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Development of a greener flow-based analytical tool for the expeditious determination of total soluble proteins

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ABSTRACT

Protein hydrolysates are increasingly used in feed, underscoring the need for analytical tools for the rapid, reliable determination of total protein. This study revisited a merging-zones flow-based spectrophotometric method for minimizing reagent consumption and employs the Biuret reaction for the quantification of total soluble protein in by-products hydrolysates. The analytical method was optimized across different physical and chemical parameters such as: flowrate, reactor length, sample injection volume and reagents concentration. The use of different matrices relevant in the hydrolysis processes (acetate, phosphate, and hydrochloric acid) showed no significant interference (<10%) on the method's performance. Under optimal conditions, the method quantified hydrolysate protein within a dynamic range of 0.100–2.00 mg mL^{-1} , with LOD and LOQ of 0.069 and 0.290 mg mL^{-1} , respectively. Analyses of hydrolysates showed no significant differences compared with the reference method (<10%) while reducing analysis time from 30 min to 3 min per sample (triplicate). The method's greenness, assessed by AGREE software, showed an improved score, from 0.58 to 0.76. The optimized protocol provides a fast, robust, and greener approach for monitoring protein hydrolysates in the food industry.

1. Introduction

Protein hydrolysates are the result of protein degradation into peptides or free amino acids, achieved through enzymatic or chemical processes (Kristinsson and Rasco, 2000). They are widely used in food and feed products due to their ease of digestion, rapid absorption, and reduced allergenic potential (Koopman et al., 2009), with applications ranging from sports supplements to infant formulas and vegan products (D'Auria et al., 2021). Despite extensive work on protein extraction, hydrolysis, engineering, and bioactivity profiling (Abbasi et al., 2025; Taghizadeh et al., 2025; Gholami et al., 2025), limited attention has been given to the development of rapid and environmentally friendly analytical systems for characterizing hydrolysates. Moreover, the production of hydrolysates requires close monitoring, ensuring the evaluation of their properties, nutritional value, and functionality (Prado et al., 2020; Zhu et al., 2006). Characterization of protein hydrolysates typically involves assessing key physicochemical, biochemical, functional, and safety parameters (Clemente, 2000; Adler-Nissen, 1986), including the assessment of the total soluble protein content. Although a variety of analytical techniques are available in the literature for the quantification of protein content, many are time-consuming and, usually, display high reagent consumption. Previous investigations into bioactive peptide generation from plant and food proteins (Abbasi et al., 2025; Taghizadeh et al., 2025) emphasized that hydrolysis conditions markedly influence peptide yield and properties. However, such hydrolysates often contain complex mixtures of small peptides and amino acids that challenge conventional spectrophotometric assays, leading to inconsistent quantification and extensive reagent use. This motivates the development of greener analytical platforms capable of accurately quantifying total soluble protein in diverse hydrolysates. In this scenario, flow-based techniques, such as flow injection analysis (FIA), are appealing miniaturized analytical tools presenting some advantages over the traditional analytical methods. The main advantages associated with these methods include high precision, reduced reagent consumption, high degree of automation, and they present potential for real-time monitoring. The core principles of flow-based methodologies rely on: (i)

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reproducible sample volume injection, (ii) controlled dispersion, and (iii) consistent time between injection and detection (Bezerra et al., 2020; Ribas et al., 2017). These methods are ideal for rapid, reliable, and repeatable analyses with minimal operator intervention, making them valuable in both laboratory and industrial contexts (Zhang et al., 2021).

The present study aims to implement a classical colorimetric method, based on the Biuret reaction, within a FIA system approach for the quantification of total soluble protein in hydrolysates. Additionally, this study revisited the use of a merging zone approach within the flow-based system. Briefly, the merging zones involves the simultaneous introduction of sample and reagent into different carrier streams that, at a defined point, converge into a unique flowing stream, allowing chemical reaction before reaching the detector. This approach further improves the reagent consumption, minimizing it (Jacintho et al. 1981). The Biuret method, first described by Gornall et al. in 1949, is a spectrophotometric technique that uses sodium-potassium tartrate ($NaKC_4H_4O_6{\cdot}4H_2O$) as a stabilizing agent. This compound is crucial for stabilizing copper (II) in alkaline media, preventing precipitation and enabling complex formation with peptide bonds, producing a deep violet color. So, this method relies on cupric ion complexation in an alkaline environment, generating a violet complex measurable at the wavelength of 545 nm. Due to its simplicity and analytical robustness, the Biuret reaction remains a widely used method for protein quantification (Gornall et al., 1949). The reference protein used for the calibration of the protein quantification was bovine serum albumin (BSA), a widely used reference material for colorimetric assays including the Biuret method. Its selection relies on its high stability and well-characterized properties, which ensure accurate and reproducible calibration curves (NIST 927e). To enhance the applicability of the method, various types of samples were tested in the developed flow system. Initially, hydrolysate solutions were analysed with the primary goal of monitoring the hydrolysis process in real time. Additionally, dried protein hydrolysates were evaluated, allowing the method to be applied in post-production analysis.

Despite extensive work on protein extraction, hydrolysis, engineering, and bioactivity profiling, limited attention has been given to the development of rapid and environmentally friendly analytical systems for total protein quantification in complex hydrolysates (Gholami et al. 2025; Hashemi et al. 2025; Hoseini, et al. 2025). To address this gap, the present study develops a flow-based Biuret assay that minimizes reagent consumption, reduces analysis time, and enhances greenness, thereby providing a sustainable analytical tool for protein and peptide research.

2. Materials and methods

2.1. Reagents and solutions

All solutions were prepared with analytical grade chemicals and boiled deionized water (specific conductance of less than 0.1 μS cm^{-1}).

A Bovine Serum Albumin (BSA, Sigma Aldrich, Germany) stock solution of 20 mg mL^{-1} was prepared by dissolution of 400.0 mg of the solid in 20.0 mL of water. The working standard solutions within a range of 0.100 mg mL^{-1} to 2.00 mg mL^{-1} were daily prepared by proper dilution of the stock solution.

A 1.5 mol L^{-1} sodium hydroxide stock solution was prepared, dissolving 3.0 g of sodium hydroxide (PanReac, Spain) in 50 mL of water. A 0.25 mol L^{-1} sodium hydroxide solution was prepared by proper dilution of the 1.5 mol L^{-1} stock solution.

A Biuret reagent stock solution was prepared monthly. Firstly, 3.48 g of copper (II) sulfate ($CuSO_4$, Merck, Germany) was dissolved in 10.0 mL of water. Then, 34.8 g of trisodium citrate dihydrate ($C_6H_5Na_3O_7.2H_2O$, Merck, Germany) and 20.0 g of sodium carbonate (Na_2CO_3, Merck, Germany) were dissolved in 80 mL of water. Afterwards, both solutions were mixed and the volume made up to 200 mL with deionized water. A Biuret reagent working solution ($CuSO_4$ 0.027 mol L^{-1}; $C_6H_5Na_3O_7.2H_2O$ 0.15 mol L^{-1}; Na_2CO_3 0.24 mol L^{-1}) was daily prepared by dilution (1:4) of the Biuret reagent stock solution (1:1).

A 1 mmol L^{-1} of hydrochloric acid solution was prepared by dilution of the commercial stock solution (%(m/m) = 36%; d = 1.18).

A 30 m mol L^{-1} acetate solution (pH 4.6) was prepared, dissolving 1.87 g of potassium chloride (KCl, Merck, Germany) in 100 mL of water. Then, 430 μL of glacial acetic acid (CH_3COOH, Merck, Germany) was added to the previously prepared solution, and the pH was adjusted to 4.6 with NaOH solution.

A potassium phosphate solution (pH 8.0) was prepared by dissolving 8.2 g of potassium phosphate dibasic (K_2HPO_4, Sigma Aldrich, Germany) and 0.41 g of potassium phosphate monobasic (KH_2PO_4, Sigma Aldrich, Germany) in 500 mL of water.

2.2. Flow-based manifold and procedure

The flow-based manifold, illustrated in Fig. 1, was composed by a Gilson Minipuls 3 peristaltic pump (PP in Fig. 1) equipped with three Tygon® pumping tubes (Gilson,Villiers-le-Bel, France), an injector commutator (laboratory made) assembled with two loops to inject defined volumes (400 μL) of standard/sample and Biuret reagent (1:4), and a flow cell (Hellma 178.711-QS) with 10 mm optical path (30 μL inner volume). All the components of the manifold were connected with polytetrafluoroethylene (PTFE) tubes (inner diameter 0.8 mm, 008T16–080–20). As detection system, a USB 4000 charged coupled device (CCD in Fig. 1) detector (Oceans Optics Orlando, FL, USA) equipped with a pair of optical cables, and a light source (UV–VIS–NIR, Mikropack, DH-2000-BAL). The absorbance signal was recorded using the CCD detector connected to a computer equipped with the SpectraSuite® software.

For the analytical method, a flow-rate of 1.07 mL min^{-1} was set for all three channels. The sample/standard solution (S/St) and the Biuret reagent solution were simultaneously injected via two loops (400 μL) placed on the injector commutator. Firstly, the S/St injected was mixed with sodium hydroxide solution (first confluence; S/St-NaOH). Afterwards, this mixture was merged with the injected Biuret reagent working solution (1:4) (R) (second confluence; [S/St-NaOH]-R). Then, the resulting plugs passed through a 100 cm reaction coil (RC in Fig. 1), to promote the reaction, and were directed to the detector for absorbance measurement. The absorbance signal for each sample/standard analysis corresponds to the maximum absorbance value of the transient peak formed after injection. Each standard/sample analysis was performed in triplicate.

2.3. Samples preparation

Two different types of samples were prepared for the analysis of total soluble protein content using the flow-based method described herein.

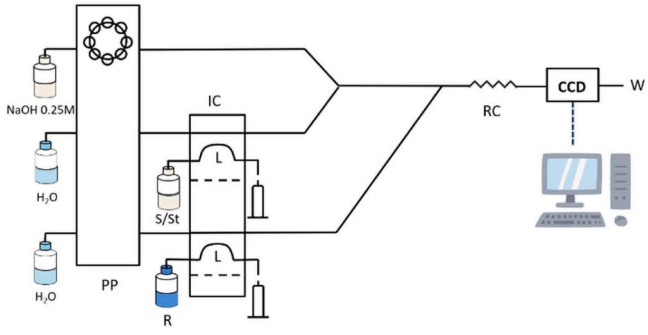


Fig. 1. Flow injection analysis manifold for the quantification of total soluble protein content; PP, peristaltic pump; IC, Injector commutator; L, Loop (400 μL); S/St, sample/standard solution; R, Biuret reagent working solution ($CuSO_4$ 0.027 mol L^{-1}; $C_6H_5Na_3O_7.2H_2O$ 0.15 mol L^{-1}; Na_2CO_3 0.24 mol L^{-1}); RC, reaction coil 100 cm; CCD, detector (λ= 554 nm); W, waste.

The first set consisted of dried hydrolysates derived from various by-products hydrolysis, including fish, pork, and insect sources (supplied by Industria Transformadora de Subprodutos - ITS). These samples were dissolved in deionized water at concentrations ranging from 3.00 to 6.00 mg mL^{-1} . The second set of samples included hydrolysate solutions obtained during the hydrolysis process of various by-products. These hydrolysates were obtained through the following enzymatic hydrolysis procedures: (i) procedure described by Borges et al., 2023 that uses the enzyme alcalase in a phosphate matrix medium (pH 8.0); (ii) procedure described by Borges et al., 2025 that uses the enzyme bromelain in an acetate buffer matrix medium (pH 4.6); and (iii) procedure described by Mak et al., 2024 that uses an acidic protease in an hydrochloric acid matrix medium (pH 3.0).

As these samples contained suspended solids, they were centrifuged (15 min; 5000 rpm), and the supernatant was collected. Subsequently, the supernatants were filtered (0.45 μm) and diluted at ratios of 1:5 and 1:10 to fit the linear range of the calibration curve.

2.4. Reference method

For validation purposes, the total soluble protein content was determined following the Biuret method described by Arora et al. (2018), as reference procedure. The method was performed as follows: 1.0 mL of the sample or standard solution (0.100–2.00 mg mL^{-1}), 1.0 mL of 1.5 mol L^{-1} NaOH, and 0.4 mL of Biuret reagent ($CuSO_4$ 0.11 mol L^{-1}; $C_6H_5Na_3O_7.2H_2O$ 0.6 mol L^{-1}; Na_2CO_3 0.96 mol L^{-1}) were mixed and incubated at room temperature (25ºC) for 30 min. After the incubation period, the absorbance of the solutions was measured using a CCD detector ($\lambda = 555$ nm), acquired using the computer SpectraSuite® software. Afterwards, if needed, the samples were diluted through a multi-step approach to ensure their composition to fit the linear range of the calibration curve. The results were compared with the results obtained with the developed FIA method.

3. Results and discussion

3.1. Development of the FIA system

The development of the FIA system for total soluble protein quantification, using the Biuret reaction, involved several preliminary studies to assess the influence that physical and chemical parameters on the method's performance. The study optimization was divided into two phases: a non-flow-based approach followed by a flow-based approach. The optimization of the FIA system was conducted using a univariate optimization approach. For each parameter, calibration curves were performed using five standard solutions, each analysed in triplicate. The overall optimization procedure was conducted in order to choose the condition that provided the highest slope of the calibration curve, as this parameter directly reflects the sensitivity of the analytical method. Additionally, a spectrum was performed, using the CCD detector, to determine the wavelength that corresponds to the maximum absorbance of this reaction product, which was 554 nm. Consequently, this wavelength was selected for the study to ensure higher sensitivity for signal acquisition.

3.1.1. Non-flow-based approach

Regarding the non-flow-based approach, the studies involved the order of addition of reagents (Biuret reagent solution – R, buffer solution – B; and standard solution – St) and the incubation time. The first one assessed different sequences of reagent addition, and the second evaluated the impact of incubation time in the stability of the colored reaction product. The tested addition sequences were St–R–B, R–St–B, and St–B–R during 5, 15, 30 min. No significant differences (< 10%) were observed in the slope for all the calibration curves indicating that the order of reagent addition and the time of incubation did not significantly influence the assay's performance. Based on these preliminary studies the design and development of the FIA system was initially set.

3.1.2. Flow-based system

For the development of the FIA system, the first study was the evaluation of different configurations of the system to improve the performance of the method. The tested manifolds are depicted in Fig. 2: (i) in Fig. 2A, an injector commutator was used for the double injection of the Biuret reagent (R) and sample/standard S/St in a continuous flow stream of deionized water (H_2O) and sodium hydroxide (NaOH) solution respectively; (ii) in Fig. 2B, the use of two confluences points allowed, first, the mixture between the S/St and the R, followed by the mixture with the NaOH solution in the second confluence; (iii) Fig. 2C, an injector commutator was used for the injection of the S/St solution, in a continuous flow stream of H_2O and, afterwards, in the first confluence, the mixture with the NaOH solution, and then, in the second confluence, the mixture with R; (iv) Fig. 2D, an injector commutator was used for the double injection of the R and S/St solutions, in a continuous flow stream of H_2O; the use of two confluences points allowed, first, the mixture between the S/St and NaOH solution and, in the second confluence, the mixture with the R. In the schemes of the Figs. 2A and 2B, no absorbance signal was obtained for the standard solutions with lower concentration.

Using the scheme depicted in Fig. 2D, with two confluences and the S/St and Biuret reagent solution injected trough the injector commutator, the sensitivity obtained was slightly higher (11%) (Fig. 3). Additionally, using this manifold, reagent consumption was lower in comparison with the scheme depicted in Fig. 2C, as the reagent is injected instead of being continuously flowing in the system, and so, a lower volume of reagent is used. For this reason, the manifold chosen to proceed with the optimization of the method was the one represented in Fig. 2D. The improved sensitivity observed when firstly the sample is mixed with NaOH and after with Biuret reagent can be explained by the peptide deprotonation of the sample in an alkaline media to, afterwards promote the complexation with copper ions (Gornall et al., 1949).

After selecting the flow manifold, the method development involved a number of studies to assess the influence of chemical and physical variables on the method's performance. The studied variables were flow-rate. reactor length, sample volume, sodium hydroxide solution concentration and the Biuret reagent solution concentration. All the studies are summarized in Fig. 4, where the full bars represent the chosen condition.

The flow-rate was a parameter of study, the tested conditions were 0.53, 1.07, and 1.47 mL min^{-1}. Although 0.53 mL min^{-1} presented a significant higher slope (> 25%), 1.07 mL·min^{-1} was selected based on its higher correlation coefficient ($R^2 = 0.994$) compared to the other tested flow-rates. (Fig. 4A).

The reactor length was also a parameter to be evaluated, and the lengths studied were 50, 100, and 150 cm, corresponding to internal volumes of 250, 500, and 750 μL, respectively. The 500 μL reactor was selected due to its significantly higher calibration curve sensitivity (> 80%) compared to the 250 μL volume. Moreover, no significant differences were observed between the 500 and 750 μL reactors. Additionally, using the 500 μL reactor instead of the 750 μL, a higher throughput was achieved because it corresponds to a shorter reaction coil, and so, this was the chosen reaction coil (Fig. 4B).

Sample volume was also studied and the volumes of 100, 200, and 400 μL were tested (Fig. 4C). Using the 400 μL sample volume, a higher slope (≃ 10%) was obtained when compared to the slope when using a 200 μL of sample. So, the 400 μL sample volume was chosen.

The sodium hydroxide solution concentration was also evaluated and, firstly, the 1.5 mol L^{-1}concentration mentioned in the procedure of the Biuret method described by Arora et al., 2018 was tested. However, using this solution concentration, an erratic absorbance signal was observed due to the high refraction index gradient produced in the flowing stream when the standard solutions were injected, making it impossible to distinguish the typical transient signal characteristic of a flow-based method. Then, lower concentrations of sodium hydroxide

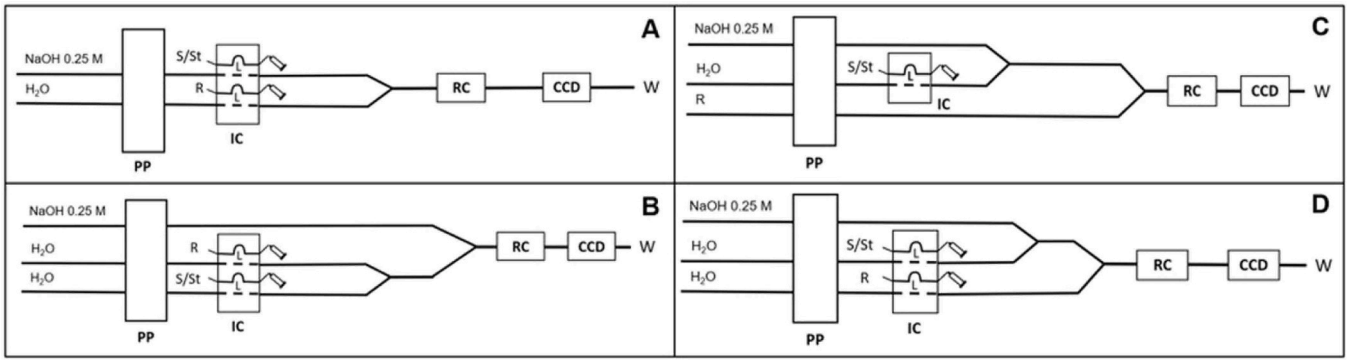


Fig. 2. Configurations evaluated for the design of the flow injection system manifold assembled with an injector commutator. (A) injector commutator assembled with two loops for S/St and reagent injections, and one confluence; (B) injector commutator assembled with two loops for S/St and reagent injection, and two confluences (firstly the sample is mixed with reagent and then mixed with NaOH); (C) injector commutator assembled with one loop for S/St injection, and two confluences (firstly the sample is mixed with NaOH and then with the reagent); (D) injector commutator assembled with two loops for S/St and reagent injections, and two confluences (firstly the sample is mixed with NaOH and then with the reagent); PP, peristaltic pump; S/St, sample/standard solution; R, Biuret reagent solution; IC, injector commutator; L, loop; RC, reaction coil; CCD, detector (λ = 554 nm); W, waste.

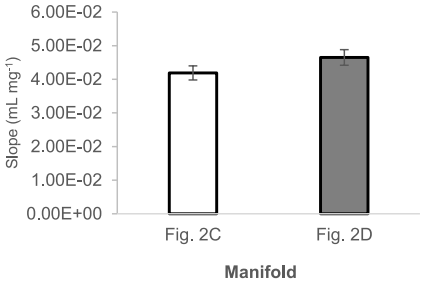


Fig. 3. Resume of sensitivities obtained with different manifolds in FIA system; error bars represent a 5% deviation related to the schemes of Figs. 2C and 2D.

solutions were tested, namely 0.50, 0.25 and 0.10 mol L^{-1}. A higher sensitivity (≃ 17%) was obtained when using a 0.25 mol L^{-1} solution (Fig. 4D), when compared to 0.50 mol L^{-1}, and an even higher sensitivity (> 80%) when compared to the use of 0.10 mol L^{-1}. Based on these results, 0.25 mol L^{-1} was selected as the concentration of sodium hydroxide.

Regarding the concentration of the Biuret reagent, the solution composition that was initially tested was the one described by Arora et al. (2018), and then several dilutions were prepared from this one, namely with a 1:2, 1:4, 1:6 and 1:8 dilution factor. After evaluating the slope of the calibration curves performed using the different concentrations of the Biuret reagent solution, a higher slope (14%) was obtained using the Biuret reagent solution diluted 1:4 (Fig. 5). With this solution, the use of the Biuret reagent was reduced by 75% when compared with the method described by Arora et al. (2018), being the final concentration of the reagent ($CuSO_4$ 0.027 mol L^{-1}; $C_6H_5Na_3O_7.2H_2O$ 0.15 mol L^{-1}; Na_2CO_3 0.24 mol L^{-1}).

Table 1 presents a summary of the above-mentioned optimization studies, including the variables assessed and the respective chosen condition.

3.2. Features of the FIA method

Under optimal conditions, the features of the developed method are summarized in Table 2 (Figure S1 – ESI). The dynamic range was set at 0.100–2.00 mg mL^{-1}. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated following the IUPAC recommendations (Currie, 1995) as the concentration equivalent to three and ten times the standard deviation of the intercept divided by the slope, respectively. The method's precision was assessed by analysing the RSD of two different samples quantification (10 measurements each). Regarding the analysis throughput, the herein described flow-based system is able to perform 20 sample analysis h^{-1} (triplicate analysis) corresponding to one sample injection per minute.

The proposed FIA method achieves a substantial reduction in reagent consumption compared to the reference procedure. The conventional method requires 0.4 mL of Biuret reagent and 1 mL of 1.5 mol L^{-1} NaOH per analysis, corresponding (in triplicate), to 21 mg $CuSO_4$, 212 mg $C_6H_5Na_3O_7{\cdot}2H_2O$, 122 mg Na_2CO_3, and 180 mg NaOH. The herein developed FIA system uses the Biuret reagent, but with a 1:4 dilution of the one used in the reference procedure, and a 0.25 mol L^{-1} NaOH solution. Consequently, this results in the reduction of the total consumption to 5.4 mg of $CuSO_4$, 53 mg of $C_6H_5Na_3O_7{\cdot}2H_2O$, 31 mg of Na_2CO_3, and 32 of mg NaOH. This represents a 75% decrease in Biuret reagent, evidencing the method's efficiency and its potential to minimize chemical waste and costs while maintaining analytical reliability.

Sustainability was further assessed using the AGREE tool (Analytical GREEnness Metric Approach and Software; Pena-Pereira et al., 2020) (Table 3) that revealed that the proposed analytical tool achieved an improved greenness score when compared with the reference procedure. The reference method scored 0.58 whereas the FIA method reached 0.76, reflecting its stronger compliance with the Green Analytical Chemistry principles. The reference method's lower score has is major weaknesses in criteria 1 (Sample Treatment), 5 (Automation, Miniaturization) and 8 (Analysis throughput). Regarding the reference method and the mentioned criteria, the method involves a large number of manual and time-consuming steps, and batch analysis. In contrast, the developed FIA method revealed a higher score in the abovementioned criteria due to its on-line analysis with minimal manual intervention and contamination risks. Additionally, criterion 8 highlighted the substantial improvement in analysis throughput, 20 samples min^{-1} for the FIA system against 2 samples h^{-1} for the reference method.

Overall, the FIA system higher score is consistent with the significant reduction in reagent use, on-line analysis and the shorter analysis time), confirming the proposed method as a more sustainable alternative without compromising analytical performance.

3.3. Interference studies

During the enzymatic hydrolysis of proteins, various enzymes can be employed, each associated with a distinct hydrolysis matrix (Borges et al., 2023; Borges et al., 2025; Mak et al., 2024). Thus, an interference study was performed to evaluate the interference of using different matrices on the method's performance. For that, different calibration curves, using BSA standards prepared in different solvents (deionized water, acetate, phosphate, and hydrochloric acid), were performed. The

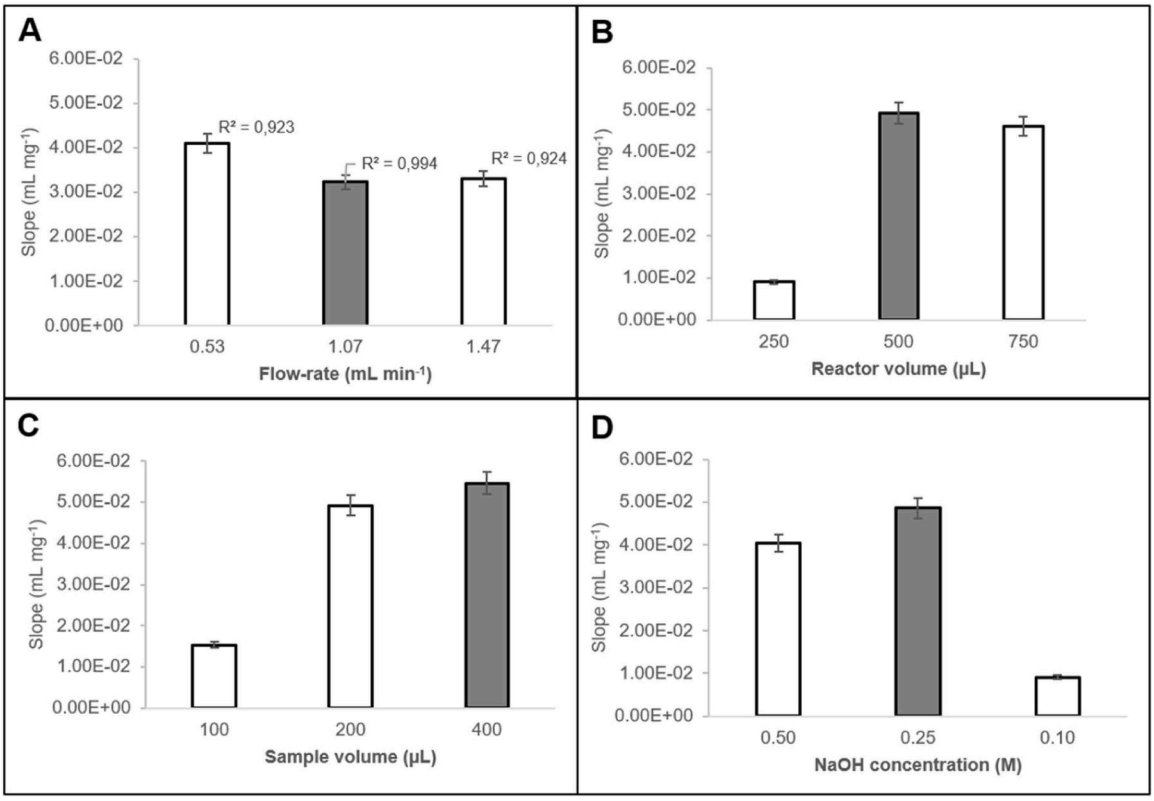


Fig. 4. Sensitivities achieved with (A) different flow-rates, (B) different reactor volumes (C) different sample volumes and (D) different concentrations of NaOH; error bars represent a 5% deviation.

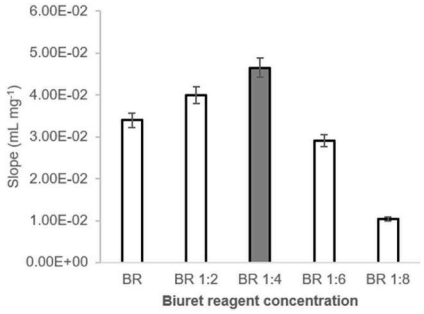


Fig. 5. Sensitivities achieved with Biuret reagent (BR) with the concentration described by Arora et al. (2018) ($CuSO_4$ 0.11 mol L^{-1}; $C_6H_5Na_3O_7.2H_2O$ 0.6 mol L^{-1}; Na_2CO_3 0.96 mol L^{-1}), and different concentrations obtained by dilution of the Biuret reagent (1:2; 1:4; 1:6 and 1:8); error bars represent a 5% deviation).

Table 1
Optimization of FIA variables.

Variable	Studied conditions	Chosen condition
Flow-rate (mL min^{-1})	0.53–1.47	1.07
Reactor length (cm)	50–150	100
Sample volume (µL)	100–400	400
Sodium hydroxide concentration (mol L^{-1})	0.10–0.50	0.25
Biuret concentration (%)	12.5–100	25.0

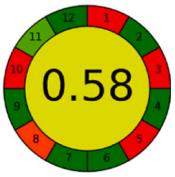
calibration curve parameters (slope and intercept) derived from standard solutions prepared in deionized water (standard calibration curve) were compared with those obtained with standards prepared in acetate,

Table 2
Features of the developed FIA system for total soluble protein quantification in hydrolysates.

Dynamic range	0.100–2.00 mg mL^{-1}
LOD	0.0687 mg mL^{-1}
LOQ	0.290 mg mL^{-1}
RSD sample[a]	3.2% (1.00 mg mL^{-1}); 3.4% (2.00 mg mL^{-1});
Calibration curve[b]	$A = 6.5 \times 10^{-2}$ [BSA] ($\pm 3 \times 10^{-3}$) + 2.9×10^{-1} ($\pm 2 \times 10^{-2}$)

[a] n = 10.
[b] n = 4

Table 3
Comparative AGREE assessment between the reference method and the developed FIA method.

Method	AGREE pictogram	Score
Reference		0.58
FIA		0.76

phosphate and hydrochloric acid matrices. No significant interferences (< 10%) were observed (Fig. 6), indicating the absence of matrix interferences in the quantification of total protein content in hydrolysates. Nevertheless, to further ensure the applicability of the methodology across additional hydrolysates, produced under different conditions from those stated above, an evaluation of the accuracy of the methodology should be considered.

3.4. Method validation – application to protein hydrolysates

To evaluate the accuracy of the herein developed FIA method, two types of hydrolysate samples were analysed (in triplicate) for the determination of total soluble proteins. The first type of samples were dried hydrolysates, obtained from different by-products hydrolysis, which were subsequently dissolved in deionized water (Table 4; Sample ID: A). The second type comprised samples obtained during the hydrolysis process of different by-products. These hydrolysis processes used different matrices (Table 4, Sample ID: B), namely acetate solution (pH 4.6), phosphate solution (pH 8.0), and hydrochloric acid (pH 3.0), depending on the enzyme used for the procedure. The determination of total soluble protein concentration in these samples (#19) was performed through the developed FIA method, and the results were compared with those obtained using the reference analytical procedure (Arora et al., 2018). The concentration calculation was performed by interpolation of the calibration curve. Whenever the concentration exceeded the linear range of the calibration curve, the samples were diluted in a multistep approach (1:5 and 1:10) to ensure the absorbance signal fit within the defined concentration range.

The concentrations obtained with the two different procedures and the comparison results (relative deviation – RD) are displayed in Table 4. A linear regression equation was established between the two sets of results (Figure S2 - ESI), the ones obtained with the developed flow-based system (represented by $[BSA]_{FIA}$) and the reference method (represented by $[BSA]_{REF}$). The established linear regression equation was $[BSA]_{FIA} = 1.02\ (\pm\ 0.54)\ [BSA]_{REF} - 0.043\ (\pm\ 0.150)$ ($r^2 = 0.99$), where the values in brackets represent the 95% confidence interval. These parameters showed that the estimated slope and intercept did not differ statistically from 1 and 0, respectively, and so, there were no significant differences between the results obtained with the developed flow-based system and the reference method (Miller and Miller, 2010).

Additionally, the agreement between the proposed analytical method and the reference procedure was evaluated using the Bland-Altman analysis (Altman and Bland, 1983). The mean difference between the two methods was 0.005 mg mL^{-1}, indicating no significant systematic bias. The calculated limits of agreement were −0.296 and 0.306, encompassing 95% of the observed differences, suggesting no significant differences between the two methods across the tested concentration range.

The paired *t*-test also confirmed the absence of a statistically significant difference between the two methods. The analysis yielded a t statistic of 0.0150 with 17 ° of freedom and a two-tailed p-values of 0.9882, above the significant threshold ($p < 0.05$), indicating no statistical differences. Furthermore, the high Pearson correlation ($r = 0.995$) reinforces the strong linear correlation between the two sets of results.

All three statistical approaches consistently indicated the absence of significant differences between the results obtained with the two analytical methods.

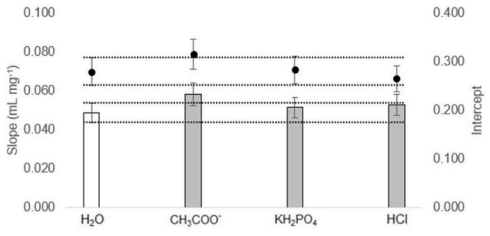


Fig. 6. Comparison between the calibration curve parameters (slope and intercept) obtained with standard solutions prepared in different matrices: deionized water (standard calibration curve) and those derived from standard solutions prepared with: acetate (pH = 4.6), phosphate (pH = 8.0), and hydrochloric acid (pH = 3.0); horizontal dashed lines represent a 10% deviation.

Table 4
Comparison of the results obtained with the developed flow system (FIA) (proposed method) and batchwise procedure (reference method) by calculating the relative error percentage (RE).

Samples ID	Matrix	Proposed method* ± SD (mg mL^{-1})	Reference method* ± SD (mg mL^{-1})	RE (%)
A1	H_2O	1.63 ± 0.13	1.54 ± 0.08	5.8
A2	H_2O	1.12 ± 0.06	1.28 ± 0.06	-12.5
A3	H_2O	1.36 ± 0.27	1.43 ± 0.07	-4.9
A4	H_2O	1.39 ± 0.11	1.54 ± 0.08	-9.7
A5	H_2O	1.24 ± 0.21	1.33 ± 0.07	-6.8
A6	H_2O	2.03 ± 0.06	1.87 ± 0.09	8.6
A7	H_2O	1.52 ± 0.09	1.41 ± 0.07	7.8
A8	H_2O	1.34 ± 0.02	1.23 ± 0.06	8.9
A9	H_2O	1.14 ± 0.01	1.17 ± 0.06	-2.6
A10	H_2O	2.00 ± 0.14	2.09 ± 0.10	-4.3
A11	H_2O	1.25 ± 0.03	1.32 ± 0.07	-5.3
B1	HCl	4.18 ± 0.62	4.59 ± 0.23	-8.9
B2	HCl	4.72 ± 0.62	4.59 ± 0.23	2.8
B3	HCl	4.68 ± 0.23	4.59 ± 0.23	2.0
B4	CH_3COO^-	2.96 ± 0.11	2.81 ± 0.14	5.3
B5	$H_2PO_4^-$	4.12 ± 1.85	3.98 ± 0.20	3.5
B6	CH_3COO^-	2.03 ± 0.11	2.11 ± 0.11	-3.8
B7	$H_2PO_4^-$	1.76 ± 0.39	1.71 ± 0.09	2.9
B8	HCl	5.66 ± 0.28	5.44 ± 0.27	4.0

* Analysis performed in triplicate. SD; standard deviation; A: dried hydrolysate sample, dissolved in deionized water (H_2O). B: hydrolysate sample obtained during the hydrolysis process;H_2O; water matrix; CH_3COO^-; acetate matrix; $H_2PO_4^-$; phosphate matrix; HCl; hydrochloric acid matrix.

4. Conclusion

A spectrophotometric FIA method, for the expeditious and low reagent consumption quantification of total soluble protein content in by-products protein hydrolysates, was successfully developed, demonstrating its potential as a robust and efficient analytical tool for the food industry. The optimized methodology not only enhances precision in quality control but also supports comprehensive nutritional profiling, reinforcing its applicability in an industrial context. As far as we know, only one study reported the development of a FIA system devoted for total protein quantification, specifically applied to clinical analysis (Shideler et al., 1980). When compared, the main difference of the herein developed method relies on the reagent consumption whereas the proposed analytical tool uses significantly less Biuret reagent as this is not continuously flowing through the system but only introduced simultaneously with the sample.

A key advantage of the proposed FIA method lies in its significant reduction in reagent consumption when compared to the reference method. The 75% reduction in Biuret consumptions highlight the method's sustainability without compromising accuracy. The higher AGREE score obtained for the FIA method (0.76 vs. 0.58 for the reference) further highlights its potential as a sustainable analytical solution for industrial applications.

By minimizing chemical waste, the FIA approach aligns with green chemistry principles and circular economy frameworks, promoting environmentally responsible analytical practices.

Beyond reagent savings, the FIA method demonstrated a substantial improvement in analysis throughput (20 sample analysis h^{-1}), whereas the conventional method requires 30 min per sample analysis and the herein developed FIA system reduces the total analysis time to just 3 min

(triplicate analysis),. This tenfold decrease in the analysis time makes FIA particularly suited for high-throughput applications and real-time monitoring, addressing the growing demand for rapid and accurate analytical solutions in the food industry.

Additionally, the FIA system exhibited robustness, as no significant interferences were observed when different matrices were tested, including acetate, phosphate and hydrochloric acid matrices. This confirms the method's applicability across a range of sample conditions without compromising accuracy or performance.

CRediT authorship contribution statement

Ribas Tânia: Writing – review & editing, Validation, Methodology, Conceptualization. **Raquel Teixeira:** Writing – original draft, Methodology, Investigation. **Rangel António:** Writing – review & editing, Supervision, Conceptualization. **Manuela Pintado:** Writing – review & editing, Supervision, Funding acquisition. **André Almeida:** Resources, Project administration, Funding acquisition.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT (OpenAI) and Copilot to assist with language polishing. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. Raquel Teixeira reports was provided by European Union. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2026.109178.

Data availability

Data will be made available on request.

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