile products. Different analytical methods like ATR-FTIR spectroscopy, SDS-PAGE electrophoresis, DLS were performed and confirmed the specific molecular weight distribution as a function of hydrolyses conditions.

MATERIALS AND METHODS

Collagen and keratin hydrolysates processed from leather and coarse wool waste, by chemical [7] and chemical-enzymatic [8] methods in the solid state (Figure 1), were dissolved in water, in a concentration of 10%, in order to be treated for molecular weight reduction and create new functional groups. These processes took place at 130°C, variable time (15, 30 and 240 minutes) and at 100°C, variable time (3, 13 and 20 hours) for collagen alkaline hydrolysate, and for keratin alkaline-

enzymatic hydrolysate, respectively. The HICLAVE HV 110 L autoclave (HIRAYAMA Manufacturing Corporation) was used for hydrolyses at 100°C and 130°C. The particle size, polydispersity and Zeta potential were measured with Nanosizer NZ (Malvern), the molecular weight distribution by SDS-PAGE electrophoresis (Mini PROTEAN 3 Cell Bio-Rad) equipped with Gel Doc EZ Imaging System and ImageLab software, and the functional groups by FTIR spectrometry with Thermo Scientific™ Nicolet™ iS50 spectrometer (Thermo Fisher).

Figure 1. Collagen (left) and keratin (right) hydrolysates

The collagen and keratin hydrolysates at initial stage and after hydrolysis at 130°C or 100°C were labeled as listed in Table 1.

Table 1: Collagen and keratin hydrolysate labels

Label	Hydrolysate
HCZ0, P1	Alkaline collagen hydrolysate, solution 10%
HCZ15; HCZ30; HCZ240	Collagen hydrolysate processed at 130°C for 15 min, 30 min and 240 min
P1, P2, P3, P4	Collagen hydrolysate processed at 100°C for 3 h, 13 h and 20 h
HKV0	Alkaline-enzymatic keratin hydrolysate, solution 10%
HKV1; HKV2	Alkaline-enzymatic keratin hydrolysate processed at 130°C for 240 min

RESULTS AND DISCUSSION

The collagen and keratin hydrolysates in initial state with concentration of 10% dry substance and after hydrolyses at 130°C and 100°C, for different times, are shown in Figure 2 and the final products in Figure 3. From Figure 3 a very different aspect of collagen hydrolysates can be seen, suggesting the substantial influence of hydrolysis temperature. The aspect of keratin hydrolysate samples processed at 130°C was very similar, suggesting a good reproducibility (Figure 3c).

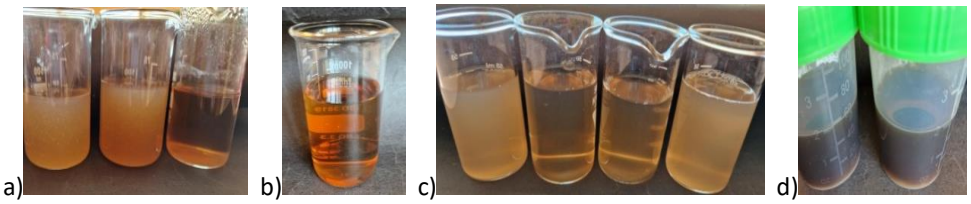


Figure 2. Collagen and keratin hydrolysates: a, b) initial collagen hydrolysate (HCZ0) and collagen hydrolysates obtained at 130°C (HCZ15; HCZ30; HCZ240); c) initial collagen hydrolysate (P1) and collagen hydrolysates obtained at 100°C (P2; P3; P4), and d) keratin hydrolysates (HKV1; HKV2)

The final products of collagen and keratin hydrolyses are shown in Figure 3.

Figure 3. Final products, collagen and keratin hydrolysates: HCZ240; P4 and HKV2

The determinations of particle sizes and Zeta potentials for the precursors, and the final collagen and keratin hydrolysates are presented in Tables 2-4 and Figures 4-6.

In Table 2 and Figure 4 an important decrease in the size of the particles can be seen as a result of hydrolysis at 130°C and a reduction in the Zeta potential, which indicates a tendency for the particles to agglomerate with the elimination of the aqueous layer that surrounds them with the decrease in polydispersity (PId), and finally, the polydispersity increases and the Zeta

potential evolves in the negative range, as a result of the hydrolysis and the reactivation of the functional groups of the collagen. The increase in the population of small particles is significantly higher at the final hydrolysis time (8.7 nm with 42.2% ratio after 240 min of treatment as compared with 9 nm with 21.2% ratio after 30 min of treatment).

Table 2: Particle size, polydispersity and Zeta potential for collagen hydrolysates treated at 130°C

Sample	Majority populations						Average,	Pld	Zeta potential,
	nm	%	nm	%	nm	%	nm		mV
HCZ0	12.7	11.0	438.3	89.0	-	-	1804.0	0.7	-10.9
HCZ 15	11.6	17.2	438.2	76.2	5308	6.7	303.2	0.5	-4.6
HCZ 30	9.0	21.2	35.3	8.3	464	63.7	272.9	0.4	3.1
HCZ 240	8.7	42.3	755.7	28.2	5180	19.4	267.2	0.9	-3.8

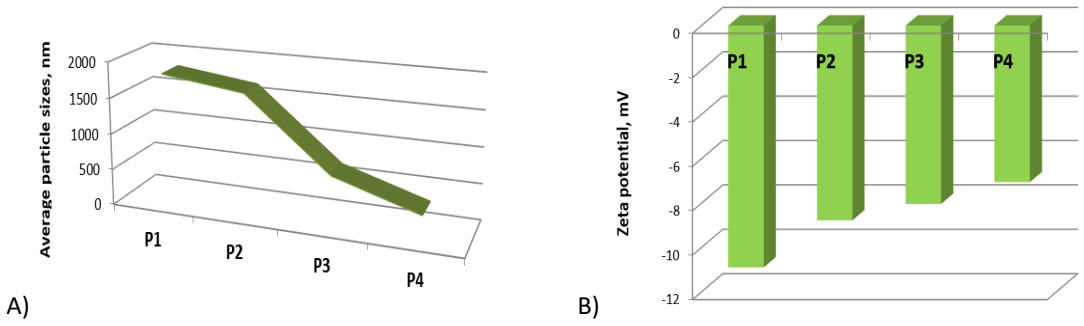
A) B)

Figure 4. A) Evolution of average particle size and B) Zeta potential over hydrolysis time at the temperature of 130°C, for collagen hydrolysates

Table 3 and Figure 5 show the particle sizes and Zeta potentials for alkaline collagen hydrolysates processed at 100°C and different time. The decrease in particle size is more evident at the maximum hydrolysis time, with insignificant variations in Zeta potential, which suggests different types of particles, without such important particle associations, compared to hydrolysis at 130°C. The proportion of small particles remains constant. Figure 5 shows the evolution of particle size and Zeta potentials.

Table 3: Particle size, polydispersity and Zeta potential for collagen hydrolysates obtained at 100°C

Sample	Majority populations						Average,	Pld	Zeta potential,
	nm	%	nm	%	nm	%	nm		mV
P1	12.7	11.0	438.3	89.0	-	-	1804.0	0.7	-10.9
P2	11.1	7.9	671.3	64.8	5301	27.3	1652.0	0.8	-8.7
P3	8.8	5.3	-	-	280.5	94.7	673.9	0.6	-8.0
P4	5.8	9.6	118.9	29.3	376.1	61.0	309.5	0.5	-7.0



A) B)

Figure 5. A) Evolution of particle size and B) Zeta potential over hydrolysis at the temperature of 100°C for collagen hydrolysates

Table 4: Particle size, Zeta potential and polydispersity for keratin hydrolysates

Sample	Majority populations						Average,	Pdl	Zeta potential,
	nm	%	nm	%	nm	%	nm		mV
HKV0	-	-	601.3	62.6	3115.0	37.4	621.4	0.5	-33.5
HKV1	66.4	7.2	544.8	59.1	3943.0	33.7	561.3	0.5	-29.0
HKV2	36.4	6.7	511.7	62.0	3924.0	31.2	509.1	0.5	-28.3

Table 4 and Figure 6 present the characteristics of keratin hydrolysate particles for two parallel samples, KKV1 and HKV2 after hydrolyses at 130°C as compared to initial keratin hydrolysate HKV0.

It can be observed that, through hydrolysis, populations of small particles appear in proportions of 6.7-7.2%, the ratio of large particle populations decreases by 3.7-6.2%, the average size of the particles decreases, the polydispersity remains constant, and the Zeta potential indicates a slight decrease in stability. The appearance of larger particles suggests particle agglomeration and correlates with the decrease in Zeta potential.

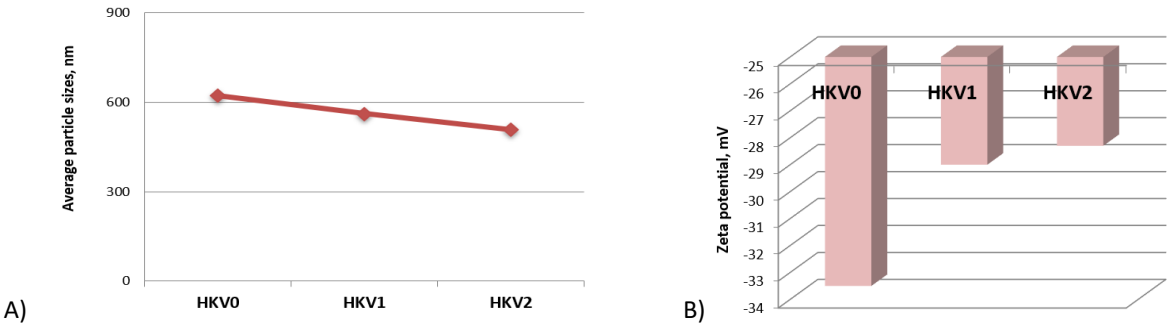


Figure 6. A) Evolution of particle size and B) Zeta potential over hydrolysis time at 130°C, for keratin hydrolysates

Hydrolysis at the temperature of 130°C leads to fragmentation of large peptide molecules into medium molecules (17-55 kDa) while preserving the share of small peptides and oligopeptides (Figure 7). The results of processing the SDS-PAGE electropherograms of P1-P4 hydrolysates are summarized in Figure 8, where the increase in the share of molecules with low weight (10-12 kDa) and the decrease of those with high weight (>150 kDa) can be clearly seen. The influence of hydrolysis conditions on molecules with medium molecular weight is small. In the case of keratin hydrolysates, the treatment at 130°C generated molecules of 10 kDa by cleavage of those with molecular weights of 60 kDa. The share of small molecules doubled by hydrolysis under pressure and at the temperature of 130°C (Figure 9).

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The results of FTIR-ATR spectrometry analyses of dried protein hydrolysates are presented in Figures 10-12. In Figure 10, the phenolic groups (1500-1000 cm^{-1}) for which the absorbance increases as a result of hydrolysis at 130°C are marked. The more intense yellow color of collagen hydrolysate (Figure 3) can be correlated with the change in phenolic groups of amino acids and generation of sensitive chromophores [9]. Other similar studies showed that the hydrolysis time does not change the polypeptide backbone (all specific amide bands are present), but they found a shifting of Amide A and Amide I to lower wavenumbers and a higher vibration of OH groups from 1037 cm^{-1} [10].

In Figure 11, a change can be observed at the level of OH groups, as a result of the hydrolysis at 100°C; the overlap of

absorbances of samples P3 and P4 in this area suggests that the hydrolysis of 13 hours generates the same products as the one at 20 hours. In Figure 12 the keratin hydrolysates showed slight intensity modifications at the level of Amide A and Amide III bands.

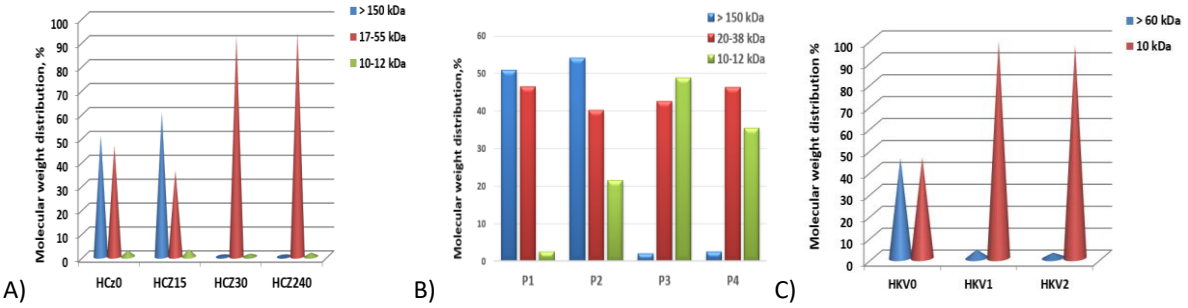

Figure 7. Molecular weight distributions of: A) collagen hydrolysates processed at 130°C; B) collagen hydrolysates treated at 100°C; C) keratin hydrolysate after processing at 130°C

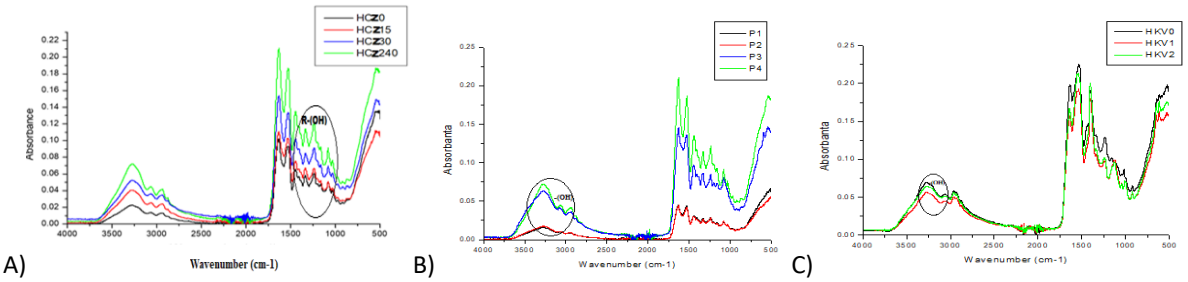

Figure 8. FTIR-ATR spectra for: A) collagen hydrolysates processed at 130°C; B) collagen hydrolysates processed at 100°C, and C) keratin hydrolysates processed at 130°C

The Amide I band region is related to the stretching vibration of the peptide carbonyl group (-C=O) involved in polypeptide backbone and represents a marker of secondary structure. The Amide A is due to N-H stretching vibration which is not dependent on protein backbone conformation and is very sensitive to intramolecular hydrogen bond strength. The absorption bands A and I signals are used in protein analysis because their signals are strong in a spectral region without other additional absorptions [11].

The ratio of the intensities of the Amide I/Amide A bands quantifies the cleavage of the collagen molecule [12], and are found in the FTIR-ATR spectra between the wave numbers 1600-1800 cm^{-1} (Amide I) and 3300-3500 cm^{-1} (Amide A) and is presented in Figure 13a-c.

The results show suggestive reductions in the AI/AA ratios, as a result of the cleavage of the collagen molecule under various conditions of temperature.

The biggest variations were recorded for keratin hydrolysate ($\Delta_{AI/AA}$=1.41), collagen hydrolysate processed at 130°C ($\Delta_{AI/AA}$=1.35) and lowest ratios were found for collagen hydrolysate processed at 100°C ($\Delta_{AI/AA}$=0.35).

The results are significant for the hydrolysates processed at 130°C, where there is a large share of particles with a size of 8.7-9.0 nm with a 31.3% increase after 140 minutes of hydrolysis compared to the initial product (11% of particles with 12.7 nm), and 21.1% increased ratio between the last two hydrolysis stages.

These particles could be populations of particles with medium molecular weights that split from particles with high molecular weights (Figure 7). Also, with these hydrolysates, the polydispersity increases significantly (from 0.4 to 0.9) and the Zeta potential changes (from 3.1 mV to -3.8 mV), confirming this hypothesis.

Instead, in the case of hydrolysates processed at 100°C, the hydrolysis step at 13 hours generates hydrolysates almost similar to

those processed for 20 hours (Figure 8), in accordance with the data in Figure 13b regarding the AI/AA amide ratio. In the case of these hydrolysates, the share of particles with sizes of 280.5-376.1 nm increases to the value of 61.0-91.7%, when the medium particle size in the initial hydrolysate is 1804 nm (Table 2). And if we take into consideration the 29.3% population of particles with 118.9 nm generated after 13 hours of hydrolyses at 100°C, we can conclude that the populations of 118.9-376.1 nm are in the same ratio of 90.1-94.7% and the hydrolyses results are similar at 13 and 20 hours.

In the case of keratin hydrolysates, the stability of the particles is high, as can be seen from the higher absolute value of the Zeta potential (-28.3 mV to 29.0 mV), unlike that of collagen hydrolysates, which are weakly negatively (-3.8 mV to -10.9 mV) charged and tend to agglomerate.

In the case of keratin hydrolysates, the appearance of a population of particles with small sizes (35.4-66.4 nm) in the proportion of 6.7-7.2% is noted. The polydispersity, medium size and Zeta potential of the particles change insignificantly, which may be due to the association properties of the protein molecules (Table 4).

The AI/AA ratios confirm the changes in molecular distribution through heat treatments for collagen and keratin hydrolysates.

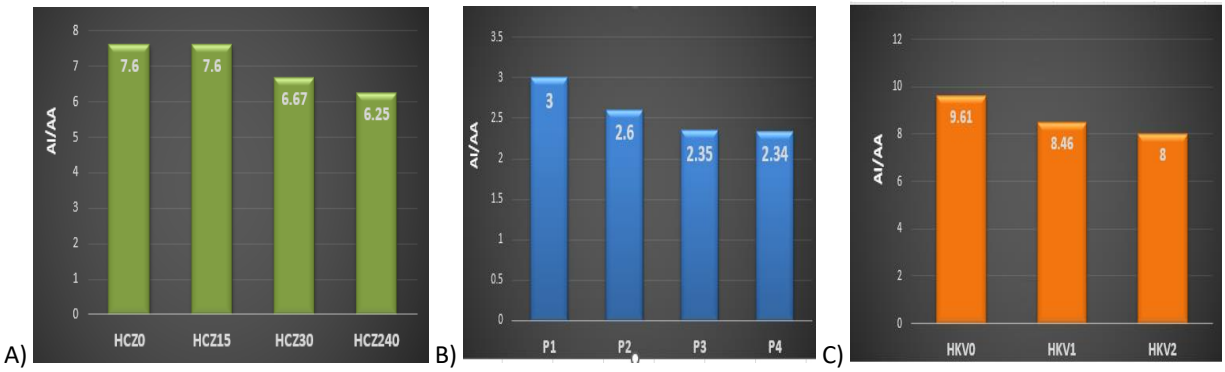

Figure 13. Decrease of the absorbance ratios of amide AI/AA bands for:
A, B) collagen hydrolysates and C) keratin hydrolysate

CONCLUSIONS

By-products from the leather and fur industry, as well as those generated from raising sheep with inferior wool quality, represent a valuable source of proteins that can be used to create new auxiliary chemicals for the leather industry, agriculture, medicine and other applications. Additional treatments under pressure and temperature can shape the molecular distribution of the composition of protein hydrolysates, which was highlighted by measurements through dynamic light scattering (DLS), gel electrophoresis (SDS-PAGE electrophoresis) and FTIR spectroscopy (ATR-FTIR). Low molecular weight peptides were generated by hydrolyses at 100°C and medium weight molecules after hydrolyses at 130°C for collagen hydrolysate. High molecular weight collagen hydrolysate ratios were decreased by hydrolysis at 100°C and 130°C. Keratin hydrolysate molecular weight decreased after hydrolyses at 130°C and molecules less than 10 kDa doubled.

Further studies on the correlation of the molecular weight and particle sizes needs to be performed as well as the separation by SDS-PAGE electrophoresis of proteins with molecular weight under 10 kDa. The potential applications of the new hydrolysates with different compositions, depending on the hydrolysis conditions (with an increased weight of components with low or medium molecular weights) will be investigated in future research.

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