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Simultaneous quantification of free amino acids and peptide fractions from keratin hydrolysates by SE-HPLC

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Title page

- **Title.** Simultaneous quantification of free amino acids and peptide fractions from keratin hydrolysates by SE-HPLC.

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Abstract

An original SE-HPLC/UV methodology was developed to quantify the main fractions of keratin protein hydrolysates – peptides and free amino acids (FAA) – from absorbance signals. The method processes SEC chromatograms assuming a boundary retention time between the two fractions before signals conversion to concentrations using key parameters: mean molecular weight (MW) by amino acid ($\overline{MW}_{aa}$) and mean molar extinction coefficient by amino acid ($\bar{\varepsilon}_{aa}$). 28 keratin protein hydrolysates produced by the acid hydrolysis of poultry feathers under various conditions of temperature and HCl/feather ratio, and sampled at multiple times were analyzed by SE-HPLC to identify the boundary retention times from the chromatograms. The hydrolysates were further assayed by a classic amino acid analyzer to obtain their free and total amino acids aminograms, capturing the concentrations of the Peptide and FAA fractions. Based on this, key parameters $\overline{MW}_{aa}$ and $\bar{\varepsilon}_{aa}$ were deduced from hydrolysate aminograms, and by nonlinear regression considering the mathematical expression of $\bar{\varepsilon}_{aa}$ as a function of concentration, respectively. Across 14 hydrolysates used for parameters estimation, key parameters calculated for each fraction remained largely constant with RSDs below 10%, leading to averaged values being used for the final implementation of the SE-HPLC method in MATLAB. Method validation against the reference method showed high accuracy (errors inferior to 15%) for 81 out of 84 quantifications of Peptide, FAA and soluble total amino acids. The validated method was ultimately applied for monitoring the peptide MW distribution and concentration kinetics of peptide classes of custom MW range, as additional capabilities.

Keywords

- Acid hydrolysis
- Free amino acids
- Keratin
- Peptides
- Protein hydrolysates
- Size-exclusion HPLC

Abbreviations

$\bar{\varepsilon}_{aa}$, mean molar extinction coefficient by amino acid; AA, amino acid; ACN, acetonitrile; AQC, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate; EDQM, European Directorate for the Quality of Medicines & HealthCare; FAA, free amino acids; FDM, feather dry matter; FMOC, 9-fluorenyl-methyl chloroformate; MW, molecular weight; $\overline{MW}_{aa}$, mean molecular weight (MW) by amino acid; OPA, O-phthalaldehyde; PITC, phenylisothiocyanate; RSD, relative standard deviation; SEC, Size-Exclusion Chromatography; SE-HPLC, Size-Exclusion High-Performance Liquid Chromatography; t_{delim}, delimitation time between Peptide and FAA fractions; TFA, trifluoroacetic acid; Tr, retention time.

1. Introduction

Keratin is an insoluble structural protein, ranked as the second most abundant naturally occurring biopolymer in animals, after collagen (Perinelli et al. 2025). Poultry feathers are keratin-rich materials (about 90 wt%) considered as recalcitrant biowastes in the poultry processing industry because of their low biodegradation rate (Lange et al. 2016). Boasting an annual production of 12 million tons in 2018 (Schieder et al. 2024), recalcitrant poultry feathers biomasses pose significant environmental and health concerns when disposed through traditional waste management streams (i.e. landfilling, incineration and composting)(Moktip et al. 2025) (Chen et al. 2022). Alternativey, keratin from poultry feathers may be extensively hydrolysed to produce amino acids or high value added amino acids derivatives interesting industrial fields as human nutrition, animal food and feed, nutraceuticals (Perinelli et al. 2025) (Moktip et al. 2025) (Banasaz and Ferraro 2024) (Hou et al. 2017) (Johns et al. 2011) (Bhati and Hayes 2025) (Apostolopoulos et al. 2021) (Jana and Paul 2024), pharmaceuticals (Moktip et al. 2025) (Banasaz and Ferraro 2024) (Apostolopoulos et al. 2021) (Jana and Paul 2024), cosmeceuticals (Moktip et al. 2025) (Banasaz and Ferraro 2024), and agricultural fertilizers and biostimulants (Moktip et al. 2025) (Banasaz and Ferraro 2024) (Bhati and Hayes 2025) (Malécange et al. 2022).

The high cystine content of keratin (7 wt%) and its cristaline structure make this protein particularly stable (Schieder et al. 2024) and resistant to enzymatic hydrolysis (Moktip et al. 2025). Hence, harsh conditions (temperature over 100 °C and acid concentration around 6 M) are required to hydrolyse peptide bonds. In these conditions, lateral chains of tryptophane, glutamine and asparagine are destroyed and even under these conditions some peptide bonds remain intact. Hence, keratin hydrolysates are complex protein hydrolysates mixtures with a predominant free amino acid fraction and a peptide fraction (Alvarez et al. 2012).

Depending on the extent of the hydrolysis reaction, more or less peptide bonds are broken, and this is reflected in the free amino acid and peptide compositions of the final hydrolysate mixture (Johns et al. 2011) (Beaubier et al. 2019) as well as the overall process performance usually indexed on yield. Besides, the molecular weight (MW) of peptides produced during the hydrolysis decreases dynamically until residual stable peptides of low MW are left in the mixture. Therefore, the online monitoring of both the composition and the peptide MW distribution of hydrolysates during conversion processes can help in the informed selection of the optimal processing conditions maximizing the yield of the fraction of interest. However, because of the complexity of the composition of such mixtures derived from the hydrolysis of biological compounds, most classical analytical procedures of protein hydrolysates quantification are laborious and impractical (Lamp et al. 2018) (Wei et al. 2024).

Standard analytical methods used for quantifying free amino acids rely on high performance liquid chromatography (HPLC) involving pre- or post-column derivatization with reagents like ninhydrin, PITC, OPA/FMOC or AQC to form UV-detectable or fluorescent amino acid derivatives (Wei et al. 2024) (E. Otter 2012). Referred to as automatic amino acid analyzers, these techniques allow single-run analysis of amino acids, with typical run times of 15-35 minutes for reverse-phase C18 columns and up to 90 minutes for Ion Exchange Chromatography columns (Wei et al. 2024). A calibration curve adapted to the specific product matrix needs to be established beforehand for every amino acid and regularly updated, which can increase analysis costs and burden. The peptide quantification is more complex. It is classically obtained by difference between total amino acids and free amino acids fraction of the sample (Hou et al. 2017) (Li and Wu 2020) (Lamp et al. 2018) (E. Otter 2012). Total amino acids quantification is obtained by the same analysis after a total hydrolysis pretreatment. As per ISO 13903:2005, the pre-treatment is a long and laborious process, comprising 16 h oxidation followed by

a standardized 24 h acid hydrolysis or digestion using 6 M HCl (International Organization for Standardization (ISO) 2005) (Commission Européenne 2009).

Size exclusion HPLC (SE-HPLC) separates protein hydrolysates constituants according to their apparent size (Johns et al. 2011). The most popular utilization of SE-HPLC/UV is to determine the MW distribution of peptides (Johns et al. 2011) (Eremeev et al. 2009). SE-HPLC can also be used for direct quantification of protein hydrolysates or peptide fractions in hydrolysates. This method exploits peptide bond and amino acids lateral chain UV absorption properties at 214 nm (Johns et al. 2011) (Kuipers and Gruppen 2007) (Bodin et al. 2015). The main challenge was to find a proper extinction coefficient to convert the UV signal into concentration. Kuipers and Gruppen (Kuipers and Gruppen 2007) showed that peptide molar absorption coefficients at 214 nm can be deduced from their amino acid composition as the contribution of peptides bonds and residual amino acids weighted by their respective number of occurrences in the polypeptide chain. Based on this, Bodin et al. (Bodin et al. 2015) have reported a simple and accurate SE-HPLC/UV method for the determination of the total peptide concentration in protein hydrolysates. In short, the protein hydrolysate mixture is assigned a mean molar extinction coefficient by amino acid ($\bar{\varepsilon}_{aa}$) derived from its aminogram (Kuipers and Gruppen 2007). Their method had a linear working range of 0.2 to 4 g/L meaning that raw hydrolysate samples must be diluted to enable quantification.

Such an approach might be used as a standalone analytical procedure for monitoring the free amino acids and peptide fraction composition of keratin hydrolysates. However two strong challenges are limiting to do so. First, the non-ideal size exclusion behavior of SE-HPLC columns offering a poor resolution in the separation of smaller peptides and free AAs (Johns et al. 2011). Second, the change in absorbance coefficient of peptide and FAA fractions due to sequence-specific absorptivity of peptides at 214 nm, and the unsteady composition in free AAs during hydrolysis, respectively. Interestingly, McDonald et al. (McDonald et al. 2024) demonstrated the predictability of the molar extinction coefficient of a wide range of substances using an advanced neural network model. The model was trained to estimate molar extinction coefficients directly from the compound molecular representation given as SMILES strings.

In this study, we propose an original methodology for using SE-HPLC chromatogram of keratin hydrolysates in order to quantify its free amino acid and peptide fraction simultaneously. This methodology is based on the conversion of absorbance into concentrations as in Bodin et al. (Bodin et al. 2015). In the proposed method, the key parameters of the conversion ($\bar{\varepsilon}_{aa}$ and $\overline{MW}_{aa}$) are obtained from experimental aminogram data collected during keratin hydrolysis under various set of conditions (hydrolysis temperatures of 95 and 106 °C, HCl/feather ratio of 4.19, and hydrolysis durations of 0.5 to 6 hours). The key parameter $\overline{MW}_{aa}$ was estimated as the molar weight of individual AAs weighted by their mass fractions in the aminograms, whereas key parameter $\bar{\varepsilon}_{aa}$ was determined by nonlinear regression minimizing the error on the concentrations of the two main fractions reported by the aminograms. The MATLAB optimization solver fminbnd and the mathematical expression of the concentrations as functions of $\bar{\varepsilon}_{aa}$ enabled the regression of this parameter. The method was then validated under new experimental conditions, identical to the previous set except for an HCl/feather ratio of 5.12. The method was also employed to precisely monitor the kinetics of peptide fractions defined by their molar weight and establish the peptide molar weight distribution of the hydrolysates.

2. Methodology

Figure 1 presents the global methodology for quantifying free amino acids and peptides. For both fractions, the same approach is applied, inspired from Bodin et al. (Bodin et al. 2015). The absorbance signal is converted into concentration using Eq. (1):

$$C_{m_x} = \frac{MW_x}{V_{inj}} \int \frac{A_x}{\varepsilon_x . l} . dV \qquad (1)$$

where A_x, ε_x and MW_x are the absorbance, the molar extinction coefficient and the molecular weight of peptide mixture x respectively, dV is a fraction of the total elution volume, V_{inj} is the injection volume, and l is the optical path length of the cell holding the sample.

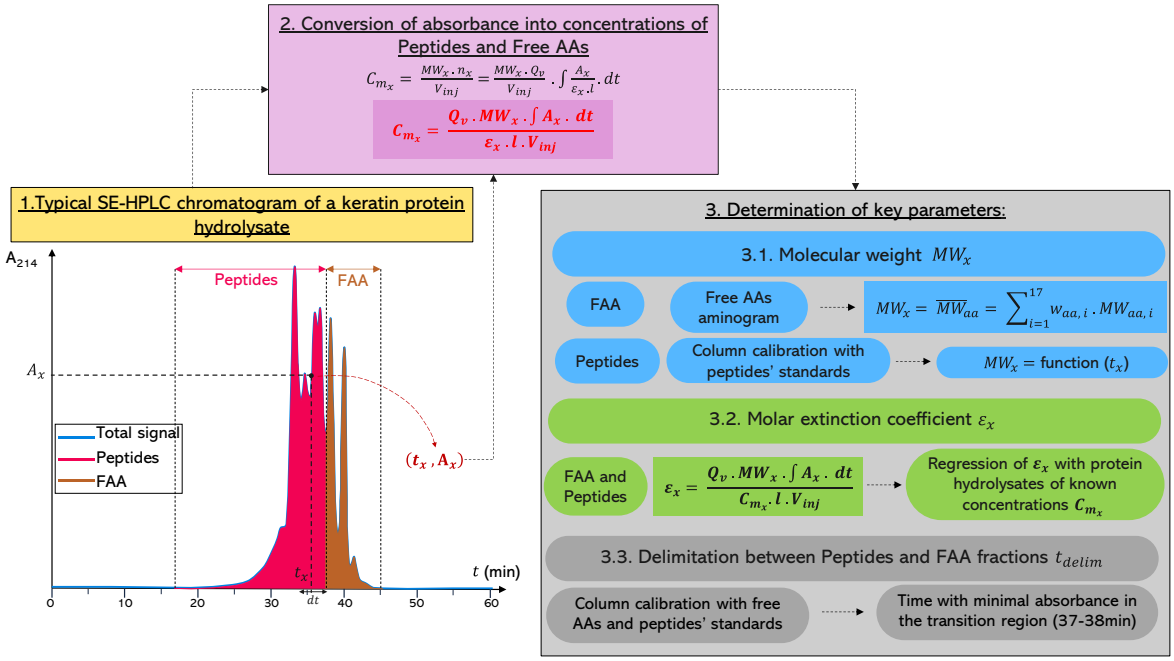


Fig. 1 *Diagram of the global methodology for the simultaneous quantification of Peptides and FAA fractions' total concentrations in keratin protein hydrolysates mixtures, based on SE-HPLC chromatograms*

Then, a numerical integration of the signal yields the total peptide or free amino acids concentration. Isocratic elution applied during the chromatographic analysis implies that the elution flowrate (Q_v) is constant. Hence, following step 2 of Figure 1, Eq. (1) may be rewritten as:

$$C_{m_x} = \frac{Q_v . MW_x . \int_{t_{lower}}^{t_{upper}} A_x . dt}{\varepsilon_x . l . V_{inj}} \qquad (2)$$

where t_{lower} and t_{upper} are the lower and upper retention times boundaries delimiting the elution range of the peptide mixture x respectively.

Apart from peak area $\int A_x$ and local key parameters ε_x and MW_x, all the terms in Eq. (2) are constants known to the operator of the analysis. Once the delimitation time between the two fractions has been elucidated, their respective peak areas $\int A_x$ can readily be obtained by numerical integration. The local key parameters ε_x and MW_x can be linked to global key parameters $\bar{\varepsilon}_{aa}$ and $\overline{MW}_{aa}$ of each major fraction. Herein, we assume these global key parameters to be constant within the elution range of each fraction. Step 3 of the methodology presented in Figure 1 outlines the procedure to determine the unknown terms of Eq. (2). The procedure is further detailed in sections 2.1 and 2.2 of the current article.

2.1. Determination of Peptides and FAA boundary retention times

Since there are only two fractions contributing to the total signal, $\int A_x$ can easily be determined by a numerical integration from time t_{lower} and to time t_{upper}. For both FAA and peptide fractions, the elution range boundaries were defined according to a procedure summarized in Table 1.

Table 1 *The upper and lower boundaries delimiting the elution range of the Peptides and FAA fractions.*

Fractions	t_{lower}		t_{upper}	
	Method of determination	**Value (min)**	**Method of determination**	**Value (min)**
Peptides	Time where Absorbance > arbitrary threshold value (0.3 mAu)	13-25	Time with minimal Absorbance in the transition region (37-38 min)	t_{delim}
Free Amino Acids (FAA)	Time with minimal Absorbance in the transition region (37-38 min)	t_{delim}	Latest eluting free AA, after column calibration with free AAs standards	44.5

$t_{lower,Peptides}$ corresponds to the elution time of the earliest eluting compound. This marks the end of the baseline. Therefore, it was defined as the time from which the absorbance signal exceeds an arbitrary baseline threshold fixed at 0.3 mAu. This value was based on chromatogram of blanks and analyzed samples.

t_{delim} corresponds to the elution time of the latest eluting peptide which matches the beginning of the elution of the FAA fraction($t_{lower,FAA}$). A first calibration of the column with free AAs standards helped us to locate this boundary. However, in raw hydrolysate samples, due to non ideal SEC separation, the signal of the smallest peptides often exhibits a trailing peak that extends past this initial boundary and overlaps with the start of the FAA signal. If the calibration boundary were used, some late-eluting peptides would incorrectly be quantified as FAA. To ensure a clean separation for accurate quantification of the two fractions, the boundary was set instead as the time where the minimum peptide signal is reached, that is just before any resurgence of the total signal due to free AAs detection. In actual chromatograms, this transition region occurs in the elution time range of 37 to 38 min wherein the delimitation retention time (t_{delim}) was consistently found.

Finally, $t_{upper,FAA}$ corresponds to the end of the elution of the FAA fraction or that of the latest eluting free AA. This is reliably found by column calibration with free AAs standards. In general, a value of either 44.0 or 44.5 min was used depending on the location of the last peak in the sample chromatogram.

2.2. Determination of Peptides and FAA key parameters ($\boldsymbol{MW_x}$ and $\boldsymbol{\varepsilon_x}$)

2.2.1. The molecular weight $\boldsymbol{MW_x}$

The molecular weight of any eluted peptide $MW_{Pep\,x}$ is determined from its elution time $t_{Pep\,x}$ injected in the following expression of the column calibration:

$$MW_{Pep\,x} = 10^{\,a\,\cdot\,t_{Pep\,x} + b} \qquad (3)$$

where a and b refer to the coefficients of the calibration curve.

The FAA fraction comprises 17 individual free AAs present in the mixture, each with well-defined MW ranging from 75 to 240 g/mol. The molecular weight of the FAA fraction is therefore expected to fall within this range and can be estimated based on the relative abundance of each AA in the mixture, as

determined from the free AAs aminogram. As a result, the expression of the mean MW per amino acid ($\overline{MW_{aa}}$) presented in Bodin et al. (Bodin et al. 2015) and Beaubier et al. (Beaubier et al. 2019) was retained to calculate this composition-dependent parameter:

$$MW_{x,FAA} = \overline{MW}_{aa,FAA} = \sum_{i=1}^{17} w_{aa,i} \cdot MW_{aa,i} \quad (4)$$

where $w_{aa,i}$ and $MW_{aa,i}$ refer to the mass fraction and the MW of individual AAs listed in the free AAs aminogram, respectively.

2.2.2. The molar extinction coefficient ε_x

Starting with the Peptide fraction, Bodin et al. (Bodin et al. 2015) and other researchers (Beaubier et al. 2019) (Kuipers and Gruppen 2007) have proposed the following equation to determine $\varepsilon_{Pep\,x}$, the molar extinction coefficient of any peptide:

$$\varepsilon_{Pep\,x} = \left(\varepsilon_{bond} \cdot \ (nb_{aa} - 1)\right) + \left(\bar{\varepsilon}_{aa,Pep} \cdot nb_{aa}\right) \quad (5)$$

where ε_{bond} is the molar extinction coefficient of peptide bonds (923 L.mol^{-1}.cm^{-1}), $\bar{\varepsilon}_{aa,Pep}$ is the mean molar extinction coefficient per peptidic AA, and nb_{aa} is the number of AAs in the peptide chain.

In the FAA fraction, the AAs exist in their free forms so that $nb_{aa} = 1$ and no additional contribution to the molar extinction coefficient other than that of the singular AA is counted. Eq. (5) is thus simplified into Eq. (6) applicable to any data point 'x' within the FAA elution range:

$$\varepsilon_{x,FAA} = \bar{\varepsilon}_{aa,FAA} \quad (6)$$

The mean molar extinction coefficient per peptidic AA ($\bar{\varepsilon}_{aa,Pep}$) or free AA ($\bar{\varepsilon}_{aa,FAA}$) is classically calculated using Eq. (7) considering the total AAs or free AAs aminogram, respectively.

$$\bar{\varepsilon}_{aa} = \sum_{i=1}^{17} x_{aa,i} \cdot \varepsilon_{aa,i} \quad (7)$$

where $x_{aa,i}$ and $\varepsilon_{aa,i}$ refer to the mole fraction and the molar extinction coefficient of individual AAs listed in the respective aminogram.

The number of AAs in the peptide chain (nb_{aa}) can be inferred from Eq. (3), assuming a mean molecular weight per peptidic AA ($\overline{MW_{aa}}$) while accounting for the fact that peptidic AAs are dehydrated so correction with water MW of 18 g/mol is required. As reported by Rutherfurd and Dunn (Rutherfurd and Dunn 2011), overlooking that peptidic AAs are 18 Da lighter than free AAs can lead to overestimation of the peptide content of 9 to 20 wt%. After due corrections, the equation worked out to calculate nb_{aa} is:

$$nb_{aa} = \frac{MW_{Pep\,x} - 18}{\overline{MW}_{aa,Pep} - 18} \quad (8)$$

2.2.3. Nonlinear regression of key parameter $\bar{\varepsilon}_{aa}$

Instead of Eq. (7), key parameter $\bar{\varepsilon}_{aa}$ was established by nonlinear regression based on the parameter mathematical expression Eq. (9). In this equation, $\bar{\varepsilon}_{aa}$ is directly expressed as a function of the concentration of the considered fraction (C_{m_x}). Thus, this approach requires a reference method for the one-shot accurate determination of the concentrations of the fractions of interest. An adaptation of the ISO 13903:2005 protocol was chosen as the reference method to establish sample aminograms

and deduce fractions concentrations. The regression consisted in testing and assessing the accuracy of candidate $\bar{\varepsilon}_{aa}$ values from a given interval by comparing the results of their predicted concentrations against the actual concentrations of the fraction of interest. The objective of the regression was to minimize the error on the actual concentrations, so that an accurate value of $\bar{\varepsilon}_{aa}$ of the fraction is obtained.

$$\bar{\varepsilon}_{aa} = \frac{Q_v \,.\, MW_x \,.\, \int_{t_{lower}}^{t_{upper}} A_x \,.\, dt}{C_{m_x} .\, l \,.\, V_{inj}} \tag{9}$$

The nonlinear regression of key parameter $\bar{\varepsilon}_{aa}$ was performed using the MATLAB built-in function `fminbnd` and a user-defined function as the objective function. This custom function calculates the total fraction concentration assuming an arbitrary epsilon value and returns the relative error with respect to the real Biochrom+ concentration in absolute value. `fminbnd` returns the optimal value of $\bar{\varepsilon}_{aa}$ over a provided interval that minimizes the error on the total fraction concentration.

2.3. Peptide MW distribution and peptide fraction quantification

To determine the MW distribution of peptides, a numerical integration of the elementary peptide concentrations $C_{m_{Pep\,i}}$ from the low MW peptides to the high MW peptides for every peptide mixture i and normalized each time with the total peptide concentration gives for any peptide mixture x the cumulative mass fraction of peptides with MW inferior or equal to that peptide. Plot of the cumulative mass fractions against the corresponding MWs determined by Eq. (3) gives the MW distribution of peptides. Here is the equation:

$$\%Pep_{MW \leq MW_{pep\,x}} = \frac{\sum_{i=1}^{x} C_{m_{Pep\,i}}}{C_{m_{Pep\,total}}} \tag{10}$$

where $\%Pep_{MW \leq MW_{pep\,x}}$ represents the cumulative mass fraction, $C_{m_{Pep\,i}}$ the concentration of an elementary peptide, and $C_{m_{Pep\,total}}$ the total peptide concentration.

The peptide signal was digitally fractionated into 10 fractions or classes (1-10) of identic elution timespan, each corresponding to a distinct and calculated MW interval. The SE-HPLC methodology was subsequently applied to quantify these peptide fractions.

3. Materials and methods

3.1. Materials

Dried chicken feathers with a dry matter content of 87.5wt%, 92wt% of which is keratin, were provided by BCF Life Sciences (Pleucadeuc, France). Hydrochloric acid (HCl 33.2%) used for the hydrolysis was purchased from Arkema (Mourenx, France). HPLC grade eluent acetonitrile (ACN) and trifluoroacetic acid (TFA) were supplied by Acros Organics (Geel, Belgium) while ultrapure water produced by the ELGA PURELAB flex water purification system of Veolia Water Solutions & Technologies (High Wycombe, UK) was used for analytical purposes. Standards of 18 individual free AAs and 3 protein standards (bovine serum albumin, carbonic anhydrase and cytochrome) were obtained from Merck Life Sciences (Saint Quentin Fallavier, France). Synthetic peptide standards used for the column calibration were acquired from BACHEM (Bubendorf, Switzerland) (12 peptides) and GeneCust (Dudelange, Luxembourg) (14 peptides).

3.2. Acid hydrolysis of poultry feathers

The hydrolysis set-up consisted of two identic hydrolysis systems, each composed of a 1L three-neck round-bottom flask mounted on a retort stand and equipped with a condenser, a temperature probe and a glass stopper at the unused neck. Each reactor was suspended in a crystallizing dish filled with silicone oil which serves as a thermostatic bath. The two crystal dishes were placed on separate magnetic stirrer hot plates, each connected to the reactor's temperature probe to enable continuous temperature regulation and maintain the reactor's temperature constant at the desired setpoint. A magnetic stir bar placed in the liquid hydrolysate within the reactor provided agitation throughout the hydrolysis experiment. The role of the condensers was to trap unexpected volatile HCl, and the two condensers were connected in series and cooled using ambient tap water.

All experiments started by loading 115 g of chicken feathers in the reactor to achieve a feather dry matter (FDM) of about 100 g. Then, water and HCl were subsequently added to reach an HCl mass concentration of 21.5% and a prescribed HCl-to-feather dry matter (HCl/FDM) ratio reported in Table 2. Afterwards, the mixture was continuously agitated and heated at a prescribed setpoint temperature also given in Table 2. The initial time (t_0) was defined as the moment when all the feathers were completely dissolved in the acidic medium. Seven samples (5-10 g) were collected at 0.5 h, 1.5 h, 2.5 h, 3.5 h, 4.5 h, 5.5 h, and 6.0 h after t_0 and were labelled H05, H15, H25, H35, H45, H55, and H60, respectively. Immediately after collection, each sample was frozen to stop the hydrolysis reaction.

A total of four hydrolysis experiments were carried out observing two HCl/FDM ratios (Ratio 1 = 4.19 and Ratio 3 = 5.12) and two temperature conditions (T1 = 106 °C and T2 = 95 °C). Table 2 indicates the experiment names and the corresponding operating conditions. It is worth noting that Ratio 3 > Ratio 1 and T2 < T1.

Table 2 *The four hydrolysis experiments conducted along with the corresponding set of applied operating conditions. Ratio 3 > Ratio 1 and T2 < T1.*

Experiments	Operating conditions	HCl/FDM ratio	Temperature (°C)
E11	(Ratio 1, T1)	4.19	106
E12	(Ratio 1, T2)	4.19	95
E31	(Ratio 3, T1)	5.12	106
E32	(Ratio 3, T2)	5.12	95

3.3. Quantification of FAA and Peptide fractions

Free and total AA analyses were performed according to ISO 13903:2005 (International Organization for Standardization (ISO) 2005) (Commission Européenne 2009), with slight adaptations described below. Analyses were conducted on a Biochrom 30+ Amino Acid Analyzer (Harvard Bioscience, USA) equipped with a Biochrom Lithium High Performance column (Part No. BC80600248) packed with Ultropac 8 cation exchange resins. For free AA determination, 150 mg of each protein hydrolysate sample was mixed with 1 mL of an internal standard solution (norleucine, 20 mM) and diluted to 50 mL with 0.2 M lithium citrate loading buffer (pH 2.2, Biochrom reference 80-2038-10). The mixture was filtered through a 0.22 µm syringe filter and 20 µL was injected into the analyzer. Amino acids were separated at 37 °C under lithium buffer gradients and detected post-column by ninhydrin derivatization at 570 nm and 440 nm for proline. For total AA determination, 300 mg of the protein hydrolysate sample was placed in a hydrolysis vial with 2 mL of norleucine internal standard. The sample was dried at 105 °C for 1 h and then hydrolyzed in 25 mL of 6 N HCl containing 1 % phenol at 110 °C for 24 h (1 h loosely capped, then 23 h sealed). In parallel, a second aliquot (300 mg of the

protein hydrolysate and 2 mL of norleucine internal standard) was oxidized at 0 °C for 16 h in 5 mL of performic acid mixture (prepared from 4.5 mL formic acid-phenol solution and 0.5 mL hydrogen peroxide, 30 %) after dessication (105 °C for 1h). 0.84 g of sodium disulfite was then added to remove excess oxidant before hydrolysis under the same conditions. After hydrolysis, the cooled solutions were adjusted to pH 2.2 with lithium hydroxide 4N, transferred to 100 mL volumetric flasks, and made up to volume with loading buffer. Samples were filtered before injection and analyzed as described for free AAs. Quantification was performed using calibration curves of amino acid standards (European Pharmacopoeia, EDQM, France) prepared in the same buffer.

Except for cystine, methionine and tyrosine, the total AA content was calculated as the average of both the unoxidized and oxidized samples. Only the unoxidized sample was used to determine the total AA content of tyrosine, whereas cystine and methionine total contents were determined exclusively from the oxidized sample. Following these analytical procedures, the results were obtained as free and total AA aminograms, providing detailed mass fractions of free and total forms for all 17 assayed AAs, respectively. To enable the conversion of these mass fractions to mass concentrations (g/L), the density of the analyzed protein hydrolysates was experimentally measured. A consistent average density of 1069 g/L was applied to all protein hydrolysates. Mass concentrations for both free and total AA fractions were determined by summing the individual concentrations of all 17 AAs within each category. Finally, as reported by (Hou et al. 2017) and (Li and Wu 2020), the total peptide concentration was inferred as the difference between total and free AA concentrations.

3.4. Analysis by size exclusion chromatography (SE-HPLC)

Hydrolysate samples were analyzed by SE-HPLC using a Superdex 30 Increase 10/300 GL SEC column (bed dimensions: 10 × 300 mm; particle size: 9 µm; fractionation range: 100 to 7000 Da) from Cytiva (Uppsala, Sweden). The column was connected to a Shimadzu model LC20 system (Shimadzu Corporation, Japan). For analysis, aliquots of the raw hydrolysate samples were diluted 100-fold to avoid detector saturation and fall within the method linearity range (0.2 – 4 g/L (Bodin et al. 2015)) , before filtration through a hydrophilic 0.45 µm PVDF syringe filter (25 mm diameter). 30 µL of the diluted samples were injected onto the column, maintained at 35 °C. Isocratic elution was performed with a mobile phase consisting of water/ACN/TFA in a 69.9/30/0.1 (v/v/v) proportion at a flow rate of 0.5 mL min^{-1}. The UV signal was recorded at 214 nm using a cell with a 1 cm optical path. The HPLC runtime was 60 minutes per analysis. Chromatogram data were exported and fed to user-defined MATLAB programs for application of the aforementioned methodology.

The column was calibrated with 26 peptide standards ranging from 132 to 1686 g/mol eluted under the same conditions. Table A.2 and Table A.3 of the supplementary materials summarize the results of the calibration with peptides and free AAs precising their MWs and obtained retention times.

Using linear regression, the following equation was obtained for the column calibration with peptides:

$$MW_{Pep\,x} = 10^{-0.0620 \times t_{Pep\,x} + 4.7235} \quad (11)$$

The correlation coefficient (R^2) was 0.84.

Hydrolysates were divided into 10 classes of peptide defined by their MW range as depicted in Table A.1 of the supplementary materials.

4. Results and discussion

4.1. Elucidation of the parameters of the SE-HPLC methodology: t_{delim} and key parameters $\bar{M}_{aa}$ and $\bar{\varepsilon}_{aa}$

4.1.1. The boundary retention time between Peptide and FAA fractions: t_{delim}

Figure 4 shows the SE-HPLC chromatograms of hydrolysates obtained in four different conditions (E11, E12, E31 and E32) at hydrolysis durations of 0.5 to 6 h (7 kinetics points). In total, there are 28 keratin hydrolysates mixtures reflecting a diversity in preparation protocols and therefore composition which is necessary to thoroughly assess the robustness of the proposed SE-HPLC methodology.

From left to right, the chromatograms display absorbance signals corresponding to compounds eluting from approximately 20 min up to 44.5 min. A broad and poorly resolved group of peaks is observed up to around 37 min, followed by three distinct, well-defined peaks. Based on the calibration of free AAs standards, these three major peaks occur at the retention times of phenylalanine (Tr = 38.26 min), tyrosine (Tr = 40.30 min), and cystine (Tr = 41.99 min). In the column calibration results (Tables A.2 and A.3), peptides and free AAs standards are listed in the ascending order of their retention time. The calibration results indicate that out of the 26 analysed peptide standards, only 4 do not elute before 37 min, but within 2 minutes past 37 min. These include low-MW peptides GGG, DY, SY and GG. At the same time, according to the same calibration results, most free AAs standards elute after 37 min with the exceptions of only 4 free AAs (leucine, isoleucine, proline and valine) that elute before that time. Overall, the calibration results suggest that the boundary retention time between Peptide and FAA fractions can be fixed at about 37 min.

Before the 37-38 min interval, the signal intensity keeps decreasing while it keeps increasing the other side of that region over the 6 hours of hydrolysis. Therefore, a delimitation time (t_{delim}) picked from that interval is also coherent with the morphologies of the 7 chromatograms within each experiment that reveal a reversal in signal intensity trends around 37-38 min. During acid-induced proteolysis, it is well documented that high MW peptides get converted into smaller peptides which eventually yield free AAs (Alvarez et al. 2012) (Bouhamed and Kechaou 2017). Using the proposed definition of t_{delim} from the 37-38 min transition region, the chromatogram reading is consistent with the kinetics of the two fractions reported in the literature. Indeed, with t_{delim} set in the transition region, as the hydrolysis reaction proceeds from H05 to H60, the entire Peptide signal diminishes while that of the FAA keeps increasing. The delimitation time of about 37-38 min is in an overlapping area of the two fractions and its exact value might fluctuate slightly in that region depending on the hydrolysate composition. Consequently, the proposed methodology numerically searches the value of t_{delim} in the transition region of individual chromatograms as the retention time with the minimal absorbance.

t_{delim} values obtained for all chromatograms are provided in Tables A.4–A.7 of the supplementary materials. Except for experiments E32 where the t_{delim} occurred at 38.0 – 38.1 min, all the other values ranged between 36.7 and 37.5 min.

The varying value of t_{delim} may introduce biases in the quantification of the Peptide and FAA fractions. We expect this bias to be mostly mitigated by some compensation effects, similar to those described by Bodin et al. (Bodin et al. 2015) for peptides rich or deprived of high-absorbing AAs. Bodin et al. (Bodin et al. 2015) demonstrated that when calculating the total peptide concentration of a mixture, the local biases caused by peptides with extremely high (e.g., aromatic AAs and histidine contributions)

or very low molar extinction coefficients tend to cancel each other out upon total signal integration. Peptide mixtures with low ε_x are underestimated, while those with high ε_x are overestimated, but the overall sum remains accurate. Interestingly, Johns et al. (Johns et al. 2011) quantitatively investigated collagen, soy and whey protein hydrolysates by classic amino acid analysis (FMOC derivatization and florescence detection) of only 8 of the 17 constitutive AAs with satisfactory results. According to the authors, these 8 AAs contributed to about 40 wt% of the hydrolysate. So, it is quite reasonable to assume that the FAA fraction can be accurately described by the 13 AAs eluting after 37 min (accounting for over 70 wt% of unhydrolyzed keratin). Eventually, the molar extinction coefficients of the 4 early AA eluted before 37 min (leucine, isoleucine, proline and valine) are very low (less than 50 $L.mol^{-1}.cm^{-1}$), so losing their negligible signals to the large signals of the latest eluting peptides would not substantially affect the total signal measured for the FAA fraction.

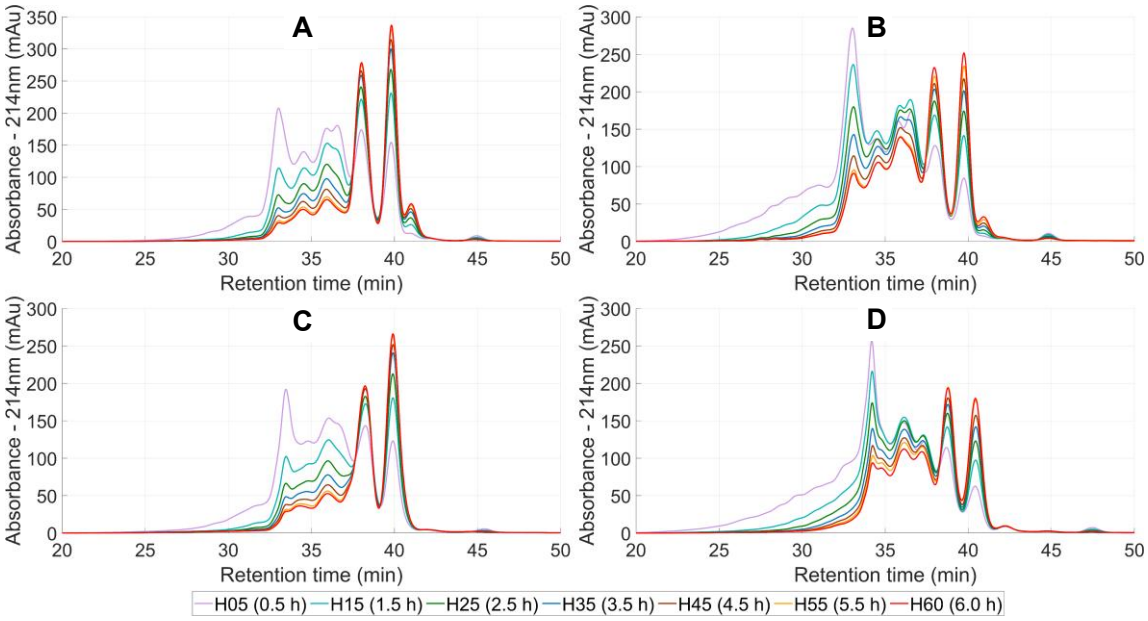


Fig. 2 *SE-HPLC chromatograms of hydrolysates produced during experiments E11 (A), E12 (B), E31 (C) and E32 (D). The chromatograms were snapped to elution time range of 20-50 min for clarity*

4.1.2. Key parameters $\bar{M}_{aa}$ and $\bar{\varepsilon}_{aa}$

Table 3 shows total and free amino acids concentrations in E11 and E12 hydrolysates. From these data, free amino acids fraction and peptides were deduced. Figure 3 shows the kinetic evolution of the fractions concentrations over the course of the hydrolysis reaction based on both aminogram data (cyan color) and application of the SE-HPLC method with optimally regressed key parameters $\bar{\varepsilon}_{aa}$ (orange color). As a reminder, key parameters $\bar{\varepsilon}_{aa}$ listed in Table 3 and required in the determination of the SE-HPLC kinetics plotted in Figure 3, were adjusted by regression of Eq. (9) based on aminogram concentration measurements. According to reference aminogram data reported in Figure 3, regardless of the applied operating conditions, the peptide concentration steadily decreases over the 6h of the hydrolysis reaction. Concomitantly, the FAA concentration keeps increasing. This denotes that, as expected, during acid hydrolysis of keratin, peptides are broken down into free AAs.

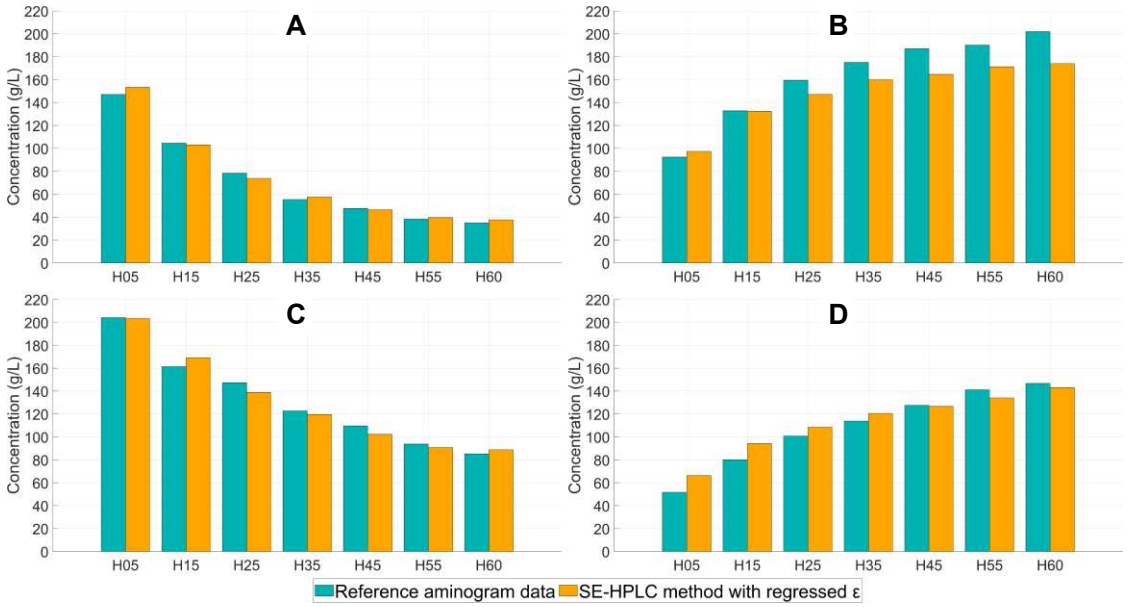


Fig. 3 *Concentration kinetics of the Peptide (A; C) and FAA (B;D) fractions during experiments E11 (A;B) and E12 (C;D) deduced from the aminogram data (cyan color) and determined for optimally regressed key parameters $\bar{\varepsilon}_{aa}$ of the SE-HPLC method (orange color)*

Table 3 also presents key parameters $\overline{M}_{aa}$ and $\bar{\varepsilon}_{aa}$ calculated from the aminogram for both main fractions. Whatever the fraction considered (Peptide or FAA) and irrespective of the hydrolysate processing conditions and sampling time, it is noticeable from this table that $\overline{M}_{aa}$ and $\bar{\varepsilon}_{aa}$ are relatively constant. The statistical analysis was performed (Table 4). The standard deviation obtained for each key parameter and per fraction across all 14 hydrolysates is remarkably low compared to its associate mean value. In fact, relative standard deviations (RSD) inferior to 3% and 10% were obtained respectively for key parameters $\overline{M}_{aa}$ and $\bar{\varepsilon}_{aa}$. This suggests that the key parameters calculated are indeed consistent and quite constants across all investigated hydrolysis conditions and irrespective of the hydrolysis time, which may justify the decision to keep them constant in the subsequent application of the SE-HPLC methodology for keratin protein hydrolysates of unknown compositions. As a result, the mean value of $\overline{M}_{aa}$ and $\bar{\varepsilon}_{aa}$ of the two fractions reported in Table 4 were considered for any future application of the SE-HPLC method for keratin acid hydrolysates.

A significant difference is observed between $\bar{\varepsilon}_{aa}$ of the Peptide fraction and $\bar{\varepsilon}_{aa}$ of the FAA fraction that is about 20% higher in average. There is also a moderate difference between $\overline{M}_{aa}$ of the two fractions marked by a FAA parameter lower by only 8% in average with respect to the Peptide fraction. These differences directly reflect the compositional disparity between the two fractions. This is explained by the known resistance of certain hydrophobic AAs, such as valine, isoleucine, and glycine, to peptide bond cleavage under acid hydrolysis (Lamp et al. 2018). Because unhydrolyzed keratin has a relatively high content of valine (7 wt%), isoleucine (4 wt%) and glycine (9 wt%), highly stable peptides resistant to the applied harsh acidic and hot hydrolysis conditions may persist. Such peptides include: Val-Val, Val-Gly, Val-Ile and Ile-Ile (Lamp et al. 2018). These resistant AAs persist in the peptide fraction, contributing less to the free pool, thus artificially diminishing $\bar{\varepsilon}_{aa}$ of the Peptide fraction while increasing that of the FAA fraction due to their low molar extinction coefficient (inferior to 50 $L.mol^{-1}.cm^{-1}$). The moderate difference noted for $\overline{M}_{aa}$ is attributable to the less dissimilar molecular weights of AAs (75 to 240 g/mol) compared to their molar extinction coefficients (21 to 5375 $L.mol^{-1}.cm^{-1}$ at 214 nm (Kuipers and Gruppen 2007)).

Table 3 *Aminograms of hydrolysates produced in experiments E11 and E12 showing the total concentrations of free AA, total AA and Peptides, and the key parameters $\bar{M}_{aa}$ and $\bar{\varepsilon}_{aa}$ deduced from these compositions using Eq. (4) and the regression methodology Eq. (9).*

	E11 - Concentrations (g/L)														E12 - Concentrations (g/L)													
	H05		H15		H25		H35		H45		H55		H60		H05		H15		H25		H35		H45		H55		H60	
	Free AA	Total AA	Free AA	Total AA	Free AA	Total AA	Free AA	Total AA	Free AA	Total AA	Free AA	Total AA	Free AA	Total AA	Free AA	Total AA	Free AA	Total AA	Free AA	Total AA	Free AA	Total AA	Free AA	Total AA	Free AA	Total AA	Free AA	Total AA
Aspartic acid	9.1	15.5	12.4	15.3	13.8	15.4	14.7	15.1	15.0	15.5	15.3	15.3	16.0	16.0	5.5	16.4	8.4	15.9	10.1	16.0	11.1	15.5	12.2	15.6	12.6	14.6	13.1	14.4
Threonine	2.1	9.5	4.0	9.3	5.5	9.5	6.7	9.2	7.4	9.4	8.1	9.4	8.6	9.7	0.9	9.9	1.7	9.9	2.5	9.7	3.1	9.4	3.8	9.3	4.5	9.3	4.8	9.4
Serine	14.4	25.4	19.9	25.4	22.6	25.5	24.1	24.6	24.8	25.3	25.8	25.8	26.2	26.2	9.1	27.3	13.3	27.1	16.3	27.0	18.1	26.1	19.9	25.2	20.0	24.2	20.5	24.8
Glutamic acid	8.5	20.2	13.0	19.7	15.3	19.9	16.8	19.2	17.6	19.7	18.1	18.7	18.7	19.1	4.5	21.4	7.5	20.8	9.8	20.6	11.2	20.0	12.6	19.9	13.0	18.5	13.5	18.6
Glycine	14.3	22.4	17.8	22.2	19.3	22.5	20.1	21.9	20.5	22.4	20.6	21.7	21.0	22.0	9.9	23.9	13.5	23.3	15.7	23.6	16.8	22.4	17.9	22.5	17.3	21.4	17.6	21.1
Alanine	6.3	12.2	8.8	12.0	10.1	12.0	10.8	11.7	11.2	12.1	12.0	12.3	12.0	12.6	3.5	12.8	5.1	12.6	6.8	12.5	8.0	12.0	8.7	12.2	9.2	12.3	9.5	12.3
Valine	3.2	16.1	4.4	16.1	5.5	16.3	6.7	15.9	7.7	16.1	8.6	15.7	9.2	16.0	2.5	17.0	2.8	16.2	3.2	16.6	3.6	16.0	4.0	15.9	5.0	15.4	5.0	15.3
Cystine	2.8	27.2	5.6	27.4	8.7	26.2	10.9	26.6	12.7	26.1	13.4	25.2	13.7	26.2	0.0	29.9	1.9	28.8	2.9	28.3	4.2	26.6	5.6	26.3	6.0	26.3	7.3	26.4
Methionine	0.7	1.1	0.8	1.0	0.9	1.0	0.8	1.0	0.9	1.0	1.1	1.1	0.9	0.9	0.9	1.0	0.7	0.7	0.5	0.8	0.6	0.7	0.7	0.7	1.2	1.2	1.2	1.2
Isoleucine	1.3	9.6	2.5	9.6	3.4	9.7	4.1	9.4	4.8	9.6	5.1	9.1	5.4	9.0	0.5	10.0	1.0	9.7	1.6	9.8	2.0	9.5	2.3	9.6	3.0	9.4	3.1	9.3
Leucine	5.2	19.2	8.7	19.1	11.1	19.1	12.9	18.7	14.1	18.9	15.5	18.3	16.0	18.2	2.6	20.2	4.4	19.6	5.9	19.6	6.9	18.9	8.1	19.0	11.2	18.9	11.6	18.7
Tyrosine	5.3	11.6	6.6	11.7	8.4	11.9	7.7	10.9	8.8	9.1	4.7	7.1	8.1	11.0	2.6	12.0	3.9	12.0	5.1	12.1	5.9	11.9	6.6	11.6	6.3	11.6	6.6	11.7
Phenylalanine	4.5	11.6	5.9	11.4	7.4	11.5	8.3	11.2	9.1	11.2	9.1	10.4	10.0	11.0	3.0	12.2	4.0	12.1	4.9	11.9	5.1	11.5	5.7	11.4	7.0	10.8	7.3	11.0
Lysine	1.1	3.3	1.8	3.3	2.1	3.4	2.3	3.3	2.6	3.3	2.5	3.0	2.6	3.1	0.5	3.5	0.9	3.4	1.2	3.4	1.4	3.2	1.6	3.2	1.9	3.2	1.9	3.1
Histidine	0.6	1.7	0.8	1.7	1.2	1.6	1.3	1.5	1.3	1.5	1.3	1.3	1.3	1.3	0.3	1.8	0.5	1.7	0.6	1.7	0.6	1.6	0.7	1.5	1.1	1.6	1.0	1.5
Arginine	3.5	12.9	5.7	12.4	7.5	12.6	8.8	12.3	9.3	12.6	9.8	11.9	10.1	12.4	1.8	13.9	3.0	13.6	4.2	13.5	5.1	12.7	5.9	12.8	6.4	12.4	6.7	12.3
Proline	9.4	19.9	14.1	19.6	16.6	19.6	18.0	18.0	18.9	20.8	19.4	22.3	22.1	22.3	3.7	22.5	7.3	14.1	9.5	21.1	10.0	18.7	11.4	20.2	15.5	23.9	15.8	20.7
Total (g/L)	**92**	**239**	**133**	**237**	**160**	**238**	**175**	**230**	**187**	**235**	**190**	**229**	**202**	**237**	**52**	**256**	**80**	**241**	**101**	**248**	**114**	**237**	**128**	**237**	**141**	**235**	**147**	**232**
Peptides (g/L)	**147**		**105**		**78**		**55**		**48**		**38**		**35**		**204**		**161**		**147**		**123**		**110**		**94**		**85**	
$\bar{M}_{aa}$ $(g.mol^{-1})$	**125**	**139**	**127**	**139**	**130**	**139**	**131**	**139**	**132**	**138**	**131**	**137**	**132**	**138**	**119**	**139**	**123**	**140**	**124**	**139**	**126**	**139**	**127**	**139**	**128**	**138**	**129**	**139**
$\bar{\varepsilon}_{aa}$ $(L.mol^{-1}.cm^{-1})$	**747**	**657**	**718**	**591**	**679**	**532**	**679**	**655**	**659**	**566**	**669**	**617**	**644**	**679**	**867**	**605**	**822**	**676**	**760**	**535**	**756**	**573**	**716**	**521**	**689**	**561**	**713**	**655**

Table 4 *Statistical analysis of key parameters values obtained for the two main fractions (FAA and Peptide) based on reference method concentration data of hydrolysates produced in experiments E11 and E12.*

Key parameters	FAA fraction			Peptide fraction		
	Mean	Standard deviation	Relative standard deviation RSD (%)	Mean	Standard deviation	Relative standard deviation RSD (%)
$\bar{M}_{aa}$ $(g.mol^{-1})$	127.4	3.8	**3.0%**	138.6	0.7	**0.5%**
$\bar{\varepsilon}_{aa}$ $(L.mol^{-1}.cm^{-1})$	722.8	63.1	**8.7%**	601.6	55.7	**9.3%**

4.2. Validation of the methodology

The methodology (with the mean parameters established previously) was applied to 28 hydrolysate chromatograms obtained in another set of conditions (95 or 106 °C and HCl/FDM ratio of 5.12) plus the original conditions (95 or 106 °C and HCl/FDM ratio of 4.19). Figure 4 displays Peptide, FAA and total AA quantification by SE-HPLC against the reference method based on aminograms. Each plot features a bisector line (y = x) and a green shaded region corresponding to absolute relative errors inferior to 15%. Data points are colour coded to differentiate training and validation datasets. Most points (81 out of 84 or 96% of the datasets) are within the ± 15% region across the 3 fractions assayed (Peptide Fraction, FAA fraction and soluble total AA). The only 3 instances of absolute relative errors above 15% by the SE-HPLC method happen for the quantification of the FAA fraction, and in these cases, the errors are still inferior to 30%. Based on these accurate estimations of the mass concentrations of the main constituents of keratin protein hydrolysates, the SE-HPLC method was deemed validated and perfectly suitable for the quantitative analysis of other keratin protein hydrolysates produced by acid hydrolysis. In line with the work of Bodin et al. (Bodin et al. 2015), the SE-HPLC method proves to be very effective and accurate for the quantification of peptides in complex protein hydrolysate mixtures. In their study focusing on the analysis of the Peptide fraction only, Bodin et al. (Bodin et al. 2015) developed and successfully validated a similar SE-HPLC method using casein and alfalfa white protein hydrolysates as the test products. They consistently measured total peptide concentrations 15% within the real concentration, with absolutely no inclination of the method to positive or negative bias. Likewise, from Figure 4.A, the quantification of the Peptide fraction reported in this study is not bias prone, as evidenced by the even distribution of data points each side of the bisector line.

The analysis of the FAA fraction with the SE-HPLC method (Figure 4.B) appears to suffer slight biases regardless of the hydrolysate origin. The bias is concentration dependent with most points above the bisector for concentration below 120 g/L (i.e., positive bias or overestimation) and most points below the bisector for higher concentrations (i.e., negative bias or underestimation). This bias probably originates from the variation of the composition of the FAA fraction over the course of the reaction which is not translated into an evolving molar extinction coefficient. The varying composition of the FAA fraction means a varying molar absorptivity not necessarily reflecting the AA composition because of the differences in absorptivity amongst individual Aas. In fact, the higher the concentration of FAA or its yield (i.e., initial keratin loading similar for all experiments), the higher the proportion of low absorptivity free Aas and inversely. Therefore, true value of $\bar{\varepsilon}_{aa}$ decreases with the advancement of the hydrolysis reaction which is not captured when using an average $\bar{\varepsilon}_{aa}$, hence the negative bias observed with time increment and inversely. To a lesser extent, the bias may be partly attributed to the absence of the 4 early eluting free AAs in the UV response processed for the FAA fraction, as concluded by Johns et al. (Johns et al. 2011) for an identic bias trend (i.e., positive bias followed by

negative bias around a mean value) in peptide MW distribution using a reference method analyzing only 8 out of 17 AAs.

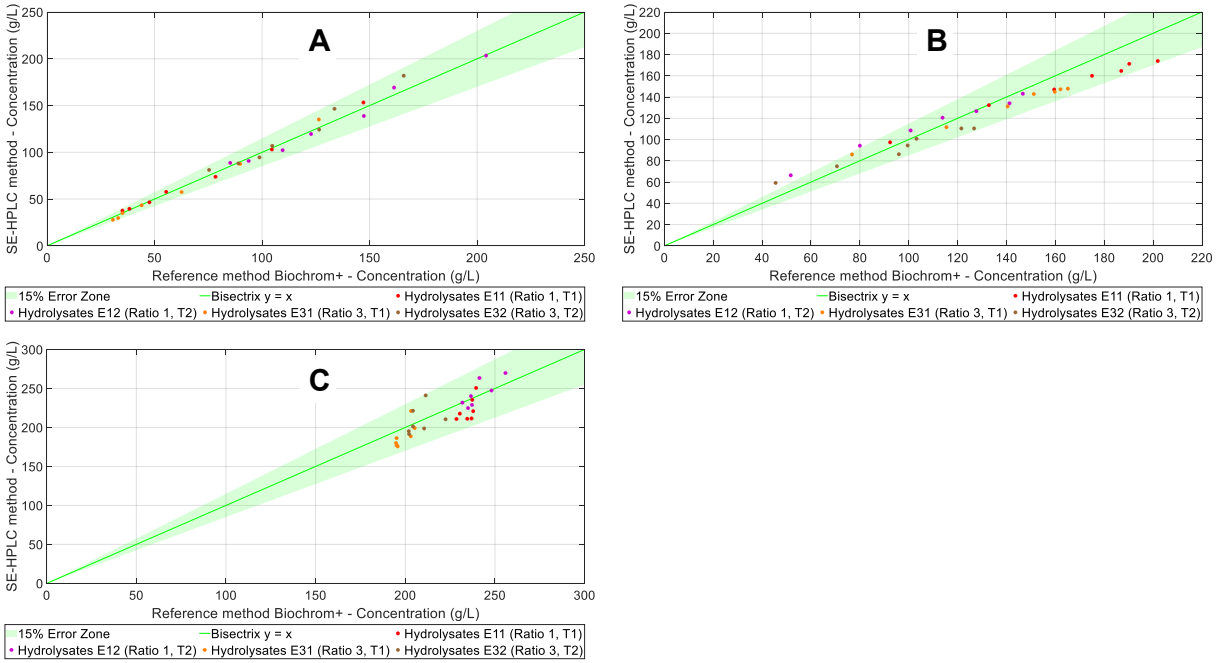


Fig. 4 *Comparison of the SE-HPLC method with the Biochrom+ reference method for validating the quantification of keratin protein hydrolysates main fractions: (A) the Peptide fraction, (B) the FAA fraction, and (C) soluble total AA*

Very few researchers have explored the possibility of quantifying the total FAA fraction of protein hydrolysates based on SE-HPLC/UV. Silvestre et al. (Silvestre et al. 1994) exploited SE-HPLC chromatograms to quantify the FAA fraction of a casein hydrolysate. They relied on a quite distinct methodology for the processing of chromatograms: removal of the large signals of aromatic AAs before absorbance conversion to mass concentration. Their methodology introduced strong negative bias leading to systematic underestimation of the FAA fraction by over 50%. Therefore, the present study is the first demonstration of a SE-HPLC/UV method for quantifying the FAA fraction simultaneously with the more documented total peptide content. Furthermore, as depicted in Figure 4.C, the soluble total AA (i.e., FAA plus Peptide fractions) is accurately quantified with the method too despite minor biases on the FAA fraction. Knowing the soluble total AA is especially valuable for monitoring insoluble fractions. For instance, in kinetic studies where the initial concentration of the hydrolyzed material is known, this would allow the indirect quantification of the insoluble fraction.

4.3. Application of the methodology for monitoring MW distribution and peptide fraction kinetics

The monitoring of the concentrations of the main fractions using the validated SE-HPLC method is reported in Figure 3 (orange bars of the histograms). The kinetic trends of the Peptide and FAA fractions obtained with the SE-HPLC method closely agree with those revealed by the reference method for the two experiments (E11 and E12). During hydrolysis, FAA concentrations gradually increase at the expense of the Peptide fraction that gets consumed in the process.

4.3.1. MW distribution of keratin hydrolysates

The MW distribution of peptides is another output of the application of the proposed SE-HPLC method. The distributions obtained for the keratin hydrolysates mixtures of experiment E12 only were drawn

on the same plot to showcase this feature of the method (Figure A.1). Each MW distribution was characterized by its weight-percentiles D_{10}, D_{50}, and D_{90}, which correspond to the MW at 10%, 50%, and 90% of the cumulative peptide content. Hereafter, they are referred to as distribution markers. According to maximum distribution marker D_{90} of 860 g/mol across all hydrolysates, all the mixtures primarily contain peptides with MW lower than 860 g/mol. Similarly, the minimum distribution marker D_{10} of 280 g/mol suggest that most E12 peptides have MW higher than 280 g/mol. So, the hydrolysate products of the keratin acid hydrolysis under condition E12 are mainly made of peptides with MW comprised between 280 and 860 g/mol. This is coherent with the extensive hydrolysis undergone by protein during acid hydrolysis, and expected to generate mostly low MW peptides and free AAs. The distribution marker D_{50} traditionally called the median MW varies only slightly on the range of 350 to 444 g/mol for all the hydrolysates, suggesting that they have a very similar composition in peptides. Still, the distribution markers D_{10}, D_{50} and D_{90} reported by the method are very distinctive and specific per hydrolysate, so they are unambiguous composition descriptors to differentiate between hydrolysates of very close compositions in a robust manner.

The individual MW distributions plotted in Figure A.1, although not perfectly superposed, largely coincide and are similar in trend. The longer the hydrolysis time is, the narrower the distributions are and the more shifted to the left (low MW peptides), they become. This confirms the dynamic evolution of the mixture composition toward a final state characterized by the predominance of low MW peptides of stable compositions. These observations imply that at this stage of the hydrolysis reaction, low MW peptides have been formed from the hydrolysis of the high MW peptides (MW > 860 g/mol), which are poorly represented in the distribution. These low MW peptides are persistent until the end of the reaction, in such a way that their proportions remain relatively unchanged during this stage of the process; only their absolute concentrations (i.e., peak intensity) might keep increasing. This was also reported by Eremeev et al. (Eremeev et al. 2009) who investigated keratin hydrolysates produced by enzymatic hydrolysis using alkaline protease C in a sodium sulphite enriched (0.5%) medium heated at 55 °C. The authors remarked that the character of the SE-HPLC MW distribution of peptides was practically identical across all studied hydrolysate samples. They concluded that the mechanism of keratin hydrolysis by this enzyme is such that peptide bond cleavages always occur at the same sites of the keratin sequence, causing the released products to be the same. The median MW measured in that study was also constant and located in the narrow range of 3.55 – 3.60 kDa, which supported their conclusion.

4.3.2. Kinetics of peptide fractions

The concentration kinetics of the fractionnated peptide classes (1-10) were determined exclusively with the validated SE-HPLC method for one of the experimental conditions (E12), as an example. They are plotted in Figure 5. In this figure, the kinetic data are formatted as 3 groups: classes 1-5, classes 6-7 and classes 8-10. This grouping is particularly effective to highlight the differences in peptides' tendency toward hydrolysis. It informs that the breakage dynamics are not the same for all peptides contained in the mixture, and that peptide size is a major factor. High MW peptides (classes 1-5) are the most prone to hydrolysis, followed by intermediate peptides (classes 6-7), which exhibit a slight resistance to complete hydrolysis, since their concentration never fully reaches zero. Finally, short peptides (classes 8-10) exhibit the strongest resistance to acid hydrolsysis, as supported by the persistence of approximately 50% of the initial peptide concentration after 6 hours. As a result, stable short peptides (classes 8-10) are also the most abundant peptides in the mixture throughout the full duration of the hydrolysis. As previously discussed, hydrophobic AAs such as valine, isoleucine and glycine are likely the main constituents of these recalcitrant short peptides (Lamp et al. 2018).

The discussion of the influence of the operating conditions on the hydrolysis kinetics is beyond the scope of this study mainly concerned with the demonstration of the utility of SE-HPLC/UV techniques for the monitoring of protein hydrolysis. As evidenced by the presented kinetics results (Figure 3 and Figure 5) and the previously conducted validation (Figure 4), the proposed SE-HPLC methodology has the desired analytical capability. In future work, the concentration kinetics determined with the SE-HPLC method may serve to derive mechanistic insight and conduct thorough kinetic studies of proteinaceous materials acid hydrolysis over a wide range of operating conditions.

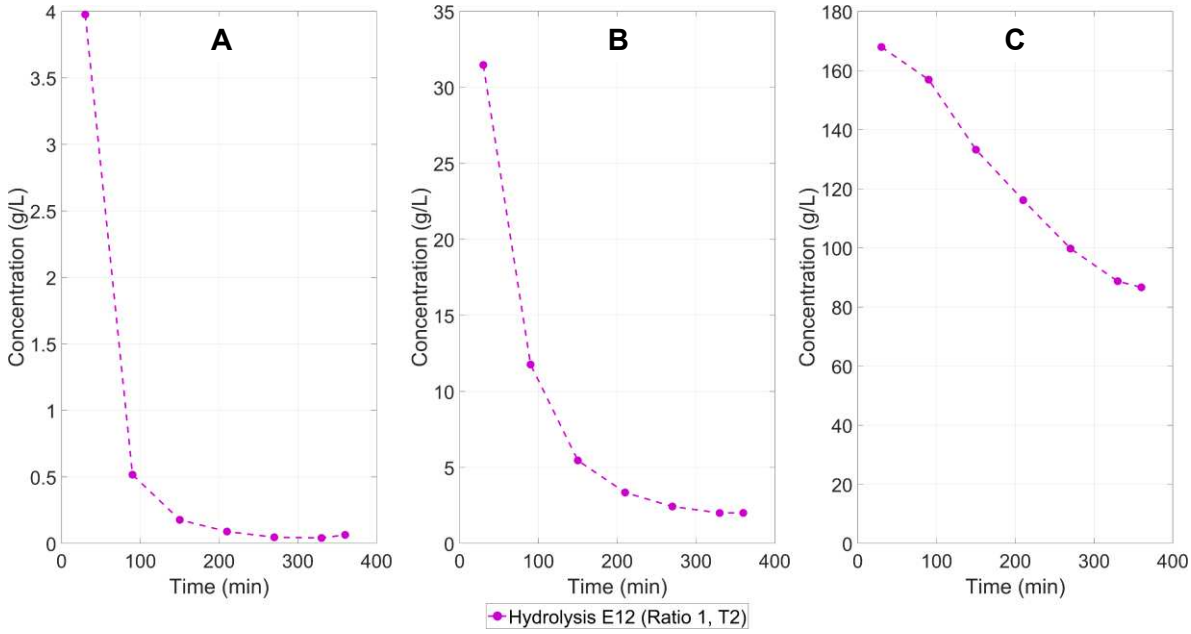


Fig. 5 *Concentration kinetics of fractionated peptide classes 1 to 10 during experiment E12 as determined with the SE-HPLC methodology. Data were formatted as 3 groups: (A) Classes 1-5 or [11.0 – 67.9 AA], (B) Classes 6-7 or [5.3 – 11.0 AA], and (C) Classes 8-10 or [1.8 – 5.3 AA]*

5. Conclusion

In conclusion, this study makes a strong case of the utility of the SE-HPLC method as a standalone analytical protocol for the complete quantification and characterization of the main fractions of complex keratin protein hydrolysate mixtures. Building on established research from the literature, we devised an original SE-HPLC methodology that can transform raw chromatographic signals into a collection of meaningful compositional data including: the mass concentrations of Peptide, FAA and soluble total AA fractions, peptide MW distribution featuring the distribution markers D_{10}, D_{50} and D_{90}, and a fractionation of the signal into 10 peptide classes. The methodology relies on the accurate determination of key parameters $\overline{M}_{aa}$ and $\bar{\varepsilon}_{aa}$, which was accomplished by using a classic amino acid analyzer as the reference method, before processing of the real concentrations data to resolve these parameters. The parameters were found to be fairly constant independently of the hydrolysate processing history, with RSD inferior to 10%. After implementation of the proposed SE-HPLC methodology in MATLAB, it was validated by comparing the quantifications of the Peptide, FAA and soluble total AA fractions to the reference method. 96% of data points had errors of the SE-HPLC method relative to the reference method inferior to 15%, which validated the methodology and demonstrated that the SE-HPLC/UV technique is an effective analytical tool to reliably quantify hydrolysate mixtures, even for their FAA fraction. The MW distribution of peptides and the kinetic study of the fractionated peptide classes proved to be valuable tools for deriving insights, otherwise hardly noticeable, from the same chromatographic signal. Altogether, SE-HPLC/UV is a powerful

analytical technique whose capabilities are probably underutilized in the growing academic and industrial ecosystem of protein hydrolysates.

Appendix A. Supplementary materials.

Supplementary materials associated with this article can be found in the online version at doi_link.

Declarations

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Competing interests

The authors have no financial or proprietary interests in any material discussed in this article.

Availability of data and material

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability

The code scripts used for SE-HPLC data processing are also available from the corresponding author on request.

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