chemistry proceedings

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Proceeding Paper

Combined Alkaline and Enzymatic Hydrolysis of Eggshell Membranes for Obtaining Ingredients for Food and Cosmetic Applications †

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† Presented at the 19th International Symposium "Priorities of Chemistry for a Sustainable Development", Bucharest, Romania, 11–13 October 2023.

Abstract: This study investigated the combined alkaline and enzymatic hydrolysis of eggshell membranes for obtaining ingredients for food and cosmetic applications. 1.25 N NaOH and Alcalase 2.4 L protease were used for eggshell membrane hydrolysis. The characterization of the hydrolysates consisted of the determination of the degree of hydrolysis, oligopeptides, and free amino acids obtained through eggshell membrane hydrolysis. The degree of hydrolysis was 14.23%. Analytical techniques such as reversed-phase liquid chromatography coupled with mass spectrometry, hydrophilic interaction liquid chromatography, and FT-IR spectroscopy revealed the presence of oligopeptides, dipeptides, and amino acids, including alanine, lysine/glutamine, glutamic acid, histidine, proline, valine, hydroxyproline, and phenylalanine. Electrophoretic analysis demonstrated protein fractions within the molecular weight range of approximately 14 kDa to 70 kDa in all samples.

Keywords: by-product; eggshell membranes valorization; enzymatic hydrolysis; food ingredients; cosmetic applications

Citation: Pasarin, D.; Enascuta, C.E.; Enache-Preoteasa, C.; Matei, C.B.; Ghizdareanu, A.-I. Combined Alkaline and Enzymatic Hydrolysis of Eggshell Membranes for Obtaining Ingredients for Food and Cosmetic Applications. *Chem. Proc.* **2023**, *13*, 20. https://doi.org/10.3390/chemproc2023013020

Academic Editors: Mihaela Doni, Florin Oancea and Radu Claudiu Fierăscu

Published: 23 November 2023

1. Introduction

Recent scientific research has focused on using natural resources and by-products from the food industry to obtain active ingredients with various applications. These studies aim to develop efficient and sustainable methods, focusing on reducing the use of organic solvents and promoting an environmentally friendly approach. This opens the way for the production of food and cosmetic products from ingredients that support sustainability, resulting in products with improved properties [1]. One of these approaches involves the valorization of eggshell membranes to obtain bioactive peptides with high potential, especially in the food and cosmetics industries. The peptides obtained may have several benefits, such as antioxidant, anti-inflammatory, and moisturizing effects that help improve skin health. These benefits are achieved by combining alkaline hydrolysis with the enzymatic hydrolysis of the eggshell membranes [2,3]. Combining enzymatic hydrolysis with alkaline hydrolysis for the valorization of eggshell membranes offers numerous advantages over using these methods separately. This integrated approach proves to be more efficient and advantageous for several reasons: enzymatic hydrolysis and alkaline hydrolysis complement each other [4]. Enzymatic hydrolysis is more selective in producing peptides with specific bioactive activities, while alkaline hydrolysis can break chemical bonds in the eggshell membrane and release the bound peptides [5,6]. This

combination ensures a more complete extraction of peptides from the eggshell membranes. Combining these two methods enables the production of higher-quality end products [7]. Enzymatic hydrolysis better preserves the structure and activity of the peptides, while alkaline hydrolysis contributes to their complete release. As a result, the obtained products will have better biological properties and be more valuable in subsequent applications. By combining both hydrolysis methods, a broader range of peptides with different biological activities can be obtained [8]. This diversity is important in meeting the diverse needs of the food and cosmetic industries, which can use these peptides in various products such as anti-aging creams, dietary supplements, or functional foods. The valorization of eggshell membranes using both methods enables the more efficient use of raw materials and reduces the amount of waste generated in the production process [9].

2. Materials and methods

2.1. Reagents

Eggshell membranes used in all experiments were obtained from commercially purchased eggs. The enzyme Alcalase 2.4 L was food-grade and was obtained from Novozymes (Bagsvaerd, Denmark). The reagents used in this research were of analytical quality and were commercially available. Reagents used in electrophoretic separation were supplied by Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA.

2.2. Alkaline Hydrolysis Combined with Enzymatic Hydrolysis

Alkaline hydrolysis and enzymatic hydrolysis to obtain bioactive peptides from eggshell membranes were performed according to the method described by Marcet I. [10] and Jain S. et al. [11] with minor modifications.

An amount of 20 g of eggshells with membranes was weighed, dried, ground, and then suspended in a 200 mL solution of 1.25 N NaOH. The mixture was incubated for 24 h at 37 °C using model No. 639/70 Nahita Refrigerated Incubator (Auxilab, Beriáin, Spain). The samples were centrifuged, (Hettich Zentrifugen, universal 320, Tuttlingen, Germany) for 20 min at 5000 rpm. The supernatant was ultrasound-treated using a Sonoplus CV 334 system (Bandelin, Berlin, Germany) at a 24 kHz frequency, 80% amplitude for 30 min. The pH was adjusted to 8.5. For the removal of NaCl formed in the soluble protein solution from eggshell membranes, the supernatant was distributed into dialysis bags and dialyzed against distilled water for 24 h, changing the water several times until the conductivity of the samples reached the range of 1–2.5 mS/cm. The pH was monitored to ensure that it remained between 7 and 8.5, corresponding to the enzyme Alcalase 2.4 L activity. The samples were pre-incubated for 15 min at 55 °C, followed by the initiation of the hydrolysis reaction via the addition of 1.5:100 g/g enzyme concentration for 9 h. The samples were heated for 10 min at 95 °C to inactivate the enzymes and they were then concentrated. Protein precipitation was performed by lowering the pH from 8.5 to the isoelectric point. The protein precipitate was preserved via freeze-drying.

2.3. The Degree of Hydrolysis

The degree of hydrolysis was determined according to the method described by Kaewka et al. [12].

2.4. Reverse-Phase Liquid Chromatography with Mass Spectrometry Analysis of the Hydrolyzed Eggshell Membranes

Reverse-phase liquid chromatography with mass spectrometry was used to determine the oligopeptides resulting from the hydrolysis of eggshell membranes. A Hypersil Gold column, 250 × 4.6 mm, with 5 µm particles and a C18 precolumn was used. The mobile phase gradient consisted of 0.1% trifluoroacetic acid (TFA) in water (A) and 0.08% TFA in acetonitrile (B), starting at 0.8 mL/min with 60% A and increasing to 35/65 A/B at 1 mL/min over 20 min. Multiple detection methods were used, including mass spectrometry, ultraviolet, and fluorescence [13–15].

2.5. Free Amino Acids Analysis

Hydrophilic interaction liquid chromatography (HILIC), a type of reversed-phase chromatography with a column for normal phase chromatography, was used to analyze the presence of free amino acids. A Luna HILIC column (Phenomenex, DH Life Sciences, Washington, USA) with a size of 150 × 2 mm and 3 µm particles and a pre-column of the same material were used. The mobile phase consisted of A) 200 mM ammonium formate in water/acetonitrile 90/10, and B) 200 mM ammonium formate in water/acetonitrile 10/90, with a gradient program starting at 100% A) for 7 min, followed by variable flow rates from 0.3 mL/min to 0.5 mL/min over 7 min, returning to 100% B) at minute 10 with a flow rate of 0.5 mL/min and an isocratic phase for an additional 2 min, for a total of 12 min [16].

2.6. Determination of the MM Distribution of Protein Hydrolysate from Eggshell Membranes

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was used for the determination of the distribution of MM according to the Laemmli method with slight modifications [17] (Laemmli et al., 1970). The analysis was performed on pre-made gradient gels (Novex™ Value™ 4 to 20%, Tris-Glycine, Invitrogen by Thermo Fisher Scientific, Waltham, USA) using BoltTM MES SDS Running Buffer (20×) (Invitrogen by Thermo Fisher Scientific, USA), under reducing conditions. The samples were diluted with Sample Buffer, Laemmli (2×) (Sigma-Aldrich, St. Louis, MO, USA) at a ratio of 1:1 (v/v) and heated to 95 °C for 10 min to break the non-covalent bonds. After centrifugation for 5 min at 14,000 rpm, 10 µL of each sample solution was added to the wells. In addition, Marker Protein VI, 11 to 250 kDa (AppliChem GmbH, Ottoweg, Germany), covering a molecular weight range of approximately 11 to 245 kDa, was added to the gel to estimate the molecular weight of each protein hydrolysate. Electrophoresis was performed at a constant current of 100 V for 60 min at room temperature in a vertical electrophoresis system (Mini Gel Tank, Invitrogen, USA). To visualize the protein subunits (oligopeptides and peptides), the gel was stained with 0.1% w/v Coomassie Brilliant Blue R-250. The gel images were analyzed. Three separate tests were performed.

3. Results

Changing the pH of the environment affects the solubility of proteins by changing their electrical charge. At the isoelectric point (pI), the solubility of proteins is minimal because the electric charge is neutral, i.e., the number of positive charges equals the number of negative charges, and protein molecules no longer interact with polar water molecules. Concentrated electrolyte solutions further reduce solubility, which often leads to precipitation. Organic solvents, such as acetone, alcohol and ether, when added to a protein solution, prevent the protein molecules from interacting with the water molecules, resulting in the precipitation of the protein. Ultrasound-assisted protein extraction is considered a 'green' technology because it reduces or eliminates the need for organic solvents, many of which are considered toxic. 'Green' extraction methods generally improve extraction yield, reduce the number of process steps, and save energy [11]. Marcet I. et. al. [10], using a Box–Behnken model to determine the effect of variables on the amount of solubilized protein. They found that ultrasonication facilitates the detachment of larger protein fragments from eggshell membranes and their solubilization, especially when the amount of NaOH, reaction time, and temperature are reduced, but that it does not affect the amount of solubilized protein.

3.1. The Degree of Hydrolysis

After the combined alkaline and enzymatic hydrolysis of eggshell membranes, a protein hydrolysate with a degree of hydrolysis of 14.23% was obtained. The alkaline hydrolysis step breaks down the covalent bonds between the amino acids in the eggshell membranes. This makes the membranes more susceptible to enzymatic hydrolysis, which can then break down the amino acids into smaller molecules. Alcalase 2.4 L is a very efficient enzyme and can cleave most of the peptide bonds within a protein molecule. It

is a serine protease that has a serine amino acid group at its active site. This serine group is essential for substrate binding and cleavage. The catalytic center of Alcalase 2.4 L also contains aspartate and histidine residues. Existing studies present the alkaline or enzymatic hydrolysis of eggshell membranes without combining the two types of hydrolysis and specifying the degree of hydrolysis obtained.

3.2. Reverse-Phase Liquid Chromatography with Mass Spectrometry Analysis of the Hydrolyzed Eggshell Membranes

The chromatographic characterization of peptides is presented in Figures 1 and 2.

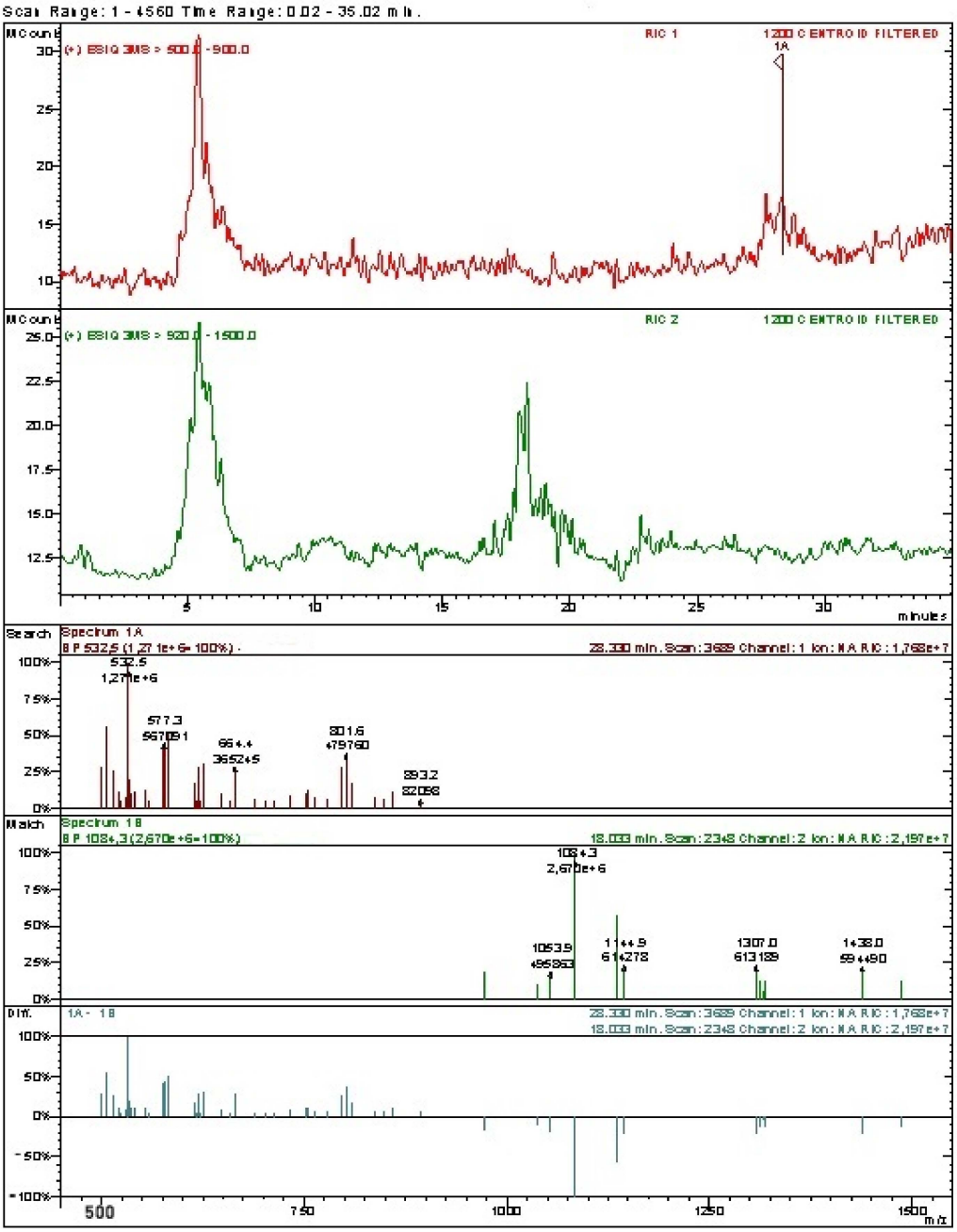

Figure 1. Chromatographic characterization of peptides.

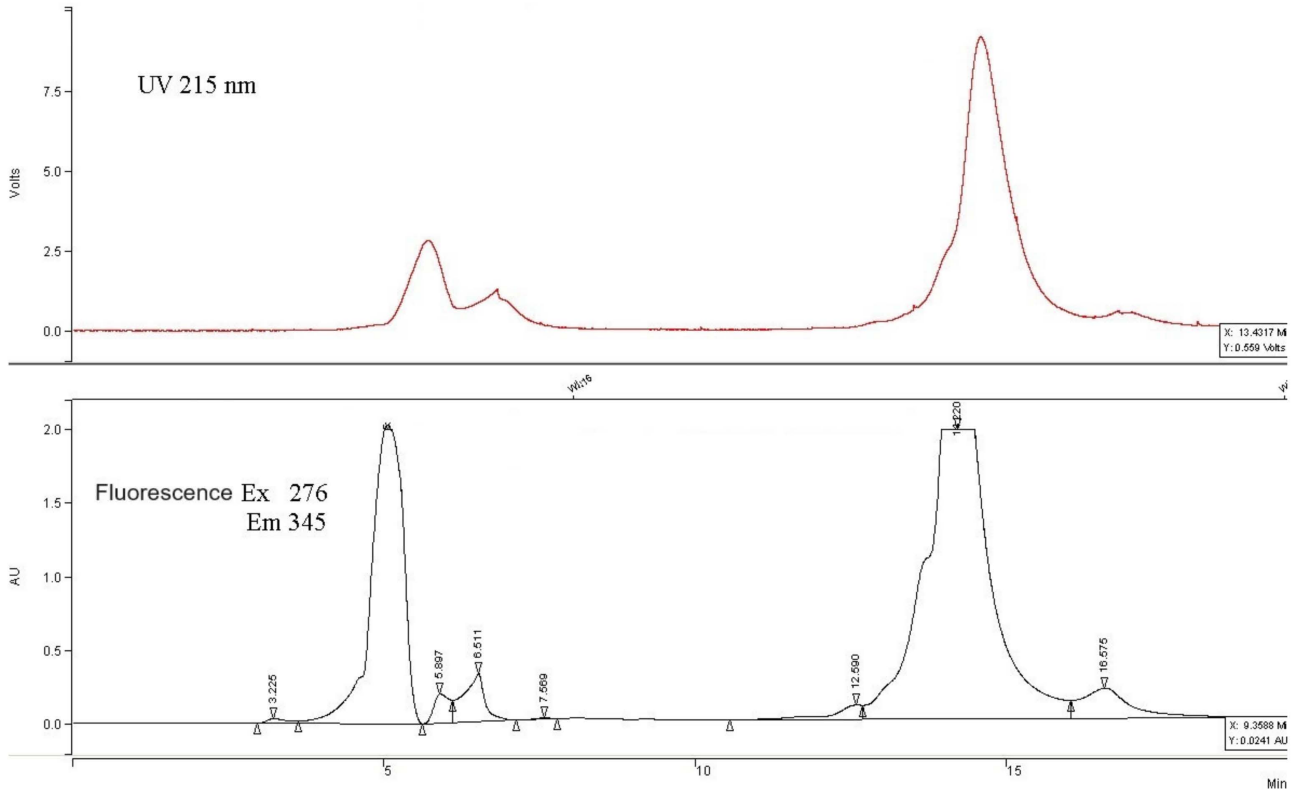

Figure 2. Comparative chromatogram using fluorescence and UV detection.

These analyses have provided evidence for the protein-based composition of the eggshell membrane, with certain similarities observed in terms of amino acids originating from collagen (hydroxyproline, alanine), as well as heterocyclic amino acids displaying native fluorescence. The results were confirmed via FT-IR spectroscopy (both in the sample before processing and during processing.

3.3. Free Amino Acids Analysis

A sample taken after the alkaline hydrolysis of eggshell membranes, a sample taken after the combined alkaline and enzymatic hydrolysis of eggshell membranes, and a synthetic mixture of amino acids for confirmation were analyzed for free amino acids. The chromatogram of the eggshell membrane hydrolysate samples shows several interferences due to their complexity.

The fragmentations for each amino acid were as follows in Multiple Reaction Monitoring (MRM) and are presented in Figures 3–7.

Hydrophobic amino acids (proline, valine, hydroxyproline, leucine and isoleucine, phenylalanine, tryptophan) known for their antioxidant activity have been shown after alkaline hydrolysis. For combined alkaline and enzymatic hydrolysates, the results were different. The amino acids detected were alanine, lysine/glutamine, glutamic acid, histidine, proline, valine, hydroxyproline, and phenylalanine.

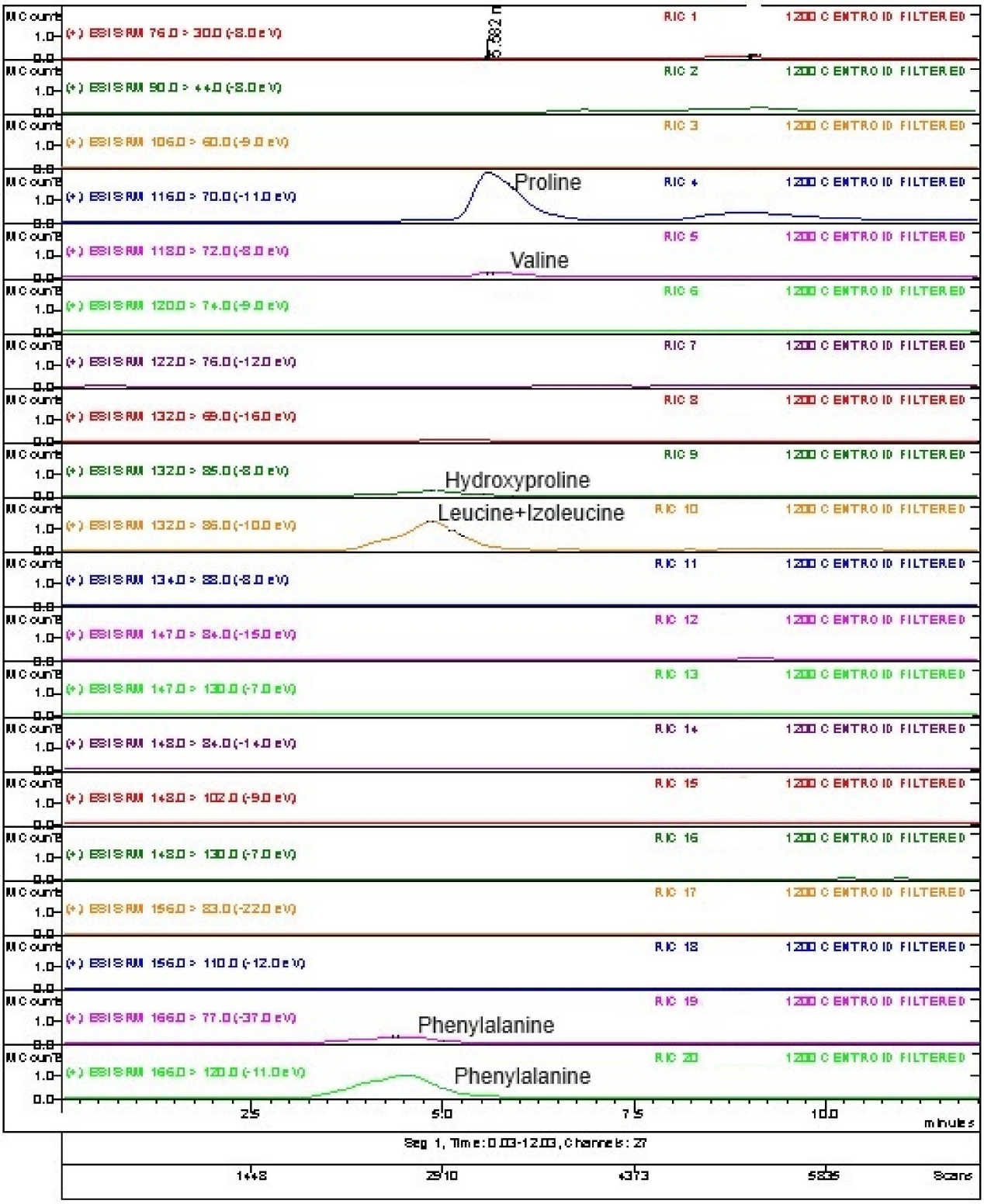

Figure 3. Chromatographic detection of free amino acids after alkaline hydrolysis RIC (reconstructed ion chromatogram) 1–20.

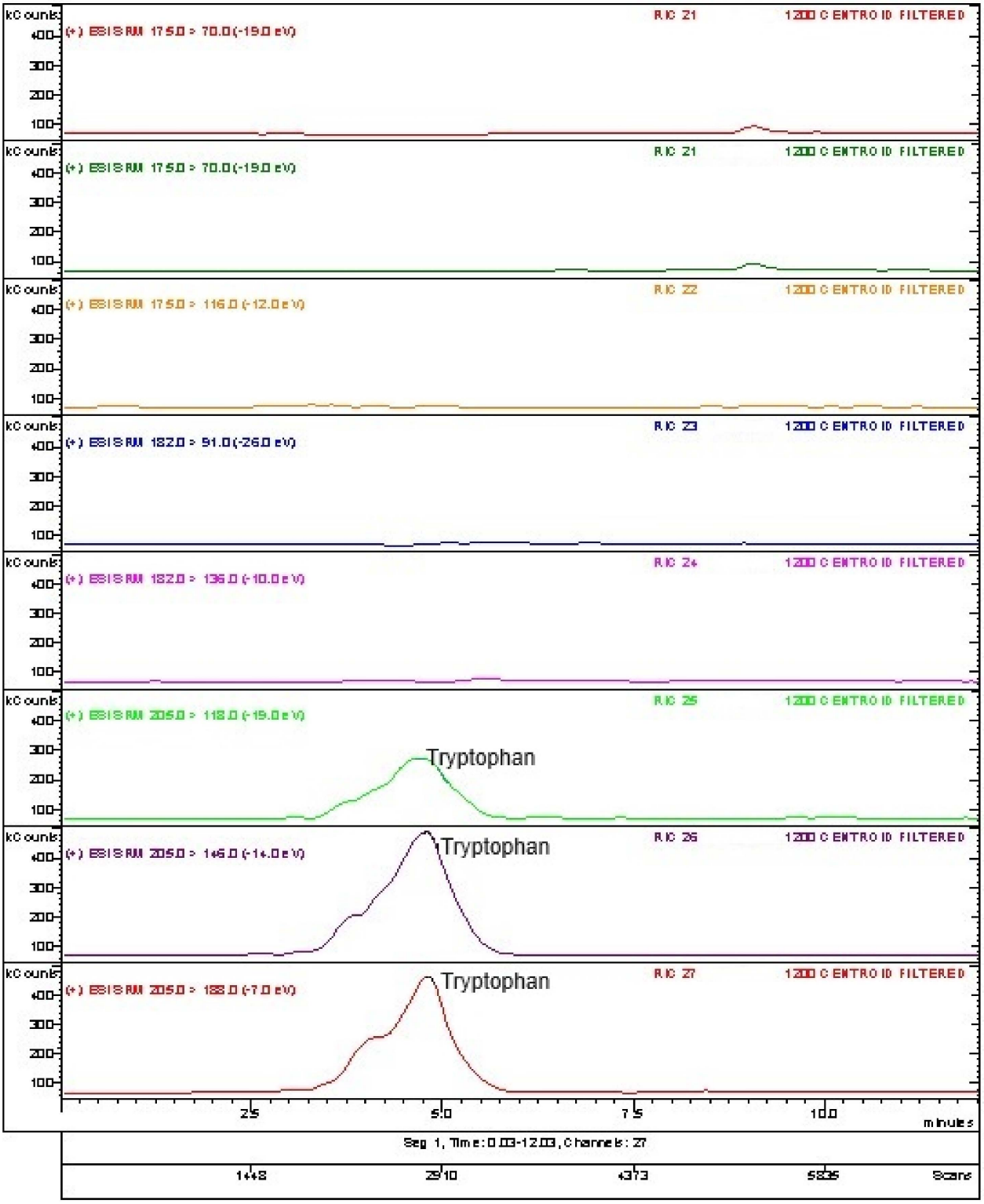

Figure 4. Chromatographic detection of free amino acids after alkaline hydrolysis RIC 21–27.

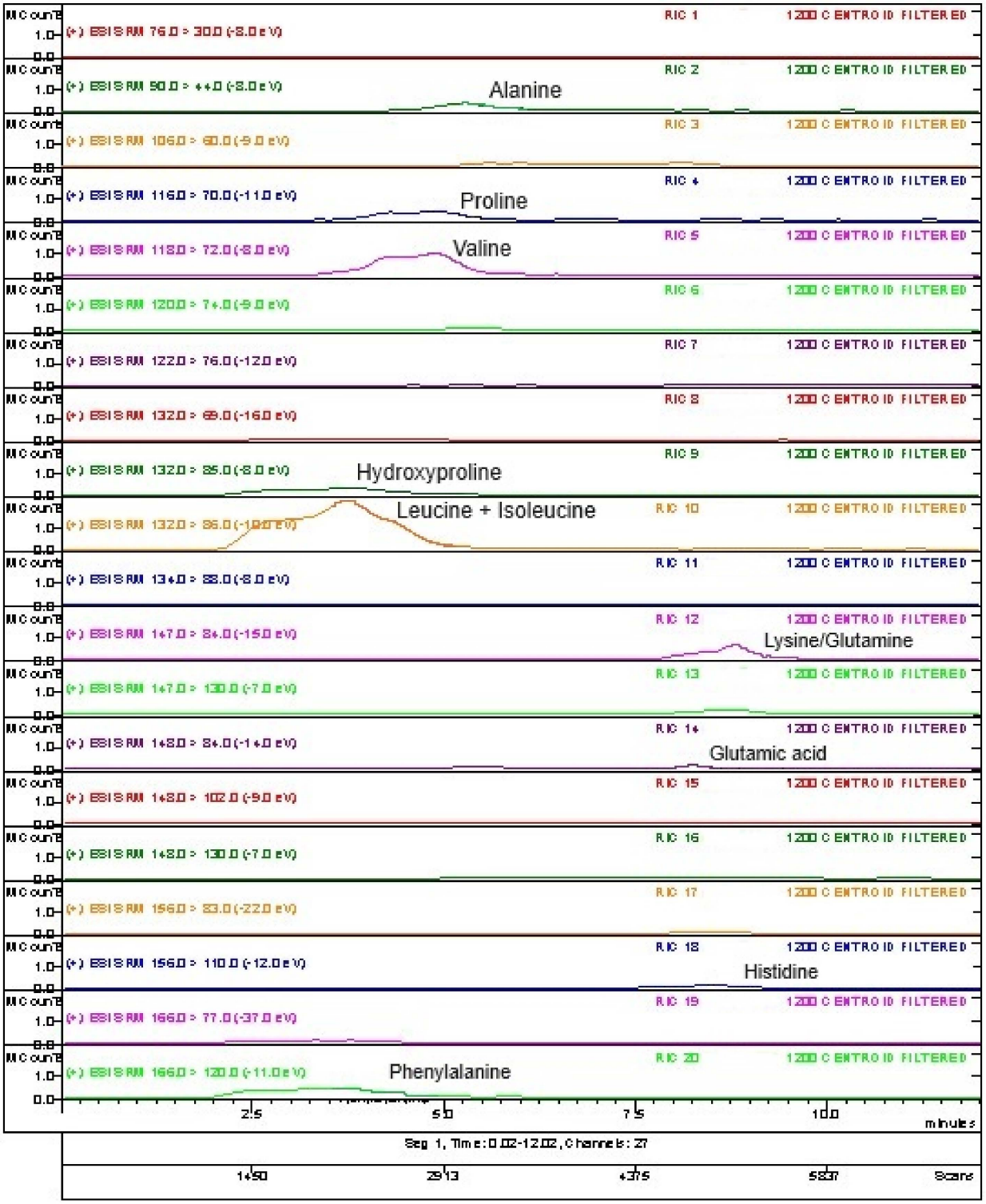

Figure 5. Chromatographic detection of free amino acids in alkaline and enzymatic hydrolysates RIC 1–20.

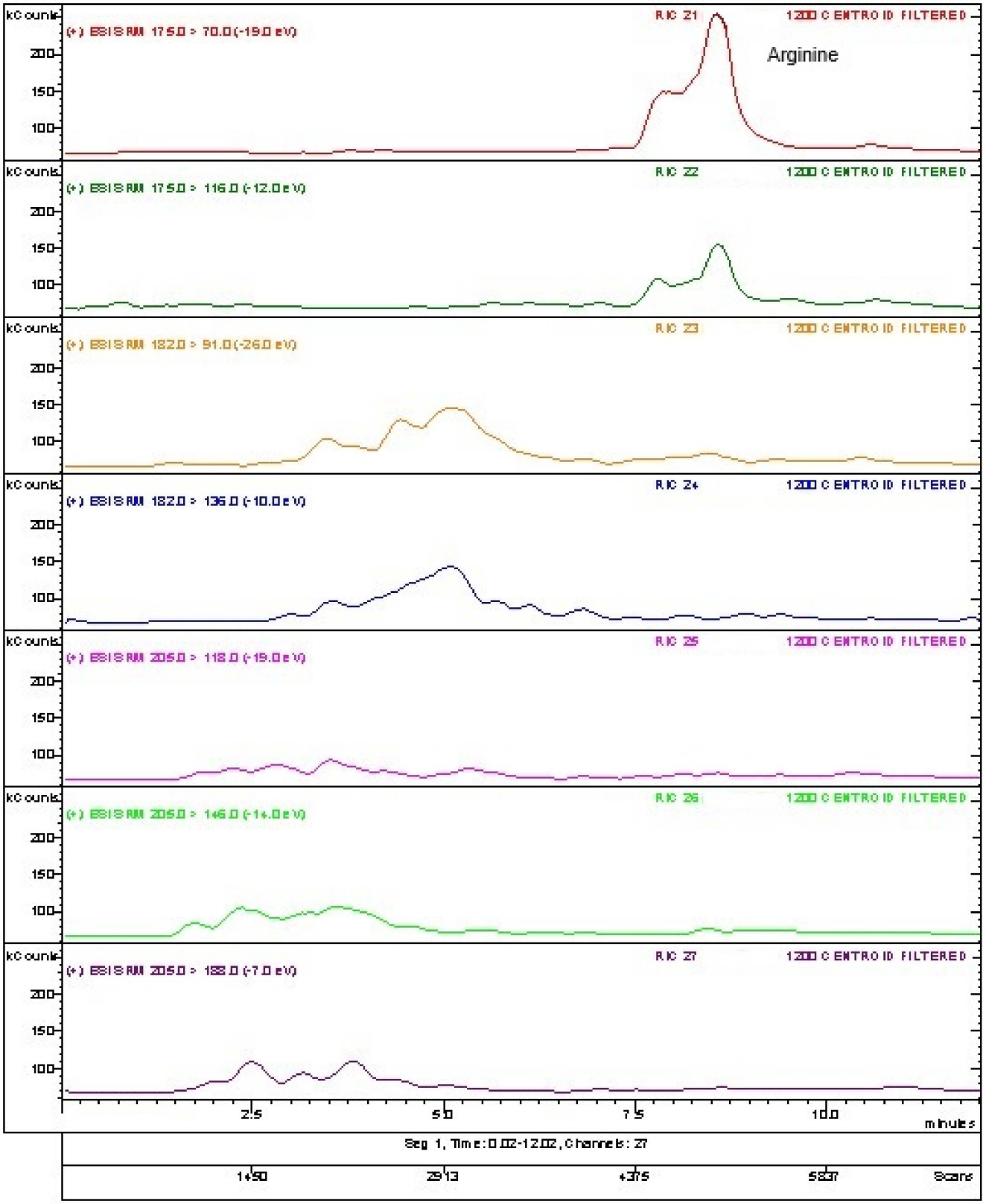

Figure 6. Chromatographic detection of free amino acids in alkaline and enzymatic hydrolysates RIC 21–27.

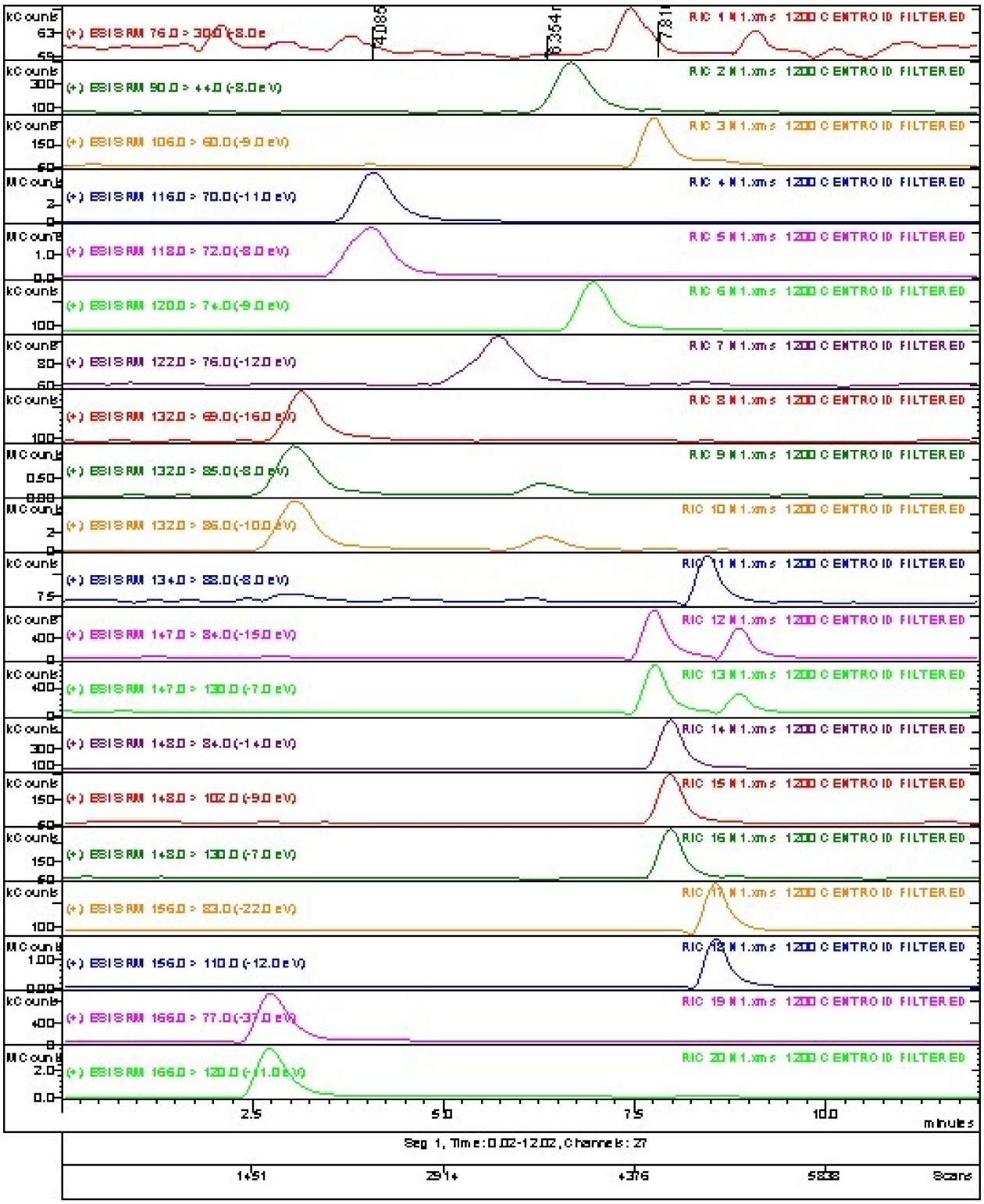

Figure 7. Chromatogram of the synthetic mixture of amino acids.

3.4. The Electrophoretic Characterization of Protein Hydrolysate from Eggshell Membranes

The electrophoretic profile of protein hydrolysate samples is presented in Figure 8.

Following the SDS-PAGE test for the samples, protein fractions ranging from approximately 14 kDa to 70 kDa were identified.

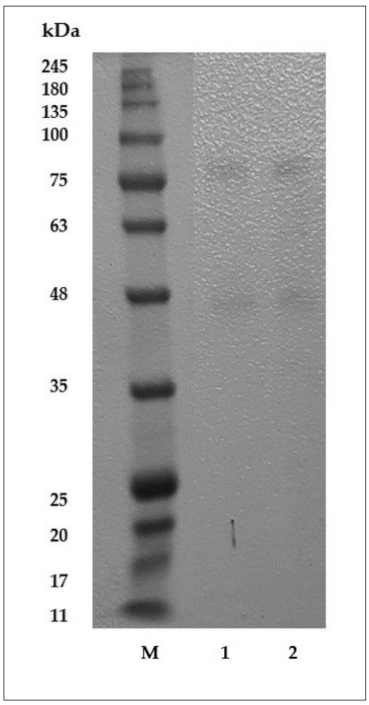

Figure 8. SDS-PAGE profiles of eggshell membrane protein hydrolysates obtained via proteases Alcalase 2.4 L. Precast gel Tris-Glycine 4–20%; 10 μL sample/well; dilution 1:1; Marker Protein VI; Lane M -molecular mass marker; lane 1- alkaline and enzymatic hydrolysate obtained with 1.25 N NaOH and Alcalase 2.4 L, 55 °C, 1.5:100 g/g concentration, 9 h; lane 2- alkaline hydrolysate obtained with 1.25 N NaOH.

4. Discussion and Conclusions

This research has confirmed the presence of collagen-derived amino acids, such as hydroxyproline and alanine, along with heterocyclic amino acids exhibiting natural fluorescence, in eggshell membrane hydrolysates with a degree of hydrolysis of 14.23%. Physico-chemical analyses of the hydrolyzed eggshell membranes revealed the existence of oligopeptides, dipeptides, and free amino acids. Amino acids such as alanine, lysine/glutamine, glutamic acid, histidine, proline, valine, hydroxyproline, and phenylalanine were identified. Amino acid profiling provided valuable insights into hydrolysis methods for optimized peptide production. FT-IR Spectroscopy was used to analyze these hydrolysates both before and during the processing steps. Electrophoretic analysis revealed protein fragments of different sizes with molecular weights ranging from approximately 14 kDa to 70 kDa in all samples analyzed. The change in the membrane structure of the eggshell improves the accessibility of the peptides in the body, which could lead to more efficient absorption compared to proteins. Subsequent research will focus on determining the biological activity of these compounds, which could have significant health-promoting benefits. In conclusion, eggshell membranes are readily available by-products from the egg industry, making them a sustainable and environmentally friendly source of bioactive compounds. The use of eggshell-membrane-derived bioactive peptides represents a promising avenue for the development of functional foods and cosmetics with potential health-promoting properties.

Author Contributions: Conceptualization, D.P. and A.-I.G.; methodology, D.P., A.-I.G., C.E.E. and C.E.-P.; formal analysis, D.P., A.-I.G., C.E.E., C.E.-P. and C.B.M.; investigation, D.P., A.-I.G., C.E.E. and C.E.-P.; writing—original draft preparation, D.P., A.-I.G., C.E.E. and C.E.-P.; writing—review and editing, D.P. and A.-I.G.; supervision, D.P. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by a grant of the Romania National Authority for Scientific Research and Innovation, CCCDI-UEFISCDI, project number PN-III-P3-3.5-EUK-2016-0041/2018. This work was carried out through the PN 23.06 Core Program—ChemNewDeal within the National Plan for Research, Development, and Innovation 2022–2027, developed with the support of the Ministry of Research, Innovation, and Digitization, project no. PN 23.06.01.01.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

Acknowledgments: This work was supported by a grant of the Romania National Authority for Scientific Research and Innovation, CCCDI-UEFISCDI, project number PN-III-P3-3.5-EUK-2016-0041/2018. This work was carried out through the PN 23.06 Core Program—ChemNewDeal within the National Plan for Research, Development, and Innovation 2022–2027, developed with the support of the Ministry of Research, Innovation, and Digitization, project no. PN 23.06.01.01.

Conflicts of Interest: The authors declare that they have no conflict of interest.

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