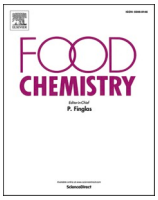
Food Chemistry 472 (2025) 142641

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Chemical composition and techno-functional properties of high-purity water-soluble keratein and its enzymatic hydrolysates

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ARTICLE INFO

Dataset link: Dataset Part 1 (Original data)

Keywords:
Alternative protein
Conventional proteolysis
Keratin reduction
Keratin isolate
Structure-properties relationship
Waste valorisation

ABSTRACT

This study compared the effectiveness of urea-containing and urea-free L-cysteine solutions in extracting high-quality feather keratin and evaluated commercial proteases for producing keratin-derived bioactive peptides. The urea-assisted extraction was crucial for achieving high structural integrity and yield of soluble keratin. The keratin isolate exhibited oil-holding capacity of 9.37 g/g, foaming capacity of up to 127 %, and emulsifying capacity of up to 49 %. Its proteolysis with trypsin, chymotrypsin, pepsin and subtilisin resulted in peptides with average molecular weight between 2.10 and 5.96 kDa and degree of hydrolysis from 6 to 36 %. The subtilisin hydrolysate had the highest degree of hydrolysis, 63 % of peptides <1 kDa, and excellent solubility across a wide pH range, but negligible water and oil-binding, foaming, and emulsifying properties. This study highlights the need to optimize each step in keratin extraction and hydrolysis processes to produce high-quality bioactive keratin preparations for diverse applications, including food and pharmaceutical.

1. Introduction

Keratins are structural proteins of the animal epidermis and its products. Their characteristic feature, distinguishing them from similar proteins such as collagen and elastin, is a high cysteine content (Ferraro et al., 2016). Keratins are extensively cross-linked with disulphide and hydrogen bonds and contain a high amount of hydrophobic amino acid residues. These properties result in a high stability of native keratins, rendering them insoluble in most solvents, indigestible and slowly biodegradable (Sharma & Kumar, 2019). α-Keratins, rich in cysteine structural proteins ranging in molecular weight (MW) between 40 and 70 kDa, consist of four α-helices coiled into a super-helix. These proteins are commonly found in wool, quills, hair, horns, fingernails, hooves and stratum corneum. β-Keratins, rich in short-chain amino acid residues protective proteins, are comprised of polypeptide chains within the 10–20 kDa MW range. These proteins, maintaining structural stability mainly through dense hydrogen bonds, are commonly found in feathers, beaks, claws and scales (Sharma & Kumar, 2019; Sinkiewicz et al., 2018; Wang, Yang, et al., 2016).

An enormous amount of keratin-rich waste biomass is generated globally, especially within the poultry industry which produces more than 8.5 billion tons of feathers annually (Jagadeesan et al., 2023). Chicken feathers contain 90 % raw keratin and constitute up to 10 % of the body mass of mature birds. Despite their abundance, the feather by-products remain substantially underutilized, presenting a considerable challenge in effective waste management and resource optimization (Sharma & Kumar, 2019). The majority of feather waste is disposed through incineration or composting, contributing to environmental pollution, while feather management methods are primarily for low-value applications, such as fertilizers and animal feed of inferior nutritional quality (feather meal). Such underutilization not only signifies a lost opportunity for the development of novel keratin-based bioproducts but also heightens environmental concerns linked to inefficient waste disposal (Ossai et al., 2022).

Abbreviations: ATR FT-IR, attenuated total reflectance Fourier transform infrared; DTT, dithiothreitol; DH, degree of hydrolysis; HP-SEC, high performance size exclusion chromatography; LGC, least gelling concentration; MW, molecular weight; OPA, o-phthaldialdehyde; pI, isoelectric point; SDS, sodium dodecyl sulphate; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

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https://doi.org/10.1016/j.foodchem.2024.142641
Received 19 September 2024; Received in revised form 12 December 2024; Accepted 22 December 2024
Available online 24 December 2024

Many techniques for keratin extraction have been developed, including chemical, physical or biological treatments and their combinations. These processes differ in the keratin yield, the physicochemical properties of protein preparations obtained and cost-effectiveness (Callegaro et al., 2019; Sinkiewicz et al., 2018). Keratin preparations containing digestible protein show great promise for use in value-added products within the food and pharmaceutical industry, such as novel dietary protein (Dias et al., 2022; Giteru et al., 2023; Houltham et al., 2014; Santos et al., 2024), source of bioactive peptides with antihypertensive and antidiabetic potential (Callegaro et al., 2019; Sinkiewicz et al., 2018) or functional additive due to its antioxidant, antimicrobial, foaming, emulsifying and fat-binding properties (Bouhamed et al., 2020; Sinkiewicz et al., 2018). However, the efficient production of keratin preparations suitable for such applications poses a challenge as native keratins are insoluble in solutions that do not trigger their degradation (Sinkiewicz et al., 2018). Thus, most feather solubilization methods yield low-quality keratin, often contaminated with unnatural amino acids or sulphur-containing odorous compounds, limiting its potential applications (Crum et al., 2018; Shavandi et al., 2017).

Reductive extraction stands out among the keratin isolation techniques. Not only for a high keratin yield but also for preserving the protein backbone through preferential cleavage of disulphide and hydrogen cross-links while minimizing peptide bond fragmentation. As a result of reductive extraction, soluble derivatives called kerateins are formed (Shavandi et al., 2017; Sinkiewicz et al., 2018). Among the most promising reducing agents that allow for keratein production is L-cysteine. Unlike most traditional reducing agents like β-mercaptoethanol, dithiothreitol (DTT) or sodium sulphide, L-cysteine is non-toxic, cost-effective, eco-friendly and widely available through sustainable industrial fermentation processes. L-Cysteine-extracted keratin could, therefore, be suitable for high-value applications, including the food and pharmaceutical industry (Ghaffari-Bohlouli et al., 2023; Xu & Yang, 2014).

Hydrolysis is a crucial tool in protein processing, releasing bioactive peptides and enhancing the protein's functional and nutritional value. Peptides produced through partial proteolysis have smaller MW and altered functional (e.g. solubility, emulsification, gelation) and biological (e.g. bioactivity, digestibility, allergenicity) attributes compared to intact proteins (Barac et al., 2012; Lorenzo et al., 2018; Tavano, 2013). While chemical hydrolysis is cost-effective and efficient, it is time-consuming, difficult to control and risks forming non-specific or toxic by-products (Momen et al., 2021; Stiborova et al., 2016). Microbial hydrolysis, though milder and more specific, is slow, and protein metabolism by microorganisms can reduce the total protein yield (Lasekan et al., 2013; Stiborova et al., 2016). Enzymatic hydrolysis provides superior control, specificity and repeatability, combined with rapid reaction speed. It produces highly nutritious hydrolysates free from toxic by-products and can be particularly cost-effective in the case of waste materials from mainstream food processing, considering processors are already charged for their disposal at landfills (Hayes, 2021; Lorenzo et al., 2018). However, commercial keratinases, while uniquely capable of cleaving native keratin, are not yet practical for industrial applications, due to the high cost and low activity (Saeed et al., 2024).

The objective of this study was to obtain a high-purity, water-soluble preparation of feather keratin and then generate bioactive peptides showing potential for food-grade applications. To address challenges in enzymatic keratin hydrolysis, this study proposes a novel two-step approach. First, soluble keratin was extracted through reduction with L-cysteine, with or without 8 M urea, and then hydrolysed using conventional proteases: trypsin, chymotrypsin, pepsin, subtilisin or papain. This approach contrasts with chemical and microbial methods typically used for keratin hydrolysis, potentially offering improved feasibility and cost-effectiveness. This investigation integrates our previous theoretical (in silico) study (Taraszkiewicz et al., 2022), which indicated that chicken feathers are a potential source of peptides with 15 bioactivities (mainly dipeptidyl peptidase IV, angiotensin-converting enzyme and prolyl oligopeptidase inhibitory and antioxidant), with experimental (in vitro) results of the keratin enzymatic hydrolysis, contributing to a comprehensive understanding of the process. Selected structural and techno-functional properties of the resulting hydrolysates, relevant to potential applications in the food or pharmaceutical industries, were also assessed.

2. Materials and methods

2.1. Materials

Commercial detergent Ludwik® was purchased from Grupa Inco S. A., Poland. L-Cysteine, urea, trypsin from porcine pancreas (1500 U/mg), α-chymotrypsin from bovine pancreas (≥40 U/mg protein), pepsin from porcine gastric mucosa (≥2500 U/mg protein), subtilisin A from *Bacillus licheniformis* (≥2.4 U/g, Alcalase® 2.4 L), papain from papaya latex (≥10 U/mg protein), acrylamide, ammonium persulphate, bromophenol blue, HCl, L-leucine, L-glycine, o-phthaldialdehyde (OPA), sodium dodecyl sulphate (SDS), sodium tetraborate, tetramethylethylenediamine, trichloroacetic acid (TCA), Tris, HPLC-grade acetonitrile, trifluoroacetic acid and protein standards were purchased from Sigma-Aldrich, USA. Acetic acid, glycerol, methanol and NaOH were acquired from POCH, Poland. Prestained Protein Marker II (6.5–200 kDa) was purchased from AppliChem, Germany. DTT and Coomassie Brilliant Blue R-250 were acquired from Fluka, USA. Food-grade refined sunflower oil was supplied by Rustica, Poland.

2.2. Obtaining keratin preparations

2.2.1. Pretreatment of feathers

White chicken feathers supplied by a local company (Drobful, Poland) were pretreated based on previous methods (Dąbrowska et al., 2022; Sinkiewicz et al., 2017). The feathers were first washed in warm tap water with detergent and rinsed with distilled water, then dried at 50 °C overnight, cut into 2–3 cm filaments and finally milled into 0.75 mm pieces using an ultra-centrifugal mill (Retsch, Type ZM 200, Germany).

2.2.2. Keratin extraction

The extraction of keratin was performed based on reduction methods reported previously (Sinkiewicz et al., 2017; Xu & Yang, 2014; N. Zhang et al., 2022). Either urea-containing or urea-free solution of L-cysteine was used for this purpose. In both cases, the pretreated feathers were mixed at a ratio of 1:20 (*w/v*) with extraction solution and shaken at 200 rpm (Thermo Forma 420 Orbital Shaker, Thermo Scientific, USA). In the case of urea-containing solution, the extraction was carried out with 1.5 or 2.0 % (w/v) L-cysteine and 8 M urea, adjusted to pH 9.0 or 10.5 with NaOH, at 30, 40, 50, 60 or 70 °C for 1, 3 or 6 h. In the case of urea-free solution, the extraction was carried out with 1.0, 1.5 or 2.0 % (w/v) L-cysteine, adjusted to pH 10.0, 11.0 or 12.0 with NaOH, at 30 °C for 24 h. After the reaction was completed, in both cases, the mixture was centrifuged (5000 rpm, 10 min) and filtered to separate the solution of soluble keratin from the feather residue. The filtrate was purified against distilled water using Spectra/Por dialysis membranes of regenerated cellulose (MWCO 3.5 kDa) at 4 °C for 3 days, freeze-dried and stored at 4 °C.

2.2.3. Keratin extraction yield

The keratin extraction yield [%] was determined as the weight loss of the raw material (feather solubilization) based on the measurement of the dry mass of undissolved feathers post keratin reduction processes (Sinkiewicz et al., 2017).

2.2.4. Enzymatic hydrolysis of keratin isolate

For the enzymatic hydrolysis only one keratin extract was chosen from the range of extracts produced, based on high yield, good structural

integrity and cost-effectiveness. This extract was obtained in the process with 1.5 % L-cysteine, 8 M urea, pH 10.5 at 30 °C for 1 h, labelled keratin isolate. This isolate was dissolved in distilled water to reach 10 mg protein/mL, and heated to the optimal temperature of each enzyme, followed by pH adjustment using either 1 M HCl or NaOH. Hydrolysis reactions with a different single enzyme: trypsin (KI-T), chymotrypsin (KI-C), pepsin (KI-P) and subtilisin (KI-S), were performed separately. For all the enzymatic reactions, the enzyme to substrate ratio was 1:20 (*w*/w) and the reaction time was 1 h. The hydrolysis was carried out in triplicate at pH 8 and 37 °C (KI-T and KI-C), at pH 2 and 37 °C (KI-P), or at pH 9 and 60 °C (KI-S). The pH was kept constant during the whole reaction using a pH-stat titrator (T70, Mettler Toledo, USA) with 0.1 M NaOH (KI-T and KI-C), 1 M HCl (KI-P), or 1 M NaOH (KI-S). After the reaction, keratin hydrolysates obtained were heated at 85 °C for 15 min to inactivate the enzymes, replicates pooled, freeze-dried and stored at 4 °C. Preliminary experiments with papain revealed that keratin hydrolysates produced by this enzyme did not reach the degree of hydrolysis (DH) > 2 % and regardless of hydrolysis conditions excessive protein aggregation occurred (data not shown).

2.3. Chemical composition and structural properties

2.3.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis was performed according to Laemmli (1970). The keratin extracts obtained with urea-free solution, the keratin isolate, and its enzymatic hydrolysates were dissolved in distilled water at 20, 10, and 30 mg/mL, respectively. Variability in the concentration was necessary to visualize the particular protein bands, due to differences in protein integrity between the samples. The stacking and separating gels were 5 and 15 %. The gels were stained with 0.25 % Coomassie Brilliant Blue R-250 dissolved in a mixture of methanol, distilled water and acetic acid (50:40:10; *v*/v) and then destained using a mixture of methanol, distilled water and acetic acid (40:53:7; v/v). Pre-stained mixture of 10–240 kDa proteins was used as an MW marker.

2.3.2. High performance size exclusion chromatography (HP-SEC)

The samples or protein standards were dissolved in a mobile phase composed of acetonitrile, Milli-Q water and trifluoroacetic acid (30:60.9:0.1; v/v) to a final concentration of 0.5 mg/mL and centrifuged (12,000 rpm, 10 min). 30 μL of supernatant was injected into ReproSil 50 SEC column (300 × 8 mm, 5 μm), with a fractionation range of 0.5–10 kDa and eluted at a flow rate of 1 mL/min. Agilent liquid chromatograph (model 1200) with a diode array detector at 215 nm was used. The column was calibrated with: aprotinin (6.5144 kDa), bovine insulin (5.7335 kDa), bovine insulin oxidized, chain B (3.4959 kDa), bacitracin A (1.4227 kDa) and bradykinin (1.0602 kDa).

2.3.3. Attenuated total reflectance Fourier transform infrared (ATR FT-IR) spectroscopy

The FT-IR spectra of the pretreated feathers, the keratin isolate and its enzymatic hydrolysates were recorded in the range of 400–4000 cm^{-1} on FT-IR spectrophotometer (Invenio-R, Bruker, USA). Sixty four scans were recorded for each spectrum, with a resolution of 4 cm^{-1}. Prior to the analysis, the samples were conditioned in a desiccator over P_2O_5 for 7 days.

2.3.4. Degree of hydrolysis

Two methods for the determination of the DH were used. DH_{OPA} was measured based on quantification of free -NH_2 groups, according to Bavaro et al. (2021) with a slight modification. 10 μL of the sample was mixed with 200 μL of reagent consisting of 0.8 mg/mL OPA, 38.1 mg/mL sodium tetraborate, 1 mg/mL SDS and 0.88 mg/mL DTT. After 15 min incubation, A_{340} was measured using a microplate reader (Synergy HT, BioTek Instruments, Inc., USA). L-Leucine was used to generate a calibration curve. DH_{OPA} was calculated as:

$$DH_{OPA}\ [\%] = (h_1/h_2) \times 100\%$$

where: h_1 and h_2 – concentration of -NH_2 groups in the sample and in the keratin isolate subjected to oxidation with performic acid followed by complete hydrolysis with HCl according to ISO 13903:2005, respectively [mmol -NH_2/g protein].

$DH_{pH\text{-}stat}$ was measured based on the volume of NaOH solution added during the hydrolysis to maintain a constant pH (Teshnizi et al., 2020) and calculated according to Adler-Nissen (1986):

$$DH_{pH-stat}\ [\%] = (V_b \times N_b)/(m_p \times h \times \alpha) \times 100\%$$

where: V_b – NaOH volume [mL], N_b – NaOH concentration [mol/L], m_p – protein mass [g], h – total number of peptide bonds [7.7 mEq/g protein], α – degree of dissociation of α-NH_2 groups.

2.3.5. Proximate composition

Moisture and ash content of the feathers, the keratin isolate and its enzymatic hydrolysates were determined using Leco TGA701 gravimetric oven (Leco Instruments UK Ltd., UK) (Pérez-Vila et al., 2024). 0.8 g of each powder was heated to 102 °C at a rate of 1 °C/min and held at 102 °C for 4 h to determine the moisture content, and then heated to 550 °C at a rate of 15 °C/min and held at 550 °C for 4 h to determine the ash content. Protein content was determined by total amino acid analysis (2.3.6.) in the feathers and the keratin isolate, and by subtraction of moisture and ash content in the enzymatic hydrolysates.

2.3.6. Amino acid analysis

Analysis of the total amino acid profile in the feathers and the keratin isolate was performed by Sciantec Analytical Services Ltd. (Cawood, UK) according to ISO 13903:2005. The analysis of the free amino acid profile (except Trp) in the keratin isolate and enzymatic hydrolysates followed the method of Mounier et al. (2007). An equal volume of the redissolved sample and 24 % (*w/v*) TCA were mixed, left for 10 min and then centrifuged (15,000 rpm, 10 min). The supernatant was removed, and the sample was diluted 1:2 with the internal standard, norleucine, to give a final concentration of 125 nmol/mL norleucine. Free amino acids were quantified using a Jeol JLC-500/V amino acid analyser (Jeol (UK) Ltd., Garden City, Herts, UK) fitted with a Jeol Na^+ high performance cation exchange column.

2.4. Techno-functional properties.

2.4.1. Solubility

The solubility of the keratin isolate and its enzymatic hydrolysates was measured according to Bouhamed et al. (2020), with a slight modification. The sample was dissolved in distilled water to a final concentration of 10 mg protein/mL, pH was adjusted to 2–9 using 0.1–2 M HCl or NaOH and then the mixture was centrifuged (5000 rpm, 30 min). The protein content of the supernatant was determined using the Biuret method. Solubility was expressed as a percentage of soluble protein content relative to the total protein content in the sample.

2.4.2. Gelling properties

The least gelling concentration (LGC) of the keratin isolate and its enzymatic hydrolysates was determined according to Taragjini et al. (2022). The samples were dissolved in distilled water to reach 1, 5, 10, 15 or 20 % (m/m), heated at 90 °C for 1 h in a heat block (Red-Hot 35, DNA Gdańsk, Poland) and then cooled down at 4 °C for 2 h. The LGC was defined as the minimal concentration at which the sample did not slip from the inverted tube.

2.4.3. Water- and oil-holding capacity

The water- and oil-holding capacity of the keratin isolate and its enzymatic hydrolysates were measured according to Ma et al. (2022), with a slight modification. The powdered samples (100 and 50 mg) were

mixed with 1 mL distilled water and 1.5 mL sunflower oil, respectively, vortexed for 1 min, left for 30 min and then centrifuged (5000 rpm, 30 min). The supernatant was eliminated and the sample was weighted.

2.4.4. Foaming properties

The foaming properties of the keratin isolate and its enzymatic hydrolysates were measured according to Bouhamed et al. (2020) with a modification. 100 mg of powdered sample was dissolved in distilled water to a final volume of 10 mL, pH was adjusted to 5, 7 or 9 using 0.1–1 M HCl or NaOH and the mixture was homogenized (12,000 rpm, 1 min).

The foaming capacity and foam stability were calculated as follows:

$$\text{Foaming capacity } [\%] = (V_B - V_A)/V_A \times 100\%$$

$$\text{Foam stability } [\%] = (V_C - V_A)/(V_B - V_A) \times 100\%$$

where: V_A, V_B and V_C – the total volume of the mixture before, directly after and 30 min after homogenization, respectively [mL].

2.4.5. Emulsifying properties

The emulsifying properties of the keratin isolate and its enzymatic hydrolysates were measured according to Taragjini et al. (2022) with a modification. 100 mg of powdered sample was dissolved in distilled water to a final volume of 10 mL, pH was adjusted to 5, 7 or 9 using 0.1–1 M HCl or NaOH and then 15 mL of sunflower oil was added. The mixtures were homogenized (12,000 rpm, 1 min), centrifuged (7000 rpm, 5 min), heated at 85 °C for 15 min, and re-centrifuged. The emulsifying capacity and emulsion stability were calculated as follows:

$$\text{Emulsifying capacity } [\%] = (V_1 - V_0)/V_0 \times 100\%$$

$$\text{Emulsion stability } [\%] = (V_2/V_1) \times 100\%$$

where: V_0 – the total volume of the mixture before homogenization [mL], V_1 and V_2 – the volume of the emulsion layer after centrifugation, heating and re-centrifugation, respectively [mL].

2.5. Statistical analysis

The experimental results were processed using SigmaPlot 11.0 (SYSTAT Software, Germany) through one-way analysis of variance (ANOVA) with a significance level of $p < 0.05$.

3. Results and discussion

3.1. Obtaining keratin preparations

3.1.1. Keratin yield obtained with urea-containing solution of L-cysteine

In the first part of this study, urea-containing solution of L-cysteine was used to extract the keratin. Urea is commonly used in reductive keratin extraction to unfold the protein and facilitate the exposure of disulphide bonds to reducing agents (Shavandi et al., 2017; Sinkiewicz et al., 2018). Initially, the effects of reaction temperature and time on keratin yield were investigated while maintaining a constant composition of the extraction solution previously proposed (Pourjavaheri et al., 2019; Xu & Yang, 2014). This solution, comprising 2.0 % (*w/v*) L-cysteine and 8 M urea at pH 10.5, consistently yielded approx. 90 % keratin, even at the mildest reaction conditions tested (Table S1). Increasing the temperature up to 70 °C and time up to 6 h did not enhance the keratin yield. Therefore, in pursuit of greater cost-effectiveness, the effects of decreasing the L-cysteine concentration from 2.0 to 1.5 % and lowering the pH from 10.5 to 9.0, on the keratin yield were examined while the temperature was 30 °C and the reaction time was 1 h. The decreased concentration of L-cysteine had no significant effect on the keratin yield ($p > 0.05$), while lowered pH led to the decrease of the keratin yield to approx. 86 % ($p < 0.05$, Table S2). Such a result likely reflects the finite number of disulfide bonds in keratin, which, once reduced, render further increases in L-cysteine concentration ineffective. The lower keratin yield at pH 9 likely results from reduced deprotonation of the -SH group, decreasing nucleophilicity and reactivity, thereby impairing disulfide bond reduction (Xu & Yang, 2014). Thus, in terms of keratin yield with urea-containing L-cysteine solutions, the optimal composition of the extraction solution was 1.5 % L-cysteine, 8 M urea, pH 10.5, and the optimal reaction conditions were 30 °C for 1 h, at which the keratin yield reached about 90 %. Xu and Yang (2014) reported a maximal feather keratin yield of 75 % under similar extraction conditions, i.e. 10 % L-cysteine based on the weight of feathers (equivalent to approx. 0.6 % in solution), 8 M urea, pH 10.5 at 70 °C for 12 h. Pourjavaheri et al. (2019) obtained a maximal yield of 66 % when treating the feathers with 2.0 % L-cysteine, 8 M urea, pH 10.5 at 40 °C for 6 h. Qin et al. (2023) achieved a maximal keratin yield of 63 % using 15 % L-cysteine, 8 M urea, pH 10.5, with ultrasonic treatment at 200 W for 4 h. In the reductive feather keratin extraction with urea and different reducing agents, Sinkiewicz et al. (2017) obtained the yield of 84, 82, 78 and 63 % with β-mercaptoethanol, sodium bisulphite, DTT and sodium m-bisulphite, respectively. The differences in keratin yield reported by these studies can be attributed to variations in the reducing agents used and their concentration, reaction conditions (e.g. temperature, pH, solid-liquid ratio), and additional treatment methods such as ultrasound, which can enhance solubilization but also lead to over-degradation under prolonged exposure. Furthermore, differences in feather pretreatment, such as grinding to increase surface area, and variations in the calculation methods for keratin yield may have also contributed to these discrepancies.

3.1.2. Keratin yield obtained with urea-free solution of L-cysteine

In the second part of the study, the keratin extraction with urea-free solution of L-cysteine was performed. According to Zhang et al. (2022), wool keratin can be effectively extracted at room temperature using only 2 % L-cysteine at pH > 10, which lowers the price by eliminating 8 M urea, but with extraction time prolonged to 24 h. According to these authors, the wool keratin yield obtained at such conditions was between 5 and 95 %, increasing with pH. In our study, the keratin yield obtained at 30 °C after 24 h reaction ranged from 14 to 88 %, increasing with L-cysteine concentration and pH of the extraction solution (Table S3). According to Dąbrowska et al. (2022) alkaline-hydrolysed feather keratin yield ranged from 11 to 41 % at room temperature and, according to Sinkiewicz et al. (2017), from 29 to 94 % at 70 °C. In both of these studies and similar to Table S3, NaOH concentration was the major factor determining the extraction yield.

3.2. Chemical composition and structural properties

3.2.1. SDS-PAGE

3.2.1.1. Electrophoretic profiles of keratin obtained with urea-containing solution of L-cysteine. SDS-PAGE was used to evaluate the keratin integrity and to estimate the MW of the peptides obtained. Only the keratin isolate produced in the optimized process with 1.5 % L-cysteine, 8 M urea, pH 10.5 at 30 °C for 1 h was chosen for analysis as it had a high keratin yield with lower temperature, less time (Table S1), and lower L-cysteine content (Table S2) than other extracts. The profile of the keratin isolate showed that it had a well-preserved protein backbone and was relatively pure (Fig. 1A, KI), as intact bands at expected MW were clearly seen. The most intense band at 10 kDa corresponded to β-keratin (the dominant component of feathers) and those at 50–70 kDa were most likely α-keratins (Sharma & Kumar, 2019).

The profiles of KI-T and KI-C indicated that hydrolysis of the keratin isolate with trypsin and chymotrypsin led to peptides of 8–13 kDa. In the profiles of KI-P and KI-S only weak bands were visible, indicating the presence of low MW peptides that are not detectable by the staining

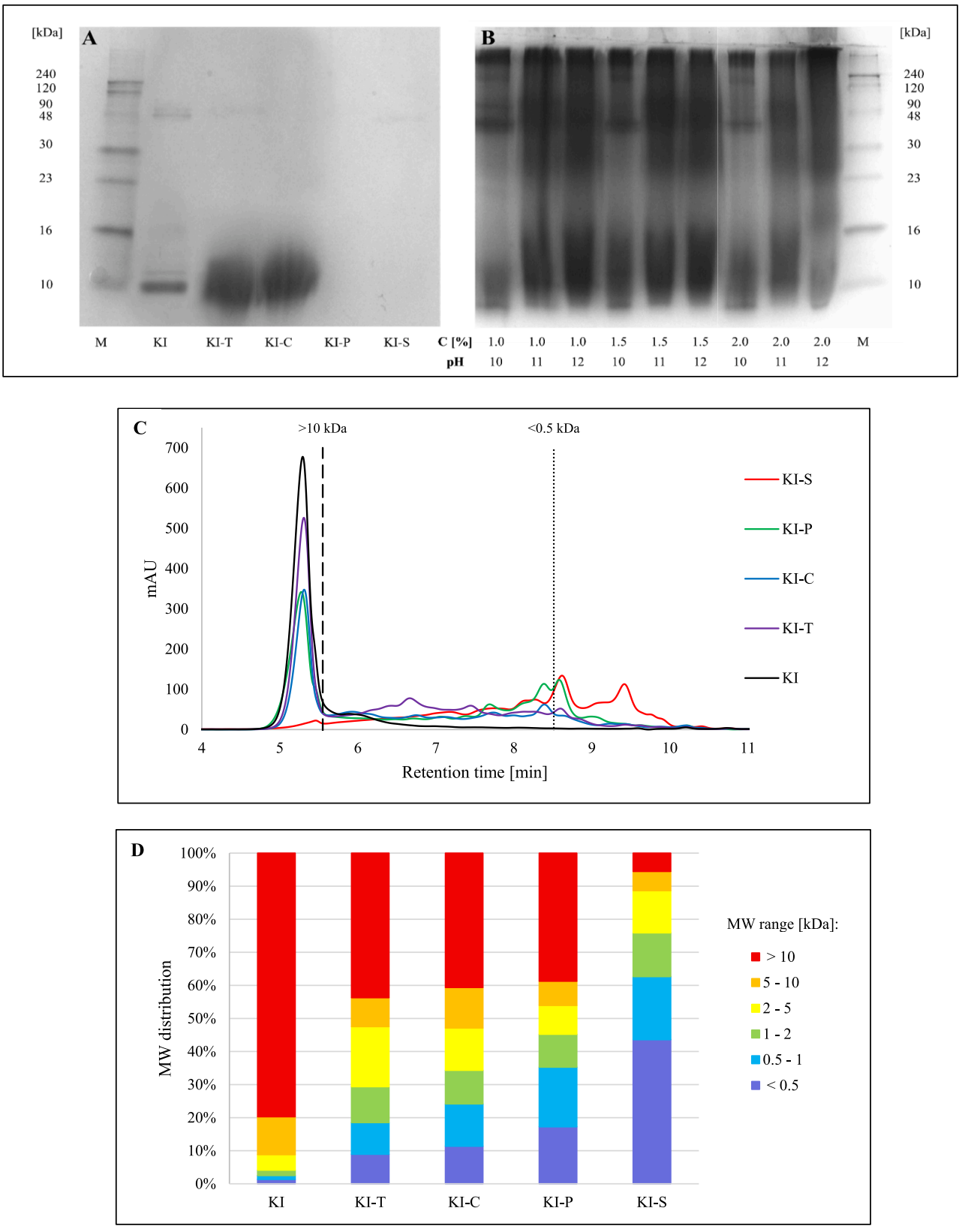

Fig. 1. MW distribution of keratin preparations: (A) SDS-PAGE electropherogram of the keratin isolate and its enzymatic hydrolysates, (B) SDS-PAGE electropherogram of keratin extracts obtained with urea-free solutions, (C) HP-SEC chromatogram of the keratin isolate and its enzymatic hydrolysates, (D) MW distribution profiles of the keratin isolate and its enzymatic hydrolysates. KI – keratin isolate.

employed. Based on the specificity of the proteases applied, such a result was expected. Trypsin has the narrowest substrate specificity, followed by chymotrypsin, having a slightly wider substrate preference and ending up with pepsin and subtilisin, both having wide substrate specificity (Minkiewicz et al., 2019).

Xu and Yang (2014) isolated feather keratin with urea-containing L-cysteine solution, visualising β-keratin only or both α- and β-keratin bands, depending on extraction conditions. Nonetheless, smears indicating protein degradation due to non-specific alkaline hydrolysis were present in all samples obtained by these authors. Pourjavaheri et al. (2019) also used urea-containing L-cysteine solution and extracted feather keratin with higher purity, as indicated by the absence of smears. However, only band corresponding to β-keratin was observed. The differences in electrophoretic keratin patterns obtained in the aforementioned studies, in comparison with the keratin isolate, may again be a result of different feather pretreatment but also keratin reduction conditions and isoelectric precipitation rather than dialysis.

3.2.1.2. Electrophoretic profiles of keratin obtained with urea-free L-cysteine solutions. As the keratin yield obtained in the urea-free extraction varied greatly (Table S3), all 9 extracts were analysed by SDS-PAGE to evaluate the protein integrity (Fig. 1B). In contrast to the electrophoretic pattern of the keratin isolate, obtained with urea-containing solution, all electrophoretic profiles of extracts produced with urea-free solutions were visible as smeared bands. This indicates that protein degradation has occurred, likely due to alkaline hydrolysis caused by high pH and long reaction time, regardless of reaction conditions tested (Fig. 1B). The degree of keratin degradation, represented by the band smearing intensity, was moderate at pH 10 and severe at pH 11 and 12. These degradation products, while likely of limited usefulness in high-value sectors, could potentially still be suitable for applications where protein integrity is less critical, such as fertilizers (Sinkiewicz et al., 2018). Zhang et al. (2022) obtained L-cysteine-extracted keratin from wool at pH ranging from 7 to 13.5 without urea. According to the authors, the keratin yield increased with rising pH levels, but the integrity of the polypeptide chain was maintained up to pH of 12, leading to the extraction yield of approx. 70 %. As Fig. 1B clearly shows, undegraded feather keratin could not be obtained without urea. The differences in the protein profiles of L-cysteine-extracted feather and wool keratins, obtained under similar reduction conditions, probably result from varying proportions of keratin types in these sources. In feathers, β-keratin, the stiffer and less extractable one, is predominant, while in wool, α-keratin, easier to solubilize, is more abundant (Sharma & Kumar, 2019; Sinkiewicz et al., 2018).

From the range of keratin extracts obtained with urea-containing and urea-free solutions of L-cysteine, only the keratin isolate was chosen for the assessment of structural and functional properties, based on the high yield, purity, structural integrity and cost-effectiveness.

3.2.2. HP-SEC

The sharpness of the keratin isolate peak (Fig. 1C, black line) and its retention time observed in the chromatogram at close proximity to the upper fractionation range of the column (10 kDa) confirm the preservation of the polypeptide backbone during extraction. A slight tail on the right side of the peak indicates the presence of only a small fraction of keratin hydrolysis products. The retention time of the peak mostly overlaps with the void volume of the column, indicating that most analytes were close to or higher than 10 kDa in size. The β-keratin of chicken feathers, the dominant component of feathers, comprises 97 amino acid residues and has the MW of 9.9 kDa (Bateman et al., 2023). The sizes of peptides were the highest in the keratin isolate and lower in the enzymatic hydrolysates, with the average MW of 9.04, 5.96, 5.79, 5.23 and 2.10 kDa for the keratin isolate, KI-T, KI-C, KI-P and KI-S, respectively (Fig. 1D). Qin et al. (2023) reported that keratin isolated by ultrasound-assisted extraction with 15 % L-cysteine, 8 M urea, pH 10.5 exhibited a broad MW distribution. Depending on ultrasonic power and time, the majority of peptides were within the 0.5–3 kDa range, reflecting significant protein fragmentation. The MW of protein hydrolysates significantly influences their bioactive properties. Low MW peptides generally exhibit reduced immunogenicity, as well as improved solubility, bioavailability, and bioactivity, except for antimicrobial activity, which is typically attributed to peptides containing 10–50 amino acid residues (Apostolopoulos et al., 2021; Malinowska-Pańczyk, 2023).

3.2.3. Degree of hydrolysis

Protein DH in the keratin preparations was measured using two methods, and the results obtained, DH_{OPA} and $DH_{pH\text{-}stat}$, were compared to theoretical DH_t values calculated in our in silico study (Taraszkiewicz et al., 2022, Table 1). The OPA assay confirmed that the DH_{OPA} value of the keratin isolate was below 1 %. The actual keratin DH may have been even lower, considering the reactivity of the OPA reagent towards residual L-cysteine remaining after extraction and ε-NH_2 groups of lysine, even in intact protein (Rutherfurd, 2010).

The effectiveness of keratin hydrolysis by the proteases applied, indicated by their DH_{OPA} values, aligned with the results from HP-SEC (Fig. 1). A strongly linear relationship ($R^2 > 0.98$) between the hydrolysates' average MW determined by HP-SEC and their DH was noted, regardless of the DH assay. This suggests that the reduction in the keratin isolate MW post-enzymatic hydrolysis resulted solely from the breakdown of peptide bonds. In contrast, according to Zhang et al. (2015), the MW decrease in duck feather keratin caused by alkaline hydrolysis resulted not only from the disruption of peptide bonds but also from the disulfide and non-covalent bonds.

$DH_{pH\text{-}stat}$ of KI-P could not be measured due to the hydrolysis at pH 2. The $DH_{pH\text{-}stat}$ values of KI-T, KI-C and KI-S showed a very strong correlation ($R^2 = 0.99$) with their respective DH_{OPA} values. However, the values of $DH_{pH\text{-}stat}$ were higher than those of DH_{OPA}, likely due to the poor reactivity of the OPA reagent with proline and cysteine in comparison with other amino acids. The values of DH_{OPA} obtained for hydrolysates rich in free cysteine and free proline, and peptides containing these amino acids, may, therefore, be underestimated (Rutherfurd, 2010).

At least one of the experimental DH values of KI-T, KI-C and KI-S aligned closely with the DH_t values (Table 1). In contrast, the value of DH_{OPA} in KI-P was much lower than its DH_t, suggesting that the peptide bonds susceptible to pepsinolysis, although present in the keratin chain, were largely inaccessible. A discrepancy between the theoretical and experimental results of protein hydrolysis is common (Iwaniak et al., 2024). The in silico proteolysis is based only on the specificity of enzymes, whereas the actual complete enzyme characteristics are more complex. The computer simulation assumes that all peptide bonds are

Table 1
DH and proximate composition of feathers, the keratin isolate and its enzymatic hydrolysates.

Parameter [%]	Feathers	KI	KI-T	KI-C	KI-P	KI-S
Moisture	9.7 ± 0.05^{A}	7.7 ± 0.03^{C}	5.6 ± 0.03^{E}	5.6 ± 0.04^{E}	8.5 ± 0.02^{B}	5.9 ± 0.01^{D}
Ash	0.6 ± 0.1^{E}	1.6 ± 0.03^{D}	4.0 ± 0.04^{B}	3.9 ± 0.06^{B}	2.0 ± 0.05^{C}	24.3 ± 0.36^{A}
Protein	83.8 ± 0.95^{C}	91.0 ± 0.08 ^{A}	90.5 ± 0.06 ^{A}	90.5 ± 0.09 ^{A}	89.5 ± 0.03^{B}	69.8 ± 0.03^{D}
DH_{OPA}	–	0.8 ± 0.05^{E}	5.9 ± 0.34^{D}	6.9 ± 0.35^{C}	7.6 ± 0.62^{B}	20.5 ± 0.55^{A}
$DH_{pH\text{-}stat}$	–	–	8.4 ± 0.19^{C}	12.8 ± 0.15^{B}	–	36.2 ± 0.11^{A}
DH_t*	–	–	6.3	14.6	54.2	36.4

The values in the columns marked with various superscript letters (A-E) differ significantly ($P < 0.05$).
KI – keratin isolate.
* Adapted from Taraszkiewicz et al. (2022).

cleavable, while under experimental conditions some of them may be resistant to hydrolysis and the protein-enzyme interactions can be impeded by the complexity of the protein 3D structure (Iwaniak et al., 2020). Additionally, in the case of feathers, the experimental DH referred to all feather proteins present in the keratin isolate, while in our theoretical study, only β-keratin was analysed (Taraszkiewicz et al., 2022).

3.2.4. FT-IR

In all the FT-IR spectra of keratin preparations obtained, bands typical for proteins were observed (Table 2, Fig. 2). The FT-IR spectrum of the keratin isolate (Fig. 2, KI) is like the spectrum of the pretreated feathers (Fig. 2, Feather), with only slightly lower absorbance at approx. 2935 and 2850 cm^{-1}, and Amide *I*-III regions. No new bands appeared in the spectrum of the keratin isolate compared to the spectrum of native keratin present in the pretreated feathers. This indicated that the former has similar functional groups to the latter (N. Zhang et al., 2022), confirming the high purity of the keratin isolate and lack of significant peptide bond scission during the extraction (Wang, Li, et al., 2016; Y. Zhang et al., 2015). However, a partial transition from α-helix to β-sheet configuration could have taken place in the keratin isolate, as evidenced by a slight shift in the Amide *I* region towards higher wavenumbers (Alahyaribeik & Ullah, 2020a; Martínez-Hernández et al., 2005; Wang, Li, et al., 2016).

In the enzymatic hydrolysates, changes in the Amide *I* and Amide II band intensity, and the right shoulder in the Amide A band indicated an increase in the amount of free -NH_2 groups, confirming the peptide bond hydrolysis. This is consistent with the results of HP-SEC and DH. A strong negative correlation ($R^2 = 0.84$) between DH_{OPA} and the band absorbance corresponding to the Amide I region was observed, while a moderate positive correlation ($R^2 = 0.69$) existed between DH_{OPA} values and the band absorbance corresponding to —COO^- group. The FT-IR spectrum of KI-P does not show a peak around 1390 cm^{-1}, unlike the remaining hydrolysates. This difference is likely due to the pH of KI-P being below the pI, causing protonation of the —COOH group, whereas in the other hydrolysates, the pH was above the pI, resulting in its deprotonation. The prominent bands at 530–540 cm^{-1} found in all keratin preparations suggest that even with extensive disruption of peptide bonds, the keratin continues to be strongly cross-linked by disulfide bonds.

3.2.5. Proximate composition

The feathers showed a moisture content of approx. 10 %, similar to previous findings (Sharma & Kumar, 2019). The keratin isolate and its enzymatic hydrolysates exhibited lower moisture level, from 5.6 to 8.5 % (Table 1), typical of powdered proteins (Rao et al., 2016) and ensuring microbiological stability.

The ash content of the feathers, initially 0.6 %, gradually increased with subsequent processing steps. In the keratin isolate, it rose by 1 %, likely due to residual NaOH remaining post-dialysis, and in the enzymatic hydrolysates, it increased proportionally with the DH ($R^2 > 0.88$), up to nearly 25 % in the case of KI-S (Table 1). The high ash content is a typical characteristic of extensively hydrolysed proteins from both plant and animal sources, as reaching high DH requires the significant addition of acids or bases for pH adjustment (Chalamaiah et al., 2012; Tawalbeh et al., 2023).

The protein content of the feathers and the keratin isolate reached approx. 84 and 91 %, respectively. High purity of the keratin isolate was confirmed, as its moisture, ash and protein content reached a total of 100 %. This result made it possible to calculate the protein content of the enzymatic hydrolysates by subtracting their moisture and ash contents from the total sample mass. The protein content in the hydrolysates decreased with the increasing DH, due to the increasing content of inorganic salts (ash) (Table 1).

3.2.6. Total amino acid composition

Table 3 details the total amino acid profile of the feathers and the keratin isolate. Published data on whey protein isolate, a complete, easily digested protein of high nutritional value is included for comparison (Norton et al., 2012). The presented profiles of the feathers and the keratin isolate were similar to the profiles of feathers and keratin preparations reported previously (Sharma & Kumar, 2019). The content of most amino acids was higher in the keratin isolate than in the feathers, except for Glx, His, Lys, Met and Tyr, indicating that these amino acid residues are likely concentrated in the parts of feathers that are more difficult to solubilize. Both the feathers and the keratin isolate contained all essential and non-essential amino acids (EAA and NEAA, respectively), however the content of His, Lys, Met and Trp was too low to consider the keratin isolate a complete protein. The total EAAs content of whey protein isolate was about 1.5 times higher than that of the keratin isolate. The keratin isolate's level of branched-chain amino acids: Ile, Leu and Val was only about 13 % lower than in whey protein isolate, indicating its potential in reducing post-exercise muscle damage and soreness (Salem et al., 2024). The keratin isolate's high Cys content no doubt contributes to it's antioxidant capacity, beneficial for cellular defence and immune functions (Callegaro et al., 2019; Iwaniak et al., 2024; Sinkiewicz et al., 2018). The high content of Arg and Cys in the keratin isolate suggests its potential use in supplements for low birth weight infants, who require elevated levels of these amino acids in their diet (Kaushik et al., 2016; M. Wang et al., 1999). A much higher Arg:Lys ratio (1:0.06 in the keratin isolate vs. 1:2.3 in whey protein isolate) indicates potential cardiovascular benefits, as a higher ratio is commonly linked to reduced cholesterolemic and atherogenic effects of food proteins (Friedman, 2018; Kaushik et al., 2016). Additionally, the high Pro content indicates the keratin isolate's potential as a precursor of bioactive peptides. Studies focusing on the structure-activity

Table 2
Band assignments in the FT-IR spectra of pretreated feathers, keratin isolate and its enzymatic hydrolysates.

Region	Position [cm^{-1}]						Band assignment	Reference
	Feather	KI	KI-T	KI-C	KI-P	KI-S		
Amide A	3281	3281	3281	3281	3279	3279	ν_{NH}, ν_{OH}, α-helix	(Martínez-Hernández et al., 2005; Pourjavaheri et al., 2015, 2019)
Amide *B*	3073	3073	3073	3061	3061	3061	asymmetric ν_{NH}	(Martínez-Hernández et al., 2005)
	2930	2961	2961	2961	2961	2959	asymmetric ν_{CH3}	(Martínez-Hernández et al., 2005; Pourjavaheri et al., 2015, 2019)
	2936	2936	2936	2936	2936	2936	symmetric ν_{CH3}	(Martínez-Hernández et al., 2005; Pourjavaheri et al., 2015, 2019)
	2853	2878	2878	2878	2878	2876	ν_{CH2}	(Martínez-Hernández et al., 2005)
Amide *I*	1630	1634	1636	1636	1638	1632	$\nu_{C=O}$, α-helix, β-sheet	(Pourjavaheri et al., 2019)
Amide II	1514	1518	1528	1526	1518	1518	δ_{NH}, ν_{CH}, β-sheet	(B. Ma et al., 2016; Martínez-Hernández et al., 2005; Pourjavaheri et al., 2019)
	1447	1449	1450	1450	1450	1450	δ_{CH2} or δ_{CH3}	(Martínez-Hernández et al., 2005)
	1389	1389	1392	1391	–	1391	δ_{CH3}, -COO^-	(Kristoffersen et al., 2019, 2020; Martínez-Hernández et al., 2005)
Amide III	1232	1234	1236	1236	1232	1238	ν_{CN}, δ_{NH}, ν_{CC}, $\delta_{C=O}$	(B. Ma et al., 2016; Martínez-Hernández et al., 2005)
	1159	1159	1157	1159	1169	1159	$\nu_{S=O}$	(Alahyaribeik & Ullah, 2020b)
	1070	1061	1072	1047	1070	1047	ν_{CC}	(Martínez-Hernández et al., 2005)
	548	548	538	536	534	542	ν_{SS}	(Alahyaribeik & Ullah, 2020b)

KI – keratin isolate.

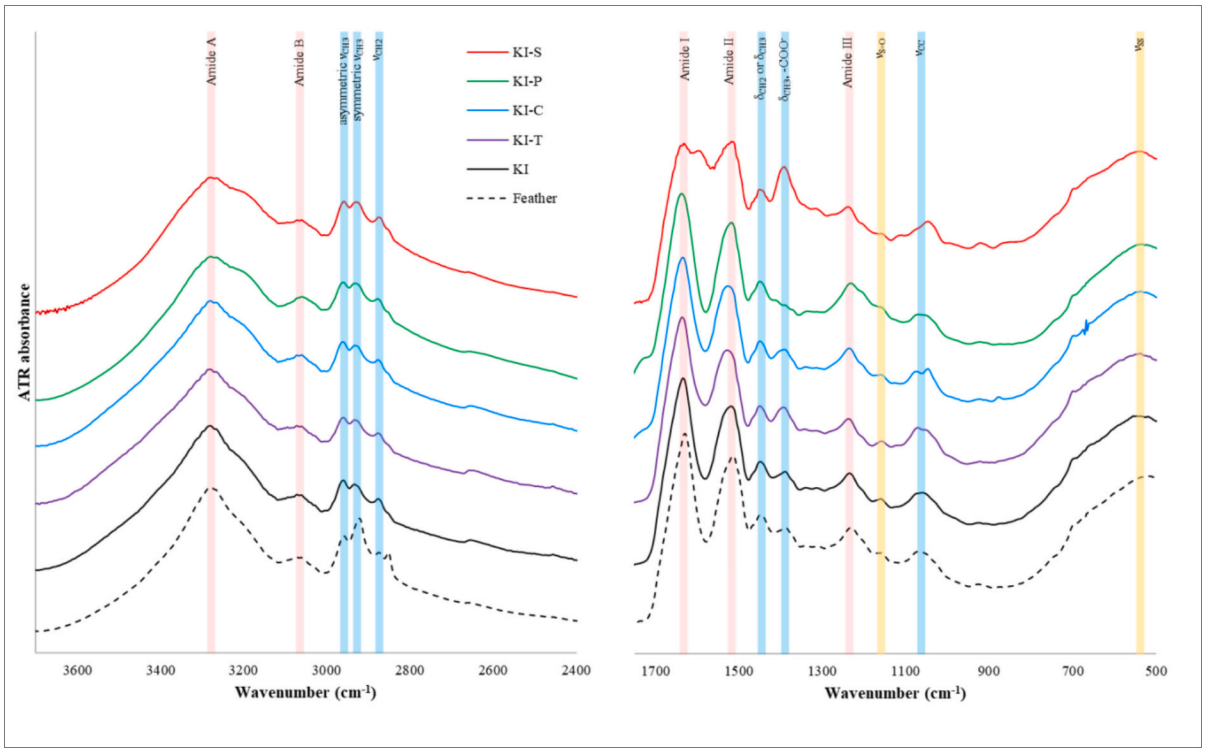

Fig. 2. FT-IR spectra of the pretreated feathers, the keratin isolate and its enzymatic hydrolysates. KI – keratin isolate.

Table 3
Total and free amino acid profiles of the feathers, the keratin isolate and enzymatic hydrolysates.

Amino acid	Total amino acid content [g/100 g]			Free amino acid content [mg/g]				
	Feather	KI	WPI	KI	KI-T	KI-C	KI-P	KI-S
Ala	3.66	4.07	4.41	0.03	0.07	0.08	0.05	1.49
Arg	5.70	6.40	2.16	0.02	3.04	0.13	0.10	0.54
Asx	5.28	5.41	9.53	0.00	0.08	0.00	0.39	0.00
Cys[a]	5.93	7.82	2.25	6.24	2.99	3.16	5.99	4.33
Cya[a]	–	–	–	0.97	4.98	8.76	11.69	11.23
Glx	8.69	8.40	15.19	0.01	0.05	0.00	0.12	2.87
Gly	6.15	7.00	1.62	0.03	0.05	0.07	0.09	0.28
His[b]	0.50	0.20	1.80	0.18	1.80	1.45	1.19	6.06
Ile[b]	4.09	4.40	5.57	0.01	0.08	0.75	1.23	6.93
Leu[b]	6.82	7.20	9.80	0.02	0.05	0.05	0.48	0.86
Lys[b]	1.50	0.40	8.18	0.00	1.06	1.72	0.16	0.85
Met[b]	0.48	0.20	1.80	0.03	0.08	0.18	0.32	1.71
Phe[b]	4.08	4.70	2.97	0.10	1.92	3.06	1.54	4.22
Pro	8.78	10.10	5.03	0.00	0.00	0.00	0.00	0.00
Ser	9.77	11.90	4.23	0.03	0.07	0.11	0.11	1.28
Thr[b]	3.96	4.20	5.75	0.01	0.05	0.12	0.09	0.66
Trp[c]	0.41	0.40	1.53	–	–	–	–	–
Tyr	1.84	1.60	2.70	0.01	0.15	0.06	0.03	0.58
Val[b]	6.16	6.50	5.39	0.00	0.00	0.55	0.00	1.74
ΣEAA	28.00	28.20	42.79	0.28	3.18	4.94	3.58	20.09
ΣNEAA	55.80	62.70	47.11	7.33	11.48	12.37	18.57	22.59
ΣBCAA	17.07	18.10	20.77	0.03	0.12	1.34	1.71	9.54
TOTAL	83.80	90.90	89.90	7.68	16.52	20.25	23.58	45.62

ΣEAA, ΣNEAA, ΣBCAA – sum of essential, non-essential and branched chain amino acids, respectively.
KI – keratin isolate.
WPI – whey protein isolate accessed from Norton et al. (2012).
[a] For the total amino acid analysis Cys content was determined after performic acid oxidation (as Cya).
[b] Essential amino acid.
[c] For the free amino acid analysis Trp could not be quantified accurately by Joel JLC-500/V.

relationship of peptides highlight Pro residue as an important contributor to their inhibition of angiotensin-converting enzyme, dipeptidyl peptidase IV (Iwaniak et al., 2018) and prolyl oligopeptidase (Taraszkiewicz et al., 2024), and antioxidant activity (Sinkiewicz et al., 2018). Furthermore, the presence of Pro residue in peptides increases their resistance to proteolytic degradation. These peptides may have longer half lives in the gut lumen increasing their opportunity to cross the gut barrier and enter the bloodstream (become bioavailable)

(Udenigwe et al., 2021).

3.2.7. Free amino acid composition

The most abundant free amino acids in the keratin isolate were Cys and Cya – its oxidized derivative (Table 3). These were likely residuals from the keratin extraction and dialysis processes. The total content of other free amino acids was below 0.5 mg/g, confirming that the keratin isolate was not significantly hydrolysed during extraction. The total content of free amino acids in the enzymatic hydrolysates was directly proportional to DH_{OPA} values ($R^2 = 0.99$). KI-T exhibited the highest content of free Arg, consistent with our prediction that Arg would be the only free amino acid released from β-keratin by trypsin (Taraszkiewicz et al., 2022). In KI-C, the total free amino acid content was about 20 mg/g, despite our predictions that chymotrypsin could only release peptides from β-keratin (Taraszkiewicz et al., 2022), suggesting these free amino acids were α-keratin-derived. The highest total content of free amino

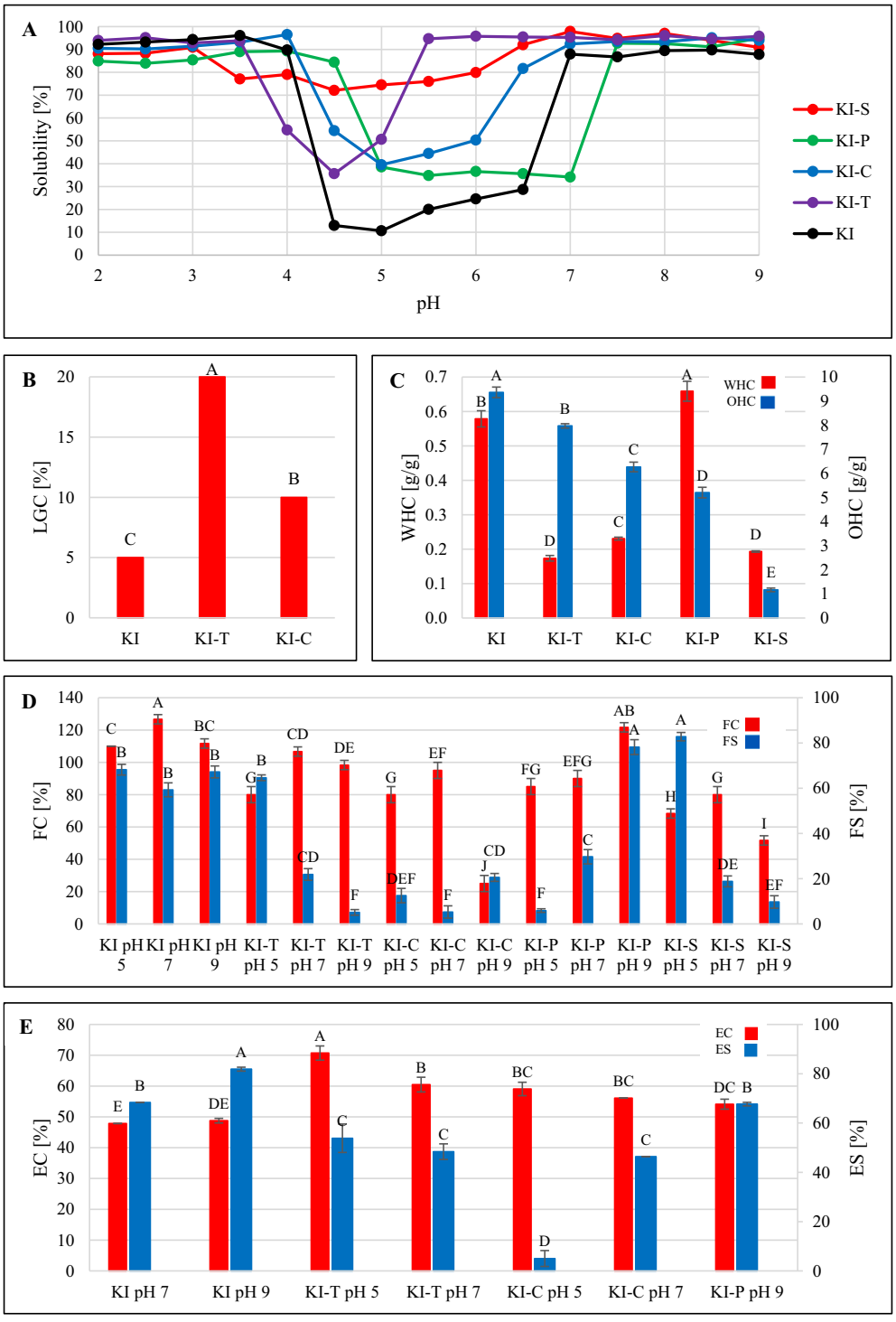

Fig. 3. Techno-functional properties of keratin preparations: (A) protein solubility [%] as function of pH; (B) gelling properties, LGC – least gelling concentration; (C) water and oil holding properties, WHC – water holding capacity, OHC – oil holding capacity; (D) foaming properties, FC – foaming capacity, FS – foam stability; (E) emulsifying properties, EC - emulsifying capacity, ES – emulsion stability. KI – keratin isolate. Values marked with different superscript letters (A-J) differ significantly ($p < 0.05$).

acids was detected in KI-S, as expected from the hydrolysate with the highest DH. However, with its MW fraction <0.5 kDa making up over 40 % (Fig. 1D), it is likely that this fraction predominantly comprised low MW peptides rather than free amino acids. None of the hydrolysates contained free Pro, even though it was the second most abundant amino acid in the keratin isolate (Table 3). This means that all Pro residues remained incorporated in peptides, indicating the keratin isolate's potential as a promising precursor of bioavailable peptides with high bioactivities (Udenigwe et al., 2021).

3.3. Techno-functional properties

3.3.1. Solubility

The keratin isolate exhibited a typical U-shaped solubility profile, with the highest amount of precipitated protein close to the isoelectric point (pI), at pH of 4.5–5.0 (Fig. 3A). The enzymatic hydrolysis of the keratin isolate caused a reaction condition-dependent shift in the pI. KI-P had a similar low-solubility pH range to the keratin isolate, although the pI shifted towards higher pH. The solubility of the remaining enzymatic hydrolysates was clearly improved in comparison with the keratin isolate, as the low-solubility pH range was narrower, and a lower amount of precipitate was noted at the pI (Fig. 3A). KI-S exhibited high solubility over the wide pH range, due to the presence of peptides with the lowest MW that are highly soluble (Fig. 1). Esparza et al. (2018) and Bouhamed et al. (2020) determined the pI of keratin at pH 4 in the case of protein resulting from DTT-assisted reduction and acid hydrolysis, respectively. Dąbrowska et al. (2022) reported the pI range at pH 3.4–4 for keratin obtained through alkaline hydrolysis. According to Xu and Yang (2014), the feather keratin extracted with the urea-containing solution of 0.6 % L-cysteine, pH 10.5 at 70 °C for 12 h, purified by isoelectric precipitation, was water-insoluble, unlike the keratin isolate extracted under very similar conditions. According to Shavandi et al. (2017), the conditions of water-soluble keratin production must be strictly controlled, as even small changes in the ratio of reducing and denaturing agents can cause protein precipitation.

3.3.2. Gelling properties

Gelling properties are crucial for proteins in food applications, where forming self-supporting gels is highly valued. Proteins with a low LGC typically exhibit strong gel-forming capability (Kamani et al., 2024; Taragjini et al., 2022). The keratin isolate, containing peptides of the highest MW (Fig. 1), had the lowest LGC at 5 %, indicating superior gel formation. Conversely, KI-P and KI-S, composed of the smallest peptides, failed to form gels even at 20 % (Fig. 3B), highlighting the necessity of large peptide size for structural integrity in gel networks (Damodaran et al., 2007). However, KI-T had the LGC of 20 %, twice that of KI-C, despite having a higher MW (Fig. 1). This suggests that peptide composition and their mutual interactions also affected gelling capacity. Moreover, gelling properties are influenced by pH, ionic strength and the presence of other solutes, which affect electrostatic and hydrophobic interactions within proteins (Kamani et al., 2024; Taragjini et al., 2022). While protein hydrolysates rarely form heat-induced gels, except for gelatine with an average MW > 20 kDa (Damodaran et al., 2007), various keratin-based gels have been reported at concentrations of 5–20 % (Shavandi et al., 2017). For comparison, the LGCs of bovine gelatine, chickpea, egg white, soybean, lentil and whey protein preparations were 3, 6, 10, 10, 13 and 14 %, respectively (Aydemir & Yemenicioğlu, 2013).

3.3.3. Water- and oil-holding capacity

Water- and oil-holding capacity are useful in determining protein powder's efficacy in preventing fluid leakage during processing and storage (Bouhamed et al., 2020; Kamani et al., 2024). The water-holding capacity of the keratin preparations obtained ranged between 0.17 and 0.66 g/g, with KI-P exhibiting the highest water retention (Fig. 3C). A clear inverse correlation was observed between water-holding capacity and solubility: the keratin isolate and KI-P, with the lowest solubility, had the highest water-holding capacity values. Conversely, the highly soluble hydrolysates – KI-T, KI-C and KI-S showed worse water retention. Such an inverse relationship is common. Highly soluble proteins tend to have more hydrophilic regions, making them more dispersible in water but less capable of forming networks that trap water (Damodaran et al., 2007). Bouhamed et al. (2020) reported similar water-holding capacity values for acid-hydrolysed feather keratin, ranging from 0.6 to 1.1 g/g. Aydemir and Yemenicioğlu (2013) reported the water-holding capacity of whey, egg white, lentil, chickpea, soybean and bovine gelatine at 0.00, 0.14, 1.04, 6.46, 7.94 and 8.84 g/g, respectively.

The keratin isolate demonstrated a remarkably high oil-holding capacity of 9.37 g/g, while the enzymatic hydrolysates showed a DH-dependent oil-holding capacity decrease (Fig. 3C). A robust inverse correlation between keratin DH and oil-holding capacity was evident ($R^2 > 0.94$), suggesting that lower peptide sizes resulted in poorer oil-binding capacities of the hydrolysates. Bouhamed et al. (2020) reported the oil-holding capacity of acid-hydrolysed feather keratin at 2–2.5 g/g, notably lower than that of the keratin isolate and most of its enzymatic hydrolysates. Wattie et al. (2018) found that feather keratin-based cryogels achieved a maximum oil-holding capacity of 10.76 g/g. According to Aydemir and Yemenicioğlu (2013), the oil-holding capacity of bovine gelatine, soybean, whey, egg white, lentil and chickpea protein preparations was 1.12, 1.16, 1.59, 6.37, 8.62 and 13.37 g/g respectively.

3.3.4. Foaming properties

All keratin preparations analysed formed foams, but their foaming properties strongly depended on pH and DH (Fig. 3D). Higher DH generally corresponded to lower foaming capacity, although not uniformly at all pH levels. At pH 5 and 7, a relatively strong linearity ($R^2 > 0.68$) was observed, but this correlation diminished at pH 9 ($R^2 < 0.24$), irrespective of the DH assay. At pH 7, the keratin isolate and all hydrolysates, except KI-P, exhibited the highest foaming capacity values. KI-P showed an increase in foaming capacity as the pH rose, peaking at pH 9 with foaming capacity of 122 %, coupled with stability of 88 %. In contrast, KI-S at pH 9 demonstrated the poorest performance, with the foaming capacity of only 52 % and stability of 67 %. At all pH levels tested, the keratin isolate outperformed the hydrolysates, maintaining some of the highest foaming capacity and achieving foam stability exceeding 60 %. For instance, the keratin isolate at pH 5 showed foaming capacity of 110 % and stability of 83 %, underscoring its superior foaming capabilities. Among the enzymatic hydrolysates, relatively high foam stability was observed only at certain pH values: at pH 5 in KI-T and KI-S, and at pH 9 in KI-P. In KI-C, the foam stability was low regardless of pH.

Bouhamed et al. (2020) reported the foaming capacity values of acid-hydrolysed feather keratin ranging from 55 % to 130 %, with stability from 0 % to 50 %, depending on hydrolysis conditions and protein concentration. Aydemir and Yemenicioğlu (2013) reported that the foaming capacity of egg white, lentil, soy, chickpea, whey and bovine gelatine protein preparations were 24, 34, 36, 44, 44 and 60 %, respectively. Their foam stability ranged between 90 and 100 %. In comparison, the keratin isolate's foaming capacity and foam stability values at pH 7 (127 % and 77 %, respectively) were remarkably high, indicating superior foaming properties. This comparison highlights the potential of keratin preparations obtained, especially the isolate, in applications requiring robust foam formation. On the other hand, the enzymatic hydrolysates displayed foaming properties ranging from moderate to poor, heavily influenced by pH.

3.3.5. Emulsifying properties

Emulsifying properties are crucial for numerous applications, where proteins stabilize oil-water mixtures. All keratin preparations obtained formed emulsions after homogenization with sunflower oil. However, only 7 out of the 15 tested pH variants formed emulsions that could be

measured after centrifugation (Fig. 3E), while in the remaining preparations, complete separation of the protein and oil layer occurred post-centrifugation. None of the preparations formed a measurable emulsion layer at all three tested pH variants, and KI-S did not form a measurable emulsion layer at any of the pH variants tested, indicating the pH sensitivity of the keratin-stabilized emulsions. The emulsifying properties of the keratin preparations were influenced by the DH and pH. The keratin isolate demonstrated a relatively high emulsifying capacity of 49 % at pH 7 and pH 9, with corresponding emulsion stability of 68 % and 82 %, but did not form a measurable emulsion layer at pH 5. In contrast, KI-T and KI-C formed measurable emulsions at pH 5 and 7, but not at pH 9, while KI-P – only at pH 9. A moderate correlation between emulsifying and foaming capacity ($R^2 = 0.61$) was noted, suggesting that proteins with good foaming abilities also stabilize emulsions effectively.

According to Damodaran et al. (2007), the emulsifying and foaming properties of proteins generally improve after partial hydrolysis, due to solubility improvement, up to DH of approx. 10 %. In this study, the keratin hydrolysates did not show a clear relationship between solubility at the given pH and the emulsifying capabilities. Despite all keratin preparations obtained, except KI-S, being largely insoluble at pH 5, KI-T and KI-C were still capable of forming measurable emulsions at this pH. In contrast, the keratin isolate and KI-P could only form emulsions in the pH range where they were highly soluble. According to Lin et al. (2022), peptides with MW above 5 kDa exhibit better emulsifying properties than smaller peptides. Our results align with these observations, as the hydrolysates with the lowest MW, exhibited significantly worse emulsifying and foaming properties.

4. Conclusions

This study significantly improved the L-cysteine-assisted feather keratin reduction method, enhancing keratin yield, quality and solubility. Urea-containing solution of L-cysteine was critical in achieving the high structural integrity of the keratin isolate, significantly reducing extraction time under alkaline conditions compared to urea-free extraction. The optimized method produced the keratin isolate with substantial potential for food and pharmaceutical applications due to its high purity, enzymatic digestibility, unique amino acid composition and desirable functional attributes, particularly gelling, oil-binding and foaming properties. The amino acid profile of the keratin isolate indicated its suitability for bioactive peptide production, with a high content of hydrophobic amino acids and Cys for high antioxidant potential, and a high Pro content enhancing peptide bioavailability. Nonetheless, supplementation with His, Lys, Met and Trp was found to be recommended for a better balanced profile of EAAs.

Enzymatic hydrolysis increased the DH of the keratin isolate up to 36 %, leading to improved solubility but limited gelling capacity and worsened oil-holding capacity in a DH-dependent manner. The foaming and emulsifying properties of KI-T, KI-C and KI-P differed to those of the keratin isolate, and these differences were a function of DH and pH. KI-S had the highest DH, the highest content of low MW peptides with potentially high bioactivity and bioavailability, and excellent solubility, but its foaming, emulsifying, water and oil-binding properties were negligible. A comparison of experimental DH and DH_t values demonstrated good agreement for trypsin, chymotrypsin and subtilisin, confirming the reliability of in silico predictions for these proteases. Conversely, pepsin and papain exhibited significantly lower experimental DH, highlighting their limited efficiency under the tested conditions. These findings validate the utility of bioinformatic approaches for enzyme performance prediction while emphasizing the need to optimize specific enzymes like pepsin and papain.

The presented study emphasizes the importance of optimizing each step in the keratin extraction and hydrolysis processes to produce high-quality, bioactive protein preparations from feather waste. Ongoing investigations evaluate the performance of the obtained keratin preparations during thermal processing, simulated gastrointestinal digestion and intestinal absorption.

Funding sources

This work was supported by the Polish national research budget, under the National Science Centre grant number DEC-2021/41/N/NZ9/04466 and Irish Department of Agriculture, Food & Marine DAFM-2019R495-INFOTECH.

CRediT authorship contribution statement

Antoni Taraszkiewicz: Writing – original draft, Visualization, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Izabela Sinkiewicz:** Writing – review & editing, Supervision, Resources, Methodology, Formal analysis. **Agata Sommer:** Writing – review & editing, Visualization, Software, Resources, Methodology, Investigation, Formal analysis, Data curation. **Barbara Kusznierewicz:** Methodology. **Linda Giblin:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Hanna Staroszczyk:** Writing – review & editing, Supervision, Project administration, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that support the findings of this study are openly available in Bridge of Knowledge at https://doi.org/10.34808/7548-pd20.

Acknowledgements

The authors are grateful to Paula O'Connor (Teagasc) for her assistance with free amino acid analysis, Piotr Bruździak (Gdańsk University of Technology) for his help with FT-IR measurements and Marika Mróz (Gdańsk University of Technology) for her aid with HP-SEC analyses.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.142641.

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