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Exploring the Functional Properties of Hydrolyzed Keratin: Filling the Knowledge Gap on Surface Active, Emulsifying, and Thickening Properties

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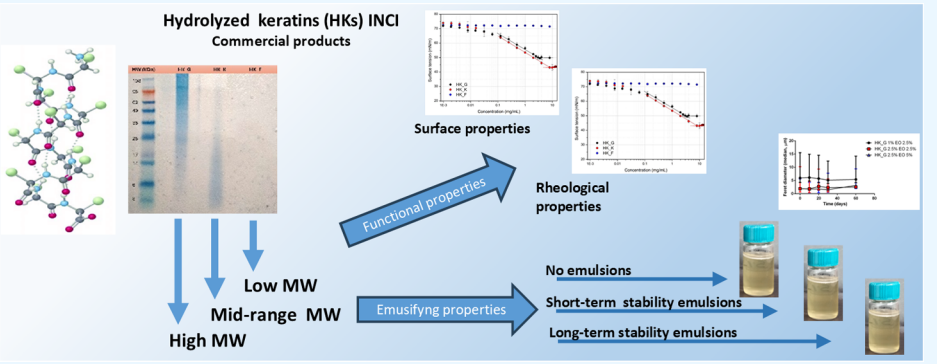

ABSTRACT: Hydrolyzed keratin (HK) refers to any hydrolysate of keratin of a different origin derived by acid, alkali, enzymatic, or other methods of hydrolysis. HK is water soluble and has distinct chemical−physical properties compared to fibrous keratin. Although HK is employed across various technological sectors, there is a notable gap in the literature regarding the detailed chemical−physical properties of commercially available HKs. This study aims to address this gap by providing a thorough analysis of the surface-active, emulsifying, and thickening properties of three commercially available HKs. The results reveal relevant differences among HKs marketed under the International Nomenclature Cosmetic Ingredient (INCI) name "Hydrolyzed Keratin," with variations in their chemical−physical properties, primarily influenced by molecular weight. Specifically, HKs with a higher average M_w (>3000 Da) and protein content demonstrate enhanced emulsifying and thickening capabilities. Conversely, HKs with low M_w (<1000 Da) do not show surface-active properties suitable for the preparation of emulsions. Therefore, this study underscores the need for the standardization of commercially available HK products to obtain biomaterials with tailored and specific chemical−physical properties, enhancing their potential applications in cosmetic and pharmaceutical topical formulations.

INTRODUCTION

Keratins constitute a large group of fibrous structural proteins that make up the cytoskeletal intermediate filaments in various epithelial cells. In animals, they are the most significant biopolymers, after collagen. Keratins are present in keratinocytes of the epidermis's outer layer and in skin appendages such as hair and nails in humans, as well as feathers, horns, claws, and hooves in animals.[1] Keratins remain insoluble in water, as they naturally organize into antiparallel, staggered tetramers. This structural stability is reinforced by disulfide bonds formed between cysteine residues and hydrogen interactions involving the carbonyl and imino groups of amino acids.[2] Keratins are divided into two classes: hard and soft keratin, which have similar peptide structures in coiled-coil conformations, but they differ in amino acid compositions.[3] The structural integrity of keratin, together with biocompatibility, biodegradability, bioactivity, low toxicity, and low costs of production, makes it an ideal polymer for several technological applications. Keratin-based biomaterials inherently possess self-assembling properties and promote cellular proliferation. Recent research on keratin has driven the development of biomaterials with applications in tissue engineering, wound healing, drug delivery, targeted release, and medical devices.[4−7]

Over the past years, there has been an increased interest in the development of green materials, including those derived from keratin.[8] Keratins can undergo chemical hydrolysis in

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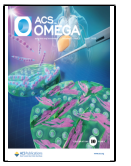

acidic or basic conditions or be processed enzymatically to yield water-soluble peptide fractions, known as keratin hydrolysates.[9] These hydrolysates have shown promising applications in cosmetics, biomedical, agricultural, and packaging sectors.[10−13] In cosmetics, they are recognized under the International Nomenclature Cosmetic Ingredient (INCI) name hydrolyzed keratins (HKs). This biomaterial can act as a skin moisturizing, hair repairing, nail hardening, and conditioning agent and can be included in the formulation of a large variety of cosmetic products.[14,15] Although widely used in cosmetics, HKs lack comprehensive chemical−physical characterization, particularly with regard to their thickening ability, surface activity, and emulsifying properties. A deeper understanding of these aspects could expand their applications, including their potential use as natural-based emulsifiers and thickening agents in pharmaceutical products. Moreover, commercially available excipients marketed with the INCI name of hydrolyzed keratins do not possess standardized chemical−physical characteristics, resulting in different functional properties in the final products.

This study aims to bridge the knowledge gap regarding the surface activity, emulsifying, and thickening properties of three commercially available products labeled under the INCI name hydrolyzed keratin, exploring their potential as natural surfactants and stabilizers for dispersed systems. At this end, the three commercially available HKs were characterized in terms of molecular weight, protein content, and ability to decrease the air−water surface tension. Then, emulsions were prepared and characterized in terms of rheology, droplet size, and physical stability over time to assess the feasibility of HKs as natural emulsifiers.

EXPERIMENTAL SECTION

Materials. Commercial products of water-soluble hydrolyzed keratin INCI NAME (HK) (N.CAS:69430−36−0) as a powder were purchased from different providers. HK, named HK_G, was purchased from Galeno srl (Comeana, Italy) and produced by Cosnaderm (Germany). According to the manufacturer, it is a hydrolysate of keratin of porcine origin obtained from acid hydrolysis. The molecular weight (M_w) of this product was not disclosed by the producer. HK, named HK_K (Keliwool), was produced and purchased by Kelisema srl (Italy). According to the manufacturer, it is a partial keratin hydrolysate obtained by gentle enzymatic hydrolysis of virgin Merino wool, with food-grade proteases at low temperatures and neutral pH. It has an optimum average molecular weight of 3000 Da and is obtained by a spray-drying process. HK named as HK_F (Vari-Ker 100) was purchased from Fagron (Italy) and produced by Variati (Italy). According to the manufacturer, it is a product obtained from feather keratin, supplied in the form of a spray-dried powder. Its composition includes free amino acids (high content of glycine and serine) and low M_w peptides (M_w < 1000 Da). Ethyl oleate was purchased from Croda (U.K.), and polysorbate 80 was purchased from Sigma-Aldrich (St. Louis, MO).

Elemental Analysis, Ashes, and Protein Content Determination. Protein content was calculated through the Kjeldahl method via the determination of the nitrogen content.[16] Protein content was obtained from the nitrogen content using the appropriate conversion factor: protein [%] = 6.25 × N [%].

Ashes were determined by using the gravimetric method. An aliquot of the sample was heated at 550 ± 10 °C until a constant weight was registered.[16]

Elemental analysis was performed using a Flash 2000 Organic Elemental Analyzer (Thermo Scientific). The percentage of carbon, hydrogen, nitrogen, and sulfur was determined by combustion of the sample at high temperatures in a pure oxygen environment.

Gel Permeation Chromatography (GPC) Analysis. Keratin molecular weight and polydispersity index (PDI, Đ) were assessed by gel permeation chromatography (GPC) analysis with a Malvern Viscotek TDA302 system (Malvern, U.K.) equipped with a refractometer (RI), a low-angle light scattering (LALS), a right-angle light scattering (RALS), and a differential viscosimeter (Visc) and thermostated at 40 °C equipped with TOSOH G4000 (10 μm, 7.8 × 300 mm^2) and G3000 (7 μm, 7.8 × 300 mm^2) PWXL columns in series eluted with 0.1 M $NaNO_3$ + 0.02% p/V NaN_3 in Milli-Q water at 0.6 mL/min flow rate. Data acquisition was performed by OmniSEC. 5.1 software using the Pullulan standard for calibration.

SDS-PAGE Electrophoresis. SDS-PAGE was performed under reducing conditions using a precast 10% acrylamide-bis(acrylamide) gel (NuPAGE 10% Bis-TRis-Gel, Invitrogen). Protein Marker "wide Range" 4 to 250 kDa (SeeBlue Plus2 prestained Protein Standard, Invitrogen) was used as the reference. The proteins were stained with Coomassie Blue (0.1% Coomassie Brilliant Blue R250 in 50% methanol and 10% acetic acid).

TGA. Thermogravimetric analysis (TGA) was performed from 30 to 700 °C at a rate of 10 °C/min under nitrogen flow using a simultaneous thermal analyzer STA 6000 (PerkinElmer Inc., Waltham, MA).

FT-IR Analysis. FT-IR spectra were acquired in the range of 4000−400 cm^{-1} using a Frontier IR/FIR instrument (PerkinElmer Inc., Waltham, MA).

Surface Tension Measurements. The surface tension of HK solutions at different concentrations in ultrapure water was measured at 25 °C according to the Du-Noüy ring method using a DCA-100 contact angle tensiometer (First Ten Angstrom). The critical micelle concentration (CMC) and the surface tension at the CMC (γCMC) values were calculated using the segmental regression segmental linear regression model (GraphPad Prism 6 software).

Aqueous Dispersions and Emulsion Preparation. Aqueous dispersions were prepared by dissolving HK at room temperature in ultrapure water at different concentrations (0.5−10% w/w). Emulsions were prepared by dispersing ethyl oleate (EO) in HK dispersions (2.5−10% w/w) under high-shear mixing (9000 rpm; 3 min, Ultraturrax T25).

Keratin dispersions were prepared by mixing commercial hydrolyzed keratin with different volumes of preserved water (0.2% w/w methyl-p-hydroxybenzoate) to allow keratin hydration. The final dispersion concentrations obtained were 2.5, 5, and 10% w/w. Emulsions were made using hydrolyzed keratin at concentrations of 2.5−10% w/w and ethyl oleate at concentrations of 2.5, 5, and 10% w/w. Samples were obtained using high-shear mixing for 5 at 9500 min^{-1}. Ethyl oleate was added to the previously formed keratin solution during the action of the high-shear mixer. After the preparation, emulsions were stored in tightly closed vials at 4 °C and protected from light.

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Rheological Analysis. Rheological properties were measured through a Kinexus Lab$^+$ rheometer (Malvern, U.K.) equipped with a plate-cone geometry CP4/40. A viscosity test was performed at 25 °C by applying a shear rate from 1 to 100 s^{-1}. An oscillation stress sweep test was carried out at 25 °C, shear stress of 1−100 Pa, and frequency of 1 Hz. A frequency test was performed at 25 °C, with a frequency of 10−0.1 Hz and shear stress of 5 Pa. Power law viscosity (PLV) and power law index (n) were calculated by fitting the experimental data using the following equation (GraphPad Prism 6 software)

$$y = \mathrm{PLV}\gamma^n$$

where y is the shear stress (Pa) and γ is the shear rate (s^{-1}).

Optical Microscopy. Microscopy analyses were performed through an MT 9200 microscope (Meji) equipped with a camera (Invenio 3s) at a 20× magnification to monitor the emulsifying properties of keratin and the stability of the emulsions. To measure droplet size, at least 1000 droplets from each emulsion were analyzed automatically after applying a Hi-Gauss filter using ImagePro-Plus 5.1 Software (Version 5.1.0.20, Media Cibernetics, Inc.). Droplet size number distribution was built using the Feret mean diameter (average caliper length of each droplet).[17] From each distribution, the median size diameter (d50) and the first (Q1) and third (Q3), and interquartile ranges were calculated.

RESULTS

Chemical Composition of Hydrolyzed Keratins. A similar chemical composition in terms of nitrogen (%), hydrogen (%), carbon (%), and sulfur (%) was found for the three analyzed commercial HKs according to elemental analysis. The ashes content determined as residue was compliant with the specifications provided by the producers and ≤10% for all samples. The calculated protein content (%) was higher for HK_G (89% on the base of the dry matter), followed by HK_K (82%) and HK_F (79%) (Table 1).

Table 1. Elemental Analysis (%), Ashes, and Protein Content Determination (%) for the Commercial Hydrolyzed Keratins

	elemental analysis[a]				ashes	protein content
	nitrogen (%)	carbon (%)	hydrogen (%)	sulfur (%)	(%)	%
HK_G	15.58	43.84	6.77	0.51	3.25 (<8)	89.0 (92−95)
HK_K	12.16	41.29	5.86	2.45	10.55 (<20)	82.0 (64−74)
HK_F	13.30	39.88	6.99	0.60	6.95 (<8)	79.0 (78−87)

[a]Numbers in the brackets refer to the ashes (%) and protein content (%) declared by the producers.

Mass Analysis. The GPC analyses were performed on HK_G to determine the mean M_w, since, different from the other two HKs, it has not been disclosed by the distributor. GPC analyses revealed a number average molecular weight (M_n) of 31825 Da and a weight average molecular weight (M_w) of 33027 Da. From these values, a dispersity index (Đ) of 1.038 was calculated, indicating the uniformity in the average size of this type of HK, according to GPC analysis. Notably, HK_G has a M_w that is more than 10 times higher than that of the other two keratins (HK_K and HK_F), as declared by the producers.

The M_w of the HKs was also confirmed by SDS-PAGE under denaturing conditions (Figure 1). HK_G displayed a large

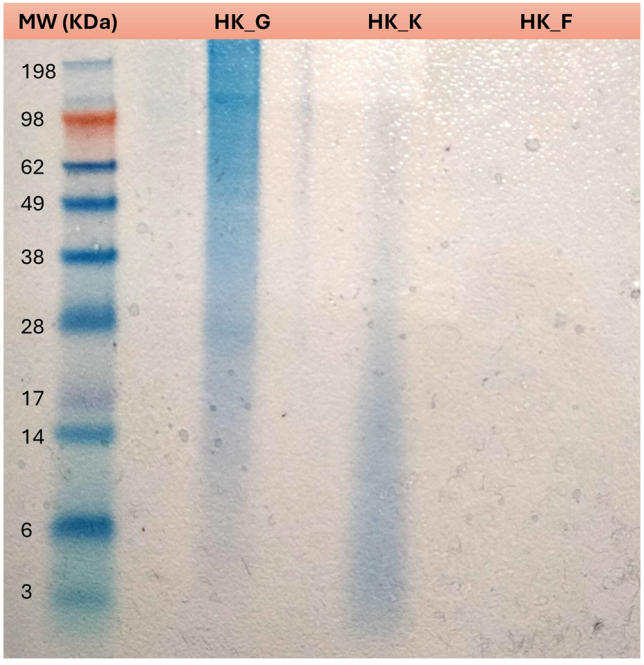

Figure 1. SDS-PAGE of commercial hydrolyzed keratins (HK_G; HK_F and HK_K). The first lane shows the protein standards.

number of dispersed bands covering a wide range of the protein standards employed between 17 and 150 kDa, suggesting that the acid hydrolysis led to a very polydisperse protein sample. HK_K exhibits only a smeared band at low M_w (<14 kDa), which is compatible with the M_w declared by the producer (average M_w ∼3 kDa) and the mild process using enzymatic hydrolysis. No bands could be observed from HK_F (M_w lower than 1 kDa from the manufacturer), indicating that this keratin undergoes a strong hydrolysis process, leading to the formation of short peptides.

Thermal Characterization. The weight (%) versus temperature plots from TGA showed two or three inflections, corresponding to the minima observed in the first derivative signal (Figure 2). The first inflection in the temperature range of 50−150 °C corresponds to the loss of adsorbed water on the powder sample. The moisture content for each sample was between 5 and 10% (3.8 ± 1.7% for HK-F, 6.0 ± 3.4% for HK_K, and 9.5 ± 1.7% for HK_G). The second or further inflections of the weight signal refer to the temperature interval in which the thermal degradation of HKs occurs. Specifically, a single-step degradation process has been recognized for HK_F and HK_G, while a multistep degradation process has been recognized for HK_K. Indeed, only one minimum value (above that referred to moisture content) can be observed for HK_F and HK_G, differently from HK_K, in the first derivate signal. From these values, the calculated denaturation temperatures are 227.3 ± 1.3 °C for HK_F, 297.7 ± 4.14 and 355.0 ± 1.1 °C for HK-K, and 334.2 ± 4.3 °C for HK_G.

FT-IR Characterization. The IR spectra of commercial HK powders are shown in Figure 3. In all spectra, the characteristic absorption bands of proteins due to peptide bond vibration (known as amide A, amide I, II, III) are visible. Specifically, the

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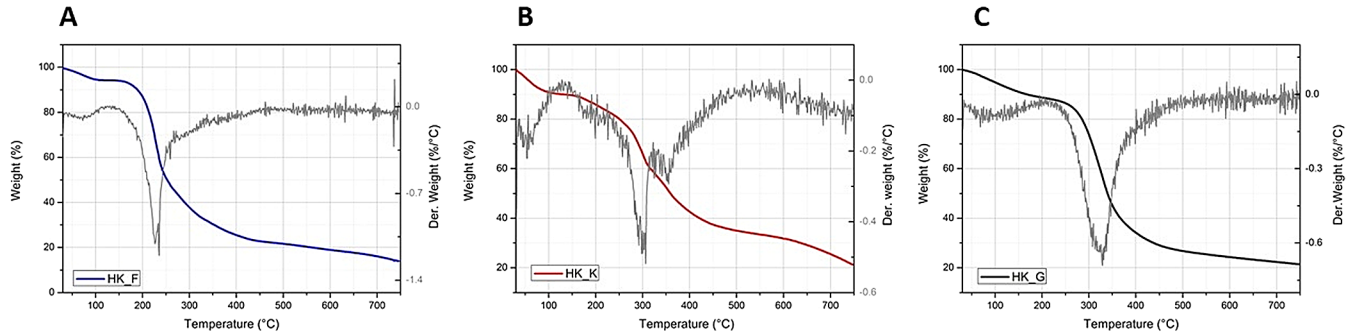

Figure 2. TGA for the commercial hydrolyzed keratins HK_F (A), HK_K (B), and HK_G (C). The gray line is the first derivative of the weight (%) vs temperature signal (°C).

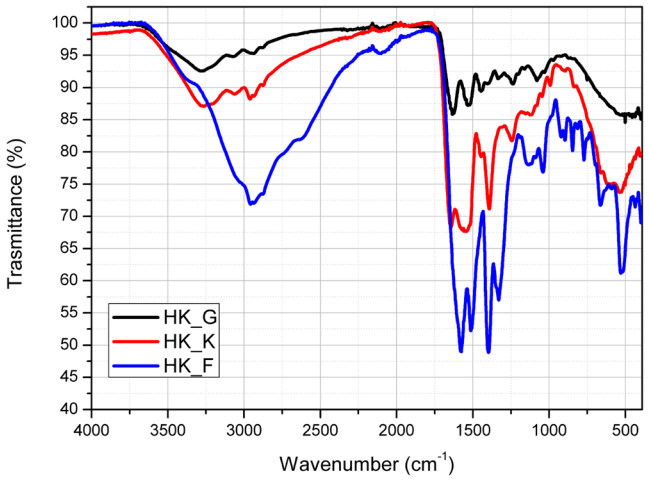

Figure 3. FT-IR analysis of commercial hydrolyzed keratins HK_F, HK_K, and HK_G.

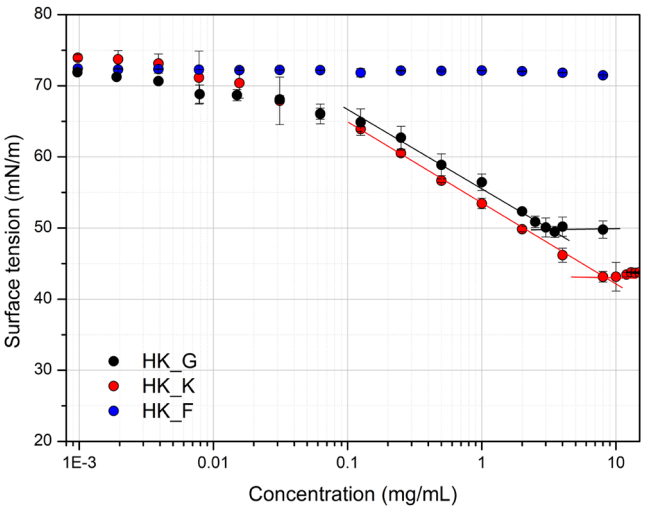

Figure 4. Surface tension (mN/m) vs concentration (mg/mL) plots for commercial hydrolyzed keratins.

absorption band of stretching vibration of N−H and −OH bonds is at around 3300 cm^{-1} and related to Amide A [12]. The O−H and N−H bands in the HK_F powder become more intense and broader, with slight shifts in position, as the molecular weight of keratin decreases and the degree of hydrolysis increases. This is due to enhanced hydrogen-bonding interactions among the hydroxyl and amino groups of smaller fragments and free amino acids.[18] Stretching vibrations of C═O bonds appear in the range from 1600 to 1700 cm^{-1}, and they are typical of the Amide I band. The C−H stretching bands within the 2800−3000 cm^{-1} range are due to the vibrational modes of C−H bonds in aliphatic hydrocarbon chains. At around 1520 cm^{-1}, the bending vibration of N−H of Amide II is observable, while stretching vibrations of C−N in combination with bending vibrations of N−H and C═O can be found at around 1220−1300 cm^{-1} and are related to Amide III. The absorption bands around 500 cm^{-1} can be attributed to the extension of the C−S bond and the S−S bond.

Surface Tension Measurement. Figure 4 shows concentration versus surface tension plots obtained from the analysis of the commercial HKs. The ability to decrease surface tension was found to be strongly dependent on the product type. Specifically, for HK_F, no decrease in surface tension was observed at any tested concentration up to 10 mM. Indeed, the measured surface tension is similar to that of pure water (∼72.8 mN/m). On the contrary, for HK_G and HK_K, the surface tension over concentration drops to values in the range of 43−49 mN/m, confirming that these types of HKs can behave as amphiphiles. From these plots, the apparent values for the critical micelle concentration (CMC) and the surface tension at CMC (γCMC) can be calculated. CMC and γCMC values are 3.50 ± 0.68 mg/mL and 49.97 ± 1.06 mN/m for HK_G and 9.20 ± 0.95 mg/mL and 43.11 ± 0.65 mN/m for HK_K, respectively.

Rheological Characterization of Keratin Dispersions in Water. A different rheological behavior was observed between the three analyzed HKs. In the range of concentrations tested (from 1 to 10% w/w) (Figure 5), HK_F and HK_K did not determine a relevant increase in the viscosity of their aqueous dispersions (viscosity range 0.001−0.002 Pa·s), which behave as Newtonian liquids (power law index, n ∼1, Table 2). On the contrary, the addition of HK_G in water led to a marked increase in viscosity as a function of keratin concentration. Indeed, the viscosity for HK_G in water increased from ∼0.004 to ∼3.61 Pa·s, moving from a concentration of 1% w/w to a concentration of 2.5% w/w. Differently, from HK_F and HK_K dispersions, HK_G dispersions display a pseudoplastic behavior as indicated by the n values that decreased from ∼0.90 to ∼0.55, moving from the concentration of 1% w/w to the concentration of 2.5% w/w (Table 2). The HK_G samples prepared at the concentration of 5% w/w and 10% w/w displayed a too-high consistency, so reliable values of dynamic viscosity cannot be measured. For this reason, these samples were analyzed through the stress sweep and frequency sweep tests (Figure

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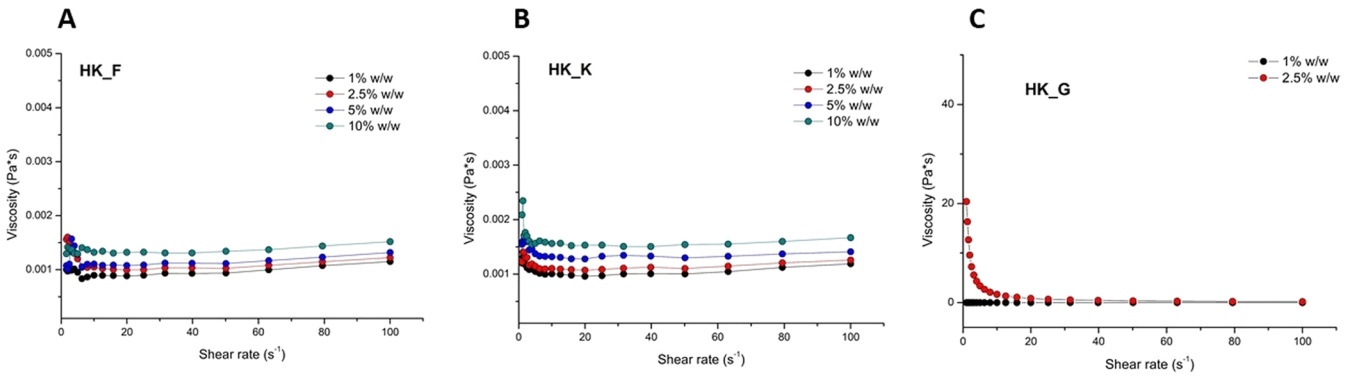

Figure 5. Viscosity vs shear rate plots for the commercial hydrolyzed keratins at concentrations of 1, 2.5, 5, and 10% w/w for HK_F and HK_K and at concentrations of 1% and 2.5% w/w, respectively, for HK_G water dispersions.

Table 2. Power Law Viscosity (PLV) and Power Law Index (n) for the Keratin (HK_F, HK_K, and HK_G) Dispersions in Water at Different Concentrations 1, 2.5, 5, and 10% w/w

	1% w/w		2.5% w/w		5% w/w		10% w/w	
	PLV (Pa·s)	n	PLV (Pa·s)	n	PLV (Pa·s)	n	PLV (Pa·s)	n
HK_F	0.0010	1.014	0.0015	0.901	0.0012	0.997	0.0013	1.009
HK-K	0.0012	0.963	0.0013	0.966	0.0016	0.955	0.0019	0.942
HK_G	0.0042	0.919	3.61	0.540	[a]	[a]	[a]	[a]

[a]no reliable PLV and n values can be calculated from viscosity measurements due to the high consistency of the sample.

Table 3. Power Law Viscosity (PLV) and Power Law Index (n) for the Keratin (HK_K and HK_G)-Based Emulsions at Different Keratin (1, 2.5, 5% w/w) and Oil (2.5, 5 and 10% w/w) Concentrations

	EO						
		2.5% w/w		5% w/w		10% w/w	
	% w/w	PLV (Pa·s)	n	PLV (Pa·s)	n	PLV (Pa·s)	n
HK_K	1	0.0013	0.983	[a]	[a]	[a]	[a]
	2.5	0.0009	1.04	0.0017	0.957	[a]	[a]
	5	0.0015	1.00	0.0014	0.975	0.0013	1.01
HK_G	1	0.008	0.954	0.007	0.934	0.006	0.946
	2.5	0.510	0.656	0.650	0.680	0.523	0.610
	5	[b]	[b]	[b]	[b]	[b]	[b]

[a]systems were not stable after preparation. [b]systems have a semisolid consistency.

SF1). The oscillatory tests confirmed the strong effect of the concentration on the consistency of the samples. Indeed, the elastic modulus (G') seems not to be much affected by the stress applied (up to 70 Pa), being ∼52 Pa for the concentration of 5% w/w and ∼420 Pa for the concentration of 10% w/w (stress sweep test). The frequency sweep test highlighted the formation of real gels from a rheological point of view, since the G' modulus is prevalent to the viscous modulus (G'') in the entire range of frequencies analyzed (1−10 Hz). Moreover, as a function of frequency, both modulus slightly increased, showing a hardening effect similar to those of other biopolymers in water.[19,20] According to these results, the thickening effect exerted by the investigated HKs was found to be strictly dependent on the molecular weight. Specifically, HK_F and HK_K, which exhibit a mean molecular weight of less than 2 kDa, have a negligible effect on the viscosity of their aqueous dispersions. On the other side, HK_G, having a much higher average molecular weight, was able to act as a thickening agent in water starting from a concentration of 2.5% w/w.

Rheological Characterization, Droplet Size, and Physical Stability of Keratin-Based Emulsions. Based on results obtained from surface tension and rheological measurements, HKs were tested for their ability to stabilize oil-in-water dispersions without using any other surfactant or stabilizer in the formulation. Indeed, there is a need for novel natural emulsifiers that can replace the nonenvironmentally sustainable surfactants derived from nonrenewable sources still largely employed so far.[21,22] At this extent, ternary systems composed of water, ethyl oleate as the model oily phase (2.5, 5, and 10% w/w), and HK as emulsifiers (1, 2.5, 5, and 10% w/w) were prepared. HK_F did not show emulsifying properties at all tested conditions, since all of the systems prepared showed an oiling-off phenomenon and phase separation in a few hours after the preparation. After 24 h, all emulsions prepared with HK_G and most of those prepared with HK_K appeared to be stable, without any eye-visible phase separation of the oil. Specifically, the visually unstable emulsions prepared with HK_K after 24 h were HK_K 1% EO 5% and HK_K 2.5% EO 10%. These results highlighted the good emulsifying ability of HK_G, which was able to provide emulsified systems at all tested compositions, and the partial emulsifying ability of HK_K, which instead was not able to form emulsified systems when the concentration of the oil was 2 times larger than that

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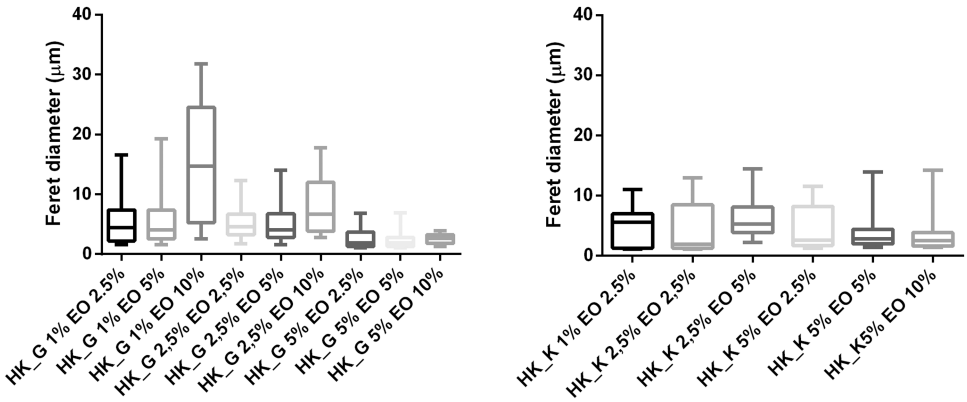

Figure 6. Box plots represent the droplet size distributions (according to number) for the keratin-based emulsions.

of the keratin. It seems that molecular weight can also play a relevant role in determining the emulsifying ability together with the surface ability of the macromolecules. Indeed, HK_F, having a very low molecular weight (<1 kDa) and no ability to decrease the air−water surface tension, does not behave as an emulsifier. On the contrary, HK_K and HK_G, having higher molecular weights and showing the ability to decrease surface tension, can behave as emulsifiers.

All emulsions that were stable after 24 h were analyzed in terms of viscosity and visually observed through optical microscopy to determine the droplet size.

The microscopy pictures of CH_G and CH_K emulsions after 24 h of preparation are included in the Supporting Information (Figure SF2).

The calculated power law viscosity (PLV) and power law index (n) or the keratin (CH_K and CH_G)-based emulsions at different keratin (1, 2.5, 5% w/w) and oil (2.5, 5 and 10% w/w) concentrations are reported in Table 3. All emulsions prepared using CH_K have very low viscosities in the range of 1−2 mPa s and behave as Newtonian fluids (n ∼1) independently from keratin concentration. An increase in viscosity from ∼5 mPa s to ∼0.5 Pa s was observed for emulsions prepared using HK_G moving from a keratin concentration of 1 to 2.5% w/w. These latter samples show also n values between 0.5 and 0.6, denoting a pseudoplastic behavior.

For all stable emulsions, droplet size distributions according to the number after 24 h of preparation are illustrated using box chart plots (Figure 6). The lower and upper borders of the box represent the 25 and 75th percentile, respectively, while the line in the middle of the box represents the median value of the distribution. The two whiskers represent the 10th and 90th percentile. For HK_G emulsions, an increase in the median values for the calculated maximum Feret diameter occurred when the oil concentration increased from 2.5 to 10% w/w at a fixed keratin concentration, generally matching with an increase in the polydispersity of the droplet size. Indeed, the median Feret values are around 4−5 μm for emulsions containing 2.5 and 5% w/w of oil, when keratin concentrations were 1 and 2.5% w/w. On the other hand, the median Feret values increased up to around 15 and 8 μm for emulsions containing 10% w/w of oil and keratin 1 and 2.5% w/w, respectively. The effect of keratin concentration on droplet size is also evident from these plots since the median Feret diameter progressively decreases moving from emulsions stabilized with 1% w/w (∼5 μm) of keratin to those stabilized with 5% w/w of keratin (∼2 μm).

For HK_K-based emulsions, similar considerations can be made for droplet size despite comparisons between samples being less straightforward since many samples were unstable after 24 h of preparation.

According to the previous results about viscosity and droplet size, three selected emulsions (HK 1% EO 2.5%, HK 2.5% EO 2.5%, and HK 2.5% EO 5%) prepared with HK_G and HK_K were observed over time at different time points (10, 20, 30, and 60 days) to assess their physical stability. All emulsions prepared with HK_K were unstable after 10 days of storage, showing oiling off on the surface and leading to phase separation (Figure SF3). On the other hand, no phase separation over time was observed for the emulsions prepared using HK_G, for at least up to 60 days, except for a slight creaming phenomenon, which was reversible upon agitation. These emulsions were observed through an optical microscope at each time point, and the median (d_{50}) values for the Feret mean diameter obtained from the number distribution are plotted in Figure 7.

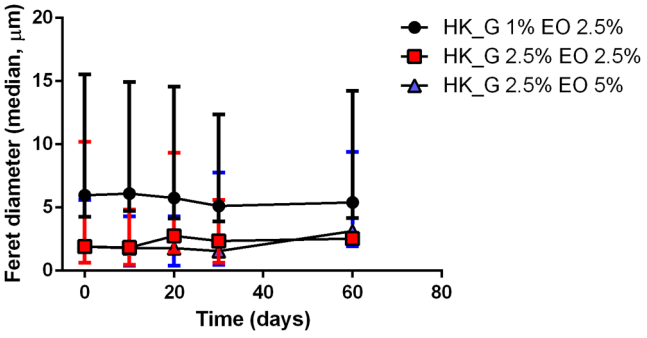

Figure 7. Variation of the calculated d_{50} values of the Feret mean diameter distribution over time for the HK_G-based emulsions. Diameter values were measured by image analysis from optical microscopy images. The error bar represents the first and third quartiles, and the distance between whiskers represents the interquartile range.

The calculated d_{50} values remain at around 5−6 μm during all of the observation time for the emulsion HK_G 1% EO 2.5% and at around 2−3 μm for the emulsions HK_G 2.5% EO 2.5% and HK_G 2.5% EO 5%. Polydispersity, as indicated by the distance between whiskers representing the interquartile range, remains almost unaffected over time, at least for up to 60 days. These results suggest that under the applied storage conditions (4 °C, in the dark, in tight closed vials), emulsions

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prepared using HK_G show satisfactory physical stability without any detectable droplet size increase and coalescence.

DISCUSSION

HKs are a family of biomaterials representing the water-soluble fraction of peptides derived from hydrolysis (acid, alkali, enzymatic, or other) of the fibrous water-insoluble keratin protein. HKs have several technological applications, mainly as a source of amino acids, in agriculture as fertilizers to improve soil structure and promote crop growth,[23,24] and in cosmetics as nutrients to smoothen and moisturize the cuticle of damaged hair and skin.[13,15] Despite the increasing interest in keratin-based biomaterials in pharmaceutics as biodegradable and biocompatible polymers for the development of scaffolds, hydrogels, and other dosage forms (e.g., films),[4,25−27] the use of HKs in pharmaceutical formulations is still very limited. Indeed, the methods for the preparation of HKs from keratins via hydrolysis have been largely reported in the literature.[8,28−30] However, little information is available regarding the chemical−physical properties of HKs, such as the ability to act as surface-active compounds and to stabilize dispersed systems, especially related to the different molecular weights and commercially available products. HKs from this study can be defined as low M_w below 1 kDa as for HK_F, midrange HKs for those having a M_w around 2−3 kDa as for HK_K, and high-M_w above 10 kDa as for HK_G, according to a classification already reported in the literature.[14] However, as evidenced by SDS-PAGE analysis, HK_G showed a smeared band at a very large M_ws range, suggesting the presence of different keratin-based ingredients in the composition of HK_G, despite being marketed with the INCI name of hydrolyzed keratin (N.CAS 69430−36−0). Large fractions of keratin ingredients can be related to partially hydrolyzed keratins as peptones or keratoses/kerateines.[31,32] Indeed, SDS-PAGE from keratoses/kerateines showed distinct bands in the M_w of 10−20 kDa, referred to as γ-kerateines, and 40−60 kDa and 100−120 kDa, referred to as α-kerateines.[31,33] The chemical−physical properties such as thickening ability in an aqueous environment and surface activity on the air−water surface were found to be strongly dependent on the M_w. Few papers reported the effect of HKs on surface tension. Bhavsar et al. showed a decrease of surface tension to around 35 mN/m in the plateau region at a concentration above 10% w/v for HKs of a M_w in the range of 3−14 kDa extracted using superheated water hydrolysis of wool.[34] Lu et al. investigated the surface behavior of keratin polypeptides obtained from alkaline and reductive extraction (8 M urea, 0.2 M SDS, and 0.5 M Na_2S_2O) from wool. The SDS-PAGE analysis of polypeptide keratins showed two main bands at around 45 kDa and 60 kDa, together with other bands at low molecular weights (6−20 kDa). These polypeptides saturated the air−water surface at a concentration above 0.1 mg/mL, reaching a surface tension value of around 55 mN/m.[35] Our results confirmed that midrange HKs seem to display higher apparent CMC and lower apparent γCMC values than the than high M_w HKs. On the other hand, low M_w HKs (M_ws < 1 kDa) are formed by short polypeptides having poor adsorption properties on the air−water surface. The emulsifying ability of HKs in relation to their M_ws has not been investigated before. Bouhamed et al. studied the emulsifying properties of feather protein hydrolysates obtained by acid hydrolysis at different times (100, 200, and 300 min) by determining the emulsion activity index (EAI) and the emulsion stability index (ESI). They found a relationship between the time of hydrolysis, but they did not investigate the effect of the MWs and did not provide any data regarding the stability of the emulsified systems.[36] In our study, a clear effect of the M_w in determining the emulsifying properties of commercially available HKs was observed since only high M_w HK (HK_G) was able to stabilize emulsifying systems over time (at least up to 60 days). Specifically, HK_G showed good emulsifying properties, since it was able to stabilize emulsions containing a percentage of oily phase also higher than those of HKs (at least up to an oil-to-surfactant ratio of 2:1) as for other natural surfactants.[37−39] From these preliminary results, it seems that an average M_w higher than 3 kDa is needed for HKs to act as an emulsifier and thickening agent. However, a comprehensive evaluation of the effect of the different MWs, protein contents, and hydrolysis processes (e.g., enzymatic, acidic, or reductive hydrolysis) on surface activity and emulsifying and thickening properties of HKs would require further systemic studies.

Definitively, HKs appear as very interesting products for different applications, including cosmetics and pharmaceutics. However, the present study highlighted an important discrepancy: despite being marketed under the same INCI name, different commercial HK products exhibit significant variability in molecular weight, protein content, and functional properties. This inconsistency complicates the selection and standardization of HK for formulation purposes, as the INCI designation alone does not provide sufficient information about the actual composition and behavior of each product. Our findings underscore the necessity for clearer standardization and quality control procedures to ensure that formulators can accurately predict product performance based on labeling.

CONCLUSIONS

This study evidenced the large variability in the chemical−physical properties of three commercial products available in the market with the INCI denomination of hydrolyzed keratin.

These chemical−physical properties seem mainly affected by the different molecular weights of the HKs analyzed. An increase in the protein content and a larger average M_w seem to improve the emulsifying and thickening ability of the HKs. Indeed, the emulsions prepared using HK_G (protein content of ∼89%, average M_w 33 kDa) have a higher consistency and much longer stability (>60 days) than emulsions prepared with HK_K (protein content of ∼82%, average M_w 3 kDa). According to our results, HKs can be a material useful as a natural emulsifier for pharmaceutical and cosmetic formulations, although the standardization of the product in terms of origin, hydrolysis method, and the average M_w is required to obtain an excipient with precise technological characteristics.

ASSOCIATED CONTENT

Data Availability Statement

All data generated or analyzed during this study are included in this published article and its Supporting Information. Data sets are available from the corresponding author upon reasonable request.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c10755.

Stress sweep and frequency sweep tests from HK_G-based hydrogels at two concentrations (10 and 5% w/w)

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(Figure SF1); optical microscopy images for HK_G-based emulsions (HK_G 1% EO 5%, HK_G 2.5% EO 5%, and HK_G 5% EO 5%) at a magnification of 20x and for HK_K-based emulsions (HK_K 1% EO 5%, HK_K 2.5% EO 5%, and HK_K 5% EO 5%) at a magnification of 10x after their preparation (Figure SF2); HK_K-based emulsions (HK 1% EO 2.5%, HK 2.5% EO 2.5%, and HK 2.5% EO 5% from left to right) after 10 days from the preparation (Figure SF3) (PDF)

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Author Contributions

D.R.P.: conceptualization, investigation, methodology, and writing of the original draft; A.C.: investigation; M.C.: data curation and visualization; A.T.: writing the review and editing; L.P.: investigation; B.S.: visualization; G.B.: writing the review and editing, supervision, and resources; G.F.P.: supervision.

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