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Dual-range colorimetric protein quantification in whey and WPC using a smart polymeric film and RGB imaging

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ABSTRACT

Whey is an important dairy by-product whose protein content must be accurately determined for quality control and fraud detection. Conventional nitrogen-based methods (e.g., Kjeldahl) require laboratory equipment and overestimate protein in the presence of non-protein nitrogen compounds. Here, we report a rapid analytical approach based on a smart polymeric film (F_{RGB}) prepared by diazotisation–azo coupling chemistry. The film reacts with aromatic residues of whey proteins, producing a colour change quantifiable from a smartphone image. The sensor displays a dual-range response: the blue channel provides high sensitivity at low concentrations, whereas the green channel remains linear at high concentrations, enabling direct analysis of whey protein concentrate without dilution. The method showed excellent linearity ($R^2 = 0.99$) in whey and commercial WPC over 0–120 mg·mL^{-1}, with a detection limit of 0.284 mg·mL^{-1} and recovery rate of 94 ± 1.46%. Non-protein nitrogen compounds did not interfere, allowing rapid screening and preliminary authenticity assessment of whey-derived products.

1. Introduction

Dairy products play a central role in global nutrition. In 2023, worldwide milk production reached approximately 965.7 million tons, of which about 160.8 million tons were produced in Europe (Dairy Market Review, 2023; University of Wisconsin–Madison Division of Extension, n.d.). A large fraction of this milk is transformed into cheese, butter or yogurt, generating whey as the main by-product. Cheese manufacture produces approximately 9–10 L of whey per kilogram of cheese and, due to its high organic load, its disposal represents an environmental concern (Besediuk et al., 2024; Iseppi et al., 2017). Once considered waste, whey is now recognised as a valuable resource because of its high-quality proteins and bioactive components, and its valorisation contributes to sustainability and circular economy strategies (Besediuk et al., 2024).

Among whey-derived products, Whey Protein Concentrates (WPC) are widely produced through ultrafiltration and diafiltration followed by pasteurisation, evaporation and drying (Mollea et al., 2013; Pires et al., 2021). WPC typically contains 25–80% protein (dry basis), with commercial grades such as WPC-35 and WPC-80, whereas Whey Protein Isolates (WPI) contain ≥90% protein according to widely adopted industry standards (ADPI). Because product classification, labelling and commercial value depend directly on the declared protein percentage, accurate protein quantification is essential for regulatory compliance, quality control and detection of economically motivated adulteration and for commercial grading and pricing of whey protein ingredients (Hayes, 2020).

Protein determination in whey and WPC is traditionally performed using nitrogen-based reference methods such as Kjeldahl and Dumas, or spectrophotometric assays including Bradford, Lowry, Biuret and A280 measurements (Bradford, 1976; Ennis, 1957; Giles et al., 2025; Nielsen, 2017). Kjeldahl and Dumas quantify total nitrogen and convert it into protein content using a fixed nitrogen-to-protein conversion factor, assuming that all nitrogen originates from proteins. Consequently, they cannot distinguish true proteins from non-protein nitrogen compounds (Hueso et al., 2022; Nobari Moghaddam et al., 2024; Saxton & McDougal, 2021b). Conversely, chromogenic assays require dilution, controlled conditions and laboratory instrumentation, which limits their

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applicability to highly concentrated WPC samples and prevents rapid analysis.

Beyond final product certification, protein concentration must also be monitored during industrial processing. During ultrafiltration and diafiltration, operators need to determine when the target concentration factor has been reached and whether blending or further concentration is required. In practice, reference methods such as Kjeldahl or Dumas are performed off-line in quality control laboratories and results are obtained hours after sampling. As a result, processing decisions are typically based on indirect parameters such as conductivity or refractive index rather than direct protein measurements.

Therefore, a practical analytical gap exists: there is currently no simple, direct chemical method capable of assessing protein concentration in both dilute whey streams and highly concentrated WPC solutions on-site, without dilution or specialised instrumentation.

Despite their widespread use, current protein quantification methods present important limitations when applied to whey protein concentrates (WPC). Nitrogen-based assays such as Kjeldahl quantify total nitrogen rather than other methods, leading to overestimation in the presence of non-protein nitrogen compounds (e.g., urea, ammonium salts, or melamine) (Hueso et al., 2022; Nobari Moghaddam et al., 2024; Saxton & McDougal, 2021b). In contrast, spectrophotometric and chromogenic assays (Bradford, Lowry, or Biuret) are solution-phase methods with limited linear ranges, typically requiring multiple dilution steps for concentrated WPC, a limitation especially relevant for highly concentrated WPC matrices and one that introduces cumulative errors and reduces practicality for rapid screening (Bradford, 1976; Ennis, 1957; Giles et al., 2025; Nielsen, 2017).

Although portable and polymer-based sensing strategies have been reported for food analysis, most operate in diluted solutions and within relatively narrow dynamic ranges; therefore, they still require pretreatment or dilution for protein-rich matrices such as WPC and often rely on controlled optical conditions or laboratory equipment. This limitation is particularly relevant in industrial contexts, where rapid decisions must be made by minimally trained personnel. In parallel, alternative strategies proposed for the authentication of whey protein supplements include mid- and near-infrared spectroscopy and smartphone imaging combined with chemometric or machine-learning analysis (Amamcharla & Metzger, 2019; De Lourdes Mendes Finete et al., 2013; Saxton & McDougal, 2021a; Tang et al., 2025). These approaches rely on multivariate pattern recognition rather than on a direct chemical interaction with protein functional groups. While effective for classification, they rely on calibration models derived from global spectral or visual fingerprints that may be sensitive to formulation variability and additives, and they do not intrinsically overcome the core limitation of nitrogen-based assays in distinguishing intact proteins from non-protein nitrogen compounds.

Whey protein products remain susceptible to economic adulteration because commercial value is directly linked to declared protein content. When protein is estimated through total nitrogen methods, different adulterants may artificially increase apparent protein content, including free amino acids (e.g., glycine and leucine), urea, ammonium salts, and other nitrogen-rich compounds, while carbohydrate fillers such as maltodextrins may dilute the product. A well-known example is melamine (2,4,6-triamino-1,3,5-triazine), historically associated with major food safety incidents (Li et al., 2019). Therefore, analytical approaches that selectively respond to protein-specific chemical structures rather than nitrogen content are desirable for whey protein authentication and fraud screening.

In this context, rapid colorimetric approaches are attractive because of their simplicity, low cost, and suitability for on-site measurements (Bordbar et al., 2018; Jia et al., 2023; Zheng et al., 2022). Our research group has developed polymer-based sensors for food matrices such as beetroots, wines, honey, and processed meats (Gaona-Ruiz et al., 2024, 2025; González-Ceballos et al., 2021; Guembe-García et al., 2022; Guirado-Moreno et al., 2023; Siripongpreda et al., 2020; Valdez et al., 2019). Building upon this experience, we developed a method to quantify protein concentration in whey and derivatives such as WPC using a smart polymeric film: an acrylic polymer bearing pendant amino groups that can be diazotised in the solid state to generate benzene diazonium functionalities. Upon exposure to whey proteins, covalent attachment via azo coupling produces a distinct colour change (Fig. 1).

The objective of the present work is to provide a complementary rapid screening approach for whey-derived products and protein concentrates. The proposed diazonium-functionalised film (F_{RGB}) provides a rapid on-site indication of relative protein concentration based on a single immersion step and smartphone image analysis and is intended as an accessible in-situ control tool for production decisions, raw-material reception, formulation adjustment and authenticity verification. Importantly, it is designed to operate across both dilute whey streams and highly concentrated WPC samples without prior dilution, and to support the detection of dilution or amino-acid-based adulteration.

2. Experimental

2.1. Materials

All materials and solvents were commercially available and used as received unless otherwise indicated. The following materials and solvents were used: 1-vinyl-2-pyrrolidone (VP) (Acros Organic, 99%), methyl methacrylate (MMA) (Merck, 99%), 4-aminostyrene (SNH_2) (TCI, 98%), urea (TCI, ≥99.0%), melamine (Sigma-Aldrich, 99%), ammonium nitrate (Sigma-Aldrich, ≥99%), methanol (VWR-Prolabo, 99.9%), ethanol (VWR, 99.96%), hydrochloric acid (VWR, 37%), sodium nitrite (Alfa Aesar, 98%), azo-bis-isobutyronitrile (AIBN, Aldrich, 98%), ethylene glycol dimethacrylate (E) (Aldrich, 98%),), β-lactoglobulin (β-Lg) from bovine milk (Merck, Approx. 90%), α-Lactalbumin from bovine milk (Merck, Type I, ≥85%), Bio-Rad Protein Assay Dye Reagent Concentrate (BioRad), commercial rennet 1:20000 (Nievi, Productos Nievi, S.A., Spain). Commercial milks: whole milk (Pascual, Calidad Pascual S.A.U, Spain), fresh whole milk (Hacendado, Mercadona S.A., Spain). Whey protein supplements: Evowhey Protein 2.0, coffee with milk flavour (HSN®, HSNstore S.L., Spain), Evowhey Protein 2.0, biscuit flavour (HSN®, HSNstore S.L., Spain).

2.2. Instrumentation and methods

The water-swelling percentage (WSP) of the films, which represents the amount of water absorbed when immersed in pure water at 20 °C until swelling equilibrium was reached, was calculated using the dry film weight (ωd) and swollen weight (ωs) using the following formula: $WSP = 100 \times [(\omega s - \omega d) / \omega d]$.

The thermal properties of the polymers were examined using thermogravimetric analysis (TGA) with a Q50 TGA analyser (TA Instruments, New Castle, DE, USA). Samples of 10–15 mg were tested under synthetic air and nitrogen atmospheres at a heating rate of 10 °C/min.

Differential scanning calorimetry (DSC) measurements were performed using a TA Instruments Q200 DSC analyser under a nitrogen atmosphere at a heating rate of 20 °C/min. The sample sizes ranged from 10 to 15 mg. Data collection began with a sampling interval of 0.10 s per point. Initially, the sample was stabilised at −80.00 °C before starting the first cycle of the experiment. The temperature was then increased to 10.00 °C/min until 150.00 °C, marking the end of the second cycle of the experiment. Cooling down to −80.00 °C followed at the same rate, completing the third cycle of the experiment. Finally, the temperature was increased to 350.00 °C at the same heating rate, completing the fourth cycle, which was used for the analysis of the results.

Fourier-transform infrared (ATR-FTIR) spectra were acquired using an FT/IR-4200 spectrometer (Jasco, Tokyo, Japan) equipped with a single-reflection ATR-PRO410-S accessory.

The tensile properties were evaluated using samples measuring 5

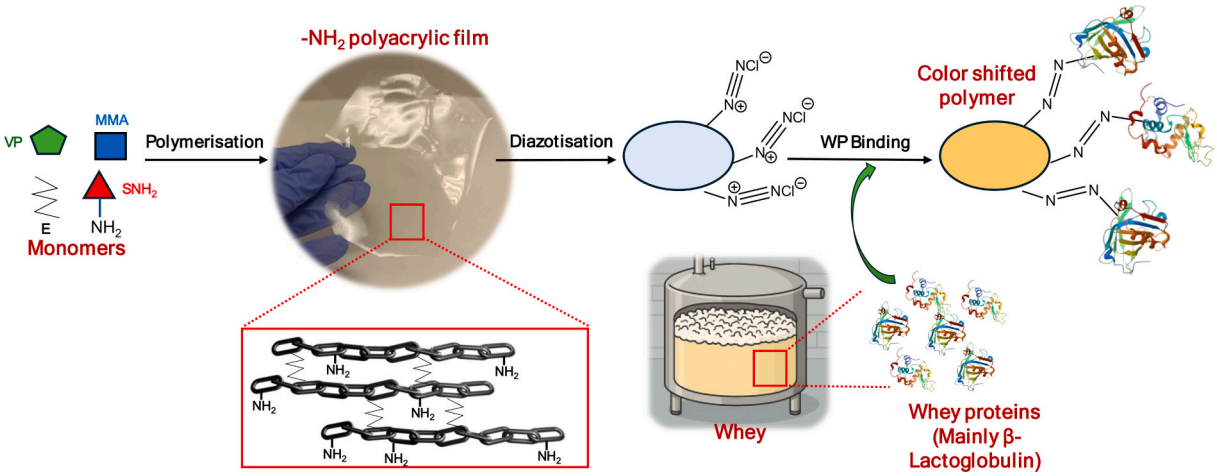

Fig. 1. Simplified scheme of film-shaped polymer sensor (F_{RGB}) synthesis and its functionality. First, a polymeric film with pendant NH_2 groups was synthesised by the bulk radical polymerisation of vinylpyrrolidone (VP), methyl methacrylate (MMA), 4-aminostyrene (SNH_2), and the cross-linking agent ethylene glycol dimethacrylate (E). Subsequently, after punching 0.6 cm of diameter disks, a diazotisation reaction was performed, yielding a material containing free benzene diazonium salts. Finally, whey proteins were covalently attached via azo coupling, resulting in a detectable colour change in the film.

mm × 9.44 mm × 0.103 mm, tested at a rate of 5 mm/min using an EZ Test Compact Table-Top Universal Tester (Shimadzu, Kyoto, Japan).

The films were polymerised between two parallel silanised glass plates separated by a calibrated 100 μm spacer, producing a uniform sheet (~7 × 7 cm). Mechanical specimens were cut from different regions of the same sheet. Thickness was measured at multiple positions using a digital micrometer, yielding an average value of 0.103 mm with minimal variation, confirming the mechanical integrity of the sensing films.

As reference protein quantification methods, protein absorbance at 280 nm (A280) was measured using a UV/Vis spectrophotometer (Hewlett Packard 8453) operated with HP ChemStation software. Measurements were performed using quartz cuvettes with a path length of 0.5 mm. The absorbance spectra of the films were recorded using a UV–Vis spectrophotometer (Hewlett Packard 8453) equipped with HP ChemStation software.

The Bradford assay, used as a comparative reference method, was performed using the Bio-Rad Protein Assay Dye Reagent, which was diluted 1:4 with distilled water. A total of 200 μL of diluted reagent and 10 μL of sample were added to each well of a 96-well microplate. Absorbance at 595 nm was measured using a PowerWave XS2 microplate reader (BioTek).

The limits of detection (LOD) and quantification (LOQ) for the three protein quantification methods (using β-Lg) were calculated using the following standard equations: LOD = 3.3 × SD/*s* and LOQ = 10 × SD/*s*, where *SD* represents the standard deviation of the blank measurements and *s* is the slope of the corresponding analytical calibration curve.

Protein quantification using the F_{RGB} sensor was carried out by immersing a fully diazotised polymer disc in the protein solution for a fixed incubation time under ambient laboratory conditions. After incubation, the discs were rinsed with distilled water to remove unbound material and dried at room temperature. Images of the films were acquired using a smartphone camera under constant lighting conditions and analysed using RGB colour extraction software. The analytical signal corresponded to the variation in the selected RGB channel intensity relative to a blank disc. The blank consisted of fully diazotised F_{RGB} discs subjected to the complete analytical protocol (washing, incubation time, image acquisition and RGB analysis) but incubated in distilled water instead of protein solution. Consequently, the calculated LOD reflects the intrinsic analytical variability of the activated sensing material and the image-analysis procedure rather than the optical background of a non-reacted polymer.

All calibration measurements were performed in triplicate and expressed as mean ± standard deviation.

2.3. Preparation of acid and sweet whey

Whey obtained by acid precipitation (acid whey) was prepared by skimming whole milk via centrifugation (8000 rpm, 20 min, 4 °C). The resulting skimmed milk was acidified to pH 4.6 using 2 N HCl to precipitate caseins, which were subsequently removed by centrifugation (1800 rpm, 15 min, 20 °C). Whey obtained by enzymatic precipitation (sweet whey) was prepared by adding 0.25 mL of commercial rennet to 500 mL of fresh whole milk that had been previously heated to 50 °C. The mixture was left undisturbed for 3 h to allow curd formation. The curd was then broken to extract more whey and left to rest for 15 min. Finally, the sample was centrifuged at 4000 rpm for 15 min at 4 °C. No additional fat separation step was applied, as the residual lipid fraction remaining after curd removal was minimal and the sensing mechanism targets soluble whey proteins.

The preparation temperatures correspond to standard procedures for acid and rennet whey production. Although mild heating during rennet coagulation may induce limited conformational relaxation of whey proteins, they remain soluble and chemically intact. Because the whey preparation conditions do not induce structural modifications in the proteins that affect the accessibility of their aromatic residues, the interaction with the F_{RGB} polymer remains unchanged and no analytical bias is introduced in the measurements.

2.4. Design and synthesis of film-shaped polymer sensor (F_{RGB})

Three different formulations of the sensory film were prepared, maintaining the structural monomers VP and MMA while varying the crosslinker (E) molar percentage (0.1 and 1 mol%) and the sensor monomer SNH_2 molar percentage (1 and 10 mol%). These compositional variations were designed to achieve an optimal balance between mechanical properties for adequate handling (both in the dry state and after swelling), a strong colorimetric response, and swelling percentage to facilitate proper interaction between proteins and the polymer. Specifically, the three formulations used to prepare 1.8 g films were: (i) 49.5/49.5/1 in molar proportion of VP (926 mg, 8.33 mmol), MMA (833 mg, 8.33 mmol), and SNH_2 (20 mg, 0.17 mmol), respectively, with 0.1 mol% of E (3.3 mg, 16.6 μmol); (ii) 45/45/10 in molar proportion of VP (832 mg, 7.5 mmol), MMA (750 mg, 7.5 mmol) and SNH_2 (198 mg, 1.7 mmol) respectively, with 0.1 mol% of E (3.3 mg, 16.6 μmol); and (iii) 45/45/10 in molar proportion of VP (818 mg, 7.36 mmol), MMA (736

mg, 7.36 mmol) and SNH_2 (195 mg, 1.6 mmol), respectively, with 1 mol % of E (32.4 mg, 0.16 mmol). The synthesis of these polymers was performed via bulk radical polymerisation, using AIBN (17.8 mg, 0.11 mmol) as a thermal initiator at 60 °C overnight, as described in previous works (Vallejo-García et al., 2023a; Vallejo et al., 2025). The solid films were demoulded and subjected to sequential solvent exchange to remove unreacted monomers and condition them for protein measurement. It was first immersed in distilled water for 15 min, then gradually transitioned to 100% acetone, and subsequently back to distilled water. This gradual exchange prevents abrupt swelling changes, which could lead to film rupture.

Once the film was synthesised, a solid-phase reaction was performed. First, 0.6 cm discs were punched from the film and diazotised through the –NH_2 groups provided by the SNH_2 monomer, leading to the formation of benzene diazonium salts and rendering F_{RGB}. For the diazotisation, ten 0.6 cm discs were immersed in 10 mL of distilled water containing 1 mL of concentrated HCl (37%) and 50 mg of sodium nitrite. The reaction was carried out at room temperature without stirring, with variable reaction times ranging from 5 to 60 min. After diazotisation, the films were washed with water three times to remove excess reagents and by-products. The resulting diazotised discs were then used in the subsequent protein incubation experiments.

2.5. Protein measurement procedure using the F_{RGB} method

The reaction between diazonium groups in the polymer and some nucleophilic amino acid residues of proteins (His, Tyr), induces a detectable colour change in the polymer matrix, which serves as the analytical signal. The same measurement protocol was applied to all tested protein systems: β-Lg standard solutions, whey obtained from acid and enzymatic milk precipitation, and commercial WPC. Each 0.6 cm diazotised F_{RGB} disc was immersed in 1 mL of protein solution and incubated statically at room temperature (22 ± 2 °C) for 15 min.

After incubation, the discs were photographed using an iPhone 16 inside a backlit lightbox to ensure consistent lighting conditions and improve reproducibility. The closed light box minimises external illumination variability and standardises image acquisition. The analytical signal is obtained from calibration curves generated under fixed acquisition conditions rather than from absolute RGB values. Therefore, similarly to spectrophotometric methods, different devices would require their own calibration but would not affect the analytical principle of the method.

Protein quantification was performed using calibration curves obtained under fixed acquisition conditions (same device, illumination and geometry). The smartphone–lightbox system therefore acts as a controlled optical reader, analogous to a spectrophotometric instrument operating under fixed settings. Quantification is based on relative variations in colour intensity rather than on absolute RGB values; consequently, each device–illumination configuration requires independent calibration, while the analytical principle remains unchanged.

Colour analysis was performed using the Colorimetric Titration app (Gaona-Ruiz et al., 2024), which extracts mean pixel intensity values from defined regions of interest (see SI-VIDEO). Colour parameters were extracted from two digital colour spaces: RGB (commonly used in electronic imaging) and HSV colour spaces. Additionally, two combinations of RGB values were evaluated, resulting in a total of eight digital colour variables analysed (Guembe-García et al., 2022). The analytical response was selected objectively: among the evaluated colour variables, the parameter showing the highest calibration linearity and the lowest residual error was chosen as the quantitative signal.

2.6. Optimisation of diazotisation time and pH on the sensor response

To optimise the diazotisation time and sample pH on the polymer's response to protein was evaluated. Discs with a diameter of 0.6 cm underwent diazotisation as described in Section 2.4 for varying durations (5, 15, 30, and 60 min), followed by three washing steps, and a 15-min incubation as described in Section 2.5 with acid whey at different pH values (4.6, 6.0, and 8.0).

2.7. Selectivity and non-protein nitrogen interference study

Selectivity of the F_{RGB} colorimetric response was evaluated by analysing the effect of non-protein components commonly present in whey systems and in economically adulterated protein products. Diazotised FRGB discs were used for β-lactoglobulin (β-Lg) determination in the presence of lactose (50 mg mL^{-1}).

Additionally, sweet whey samples were supplemented with representative non-protein nitrogen (NPN) compounds. Free amino acids (glycine, leucine and glutamine, 2 mg mL^{-1} each), urea and an ammonium salt were added while maintaining constant pH conditions. The concentrations were selected to simulate adulteration scenarios in which nitrogen content is artificially increased without a corresponding increase in true protein concentration.

All samples were analysed following the F_{RGB} analytical procedure described in Section 2.5, and the colour response was compared with that obtained for non-adulterated whey samples.

2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 9). Normality of the data distribution was evaluated using the Shapiro–Wilk test and homogeneity of variances using Levene's test.

Differences among experimental conditions (pH, diazotisation time and interference/adulteration assays) were analysed using one-way ANOVA followed by Tukey's multiple comparison test ($p < 0.05$). Pairwise comparisons were evaluated using Student's *t*-test ($p < 0.05$).

Calibration curves were obtained by linear regression analysis, and correlation coefficients (R^2) were calculated. Method comparison between F_{RGB} and A280 measurements was evaluated by regression analysis. Precision was expressed as relative standard deviation (RSD), and accuracy was assessed by spike–recovery experiments.

3. Results and discussion

3.1. Selection of film formulation and physicochemical characterization

The material was designed as the solid-phase substrate of a rapid screening analytical method rather than as a laboratory reference assay; therefore, ease of handling, short analysis time and compatibility with concentrated protein matrices were prioritised. The film format was selected because it enables direct immersion measurements without filtration or extraction steps (Gaona-Ruiz et al., 2025; Guembe-García et al., 2022). A balance between mechanical strength, colour response and controlled swelling was required to ensure effective contact between the polymer and whey proteins, allowing the diazotised film to react with aromatic amino acid residues of proteins, predominantly tyrosine and histidine, to form the coloured azo linkage (Vallejo et al., 2025).

Polymer composition was selected to control hydration, protein diffusion and accessibility of reactive diazonium groups. Vinylpyrrolidone (VP) provides a hydrophilic environment that promotes protein diffusion, methyl methacrylate (MMA) confers dimensional stability and limits excessive swelling, and aminostyrene (SNH_2) supplies the aromatic amine groups that are diazotised to generate the reactive sites responsible for azo coupling with protein aromatic residues. Consequently, sensing performance depends on the balance between hydration, mechanical stability and accessibility of the polymer–protein interface.

The F_{RGB} synthesis and protein interaction follow our previous procedure (Vallejo et al., 2025), with two modifications: omission of the sodium azide blocking step and reduction of protein incubation time to

15 min, simplifying the assay and shortening analysis time. To select the optimal formulation, three compositions were evaluated using acid whey adjusted to pH 8 (Section 2.5). A previously reported formulation (45/45/10 VP/MMA/SNH_2 with 0.1 mol% crosslinker E) (Vallejo-García et al., 2023a; Vallejo et al., 2025), tested here as Material 2 (Table 1), showed excessive swelling (83%) and poor dimensional stability, compromising handling and measurement reproducibility; therefore, it was discarded.

This behaviour represents a physicochemical limitation of the analytical sensing mechanism rather than a simple handling issue. Excessive swelling disrupts dimensional stability and generates uncontrolled mass transport within the polymer network, so the diffusion path length and local protein concentration at the interface vary between measurements, leading to poor signal reproducibility. This reflects the typical trade-off in solid-phase polymer sensors between functional group density and mass-transport control.

Two alternative formulations were evaluated (Table 1). Material 1 (1% SNH_2, 0.1% E) showed low swelling (38%) and good mechanical stability but produced weak colour signals due to insufficient protein binding. In contrast, Material 3 (10% SNH_2, 1% E) provided strong colour response with moderate swelling (58%), representing an appropriate balance between sensitivity and mechanical stability. Higher SNH_2 contents caused excessive swelling after diazotisation and washing, whereas higher crosslinker contents hindered protein diffusion toward reactive diazonium groups. Diazotisation introduces fixed ionic charges into the network, producing strong osmotic swelling; previous studies reported water swelling above 1500%, leading to poor dimensional stability and preventing reproducible measurements (Vallejo-García et al., 2023b). Therefore, these conditions limit analytical applicability rather than merely affecting handling.

Material 3 therefore represents a compromise between reactive group density, mechanical stability and diffusion accessibility rather than simply the highest monomer content. The sensing process is limited by protein diffusion and accessibility of reactive sites, not by the total number of functional groups.

From an analytical standpoint, this formulation maximizes signal-to-noise ratio: increasing reactive group density would increase colour intensity but also swelling-induced variability and diffusion heterogeneity, reducing quantitative reliability. Consequently, the final composition was selected based on calibration linearity and measurement reproducibility rather than colour intensity alone.

Based on these results, Material 3 (10% SNH_2, 1% E) was selected as the optimal formulation for protein detection. Mechanical testing showed adequate flexibility and strength (Young's modulus 1364 ± 136 N mm^{-2}), ensuring reliable handling during analysis.

TGA indicated sufficient stability ($T_5 = 314.62$ °C; $T_{10} = 331.59$ °C) for drying, storage and routine use. DSC revealed a glass transition temperature (Tg) of 162.70 °C, confirming that the polymer remains rigid under operating conditions. This is relevant because the F_{RGB} film acts as a solid-phase analytical substrate immersed in aqueous solutions; a Tg well above room temperature prevents deformation upon hydration and supports reproducible protein diffusion and colour response.

ATR-FTIR spectra of the film after protein exposure (Fig. 2a) showed clear modifications compared with the diazotised film. A new band at ~1540 cm^{-1} (amide II) and broadening in the 1640–1660 cm^{-1} region (amide I contribution) indicate incorporation of protein into the polymer matrix. These spectral changes confirm that the colour response arises from covalent azo coupling rather than reversible surface adsorption.

Table 1
Composition of the three tested F_{RGB} formulations. Material 1: 49.5 VP (vinylpyrrolidone) / 49.5 MMA (methyl methacrylate) / 1 SNH_2 (4-aminostyrene) // 0.1 E (ethylene glycol dimethacrylate) mol%; Material 2: 45 VP / 45 MMA / 10 SNH_2 // 0.1 E mol%; Material 3: 45 VP / 45 MMA / 10 SNH_2 // 1 E mol%. Last column shows representative images of the F_{RGB} films from the three formulations after 15 min incubation with acid whey adjusted to pH 8 with NaOH 0.1 M, keeping the same relative size proportions as in the original samples.

Material	%SNH_2	%E	WSP	Real Image
1	1	0.1	38	
2	10	0.1	83	
3	10	1	58	

3.2. Optimisation of diazotisation time and pH on the sensor response

At pH 4.6 the colour response was negligible and comparable to the control (Fig. 2b). A weak response was observed at pH 6, whereas at pH 8 the signal was clearly developed. No significant differences were detected among diazotisation times (5–60 min), indicating that diazonium formation occurs rapidly and that extending the reaction time does not influence subsequent azo coupling or colour development (Hu et al., 2016; Patai, 2010).

The pH dependence can be attributed to the reactivity of aromatic residues of proteins toward diazonium groups. At acidic pH these residues are protonated and poorly reactive, whereas under mildly alkaline conditions their nucleophilicity increases, favouring azo bond formation (R. Liu et al., 2019; Paik et al., 1979; Phillips et al., 1965).

In particular, tyrosine residues become increasingly reactive under mildly basic conditions (pH 6–8), promoting azo coupling with the diazonium groups (Godovikova et al., 1983; Y. Liu et al., 2019; Sun et al., 2021).

In addition to acid–base effects, mildly alkaline conditions may slightly relax whey protein tertiary structure (e.g., β-lactoglobulin), increasing solvent exposure of aromatic residues and their accessibility to the diazonium-functionalised surface. The higher signal at pH 8 therefore reflects both increased nucleophilicity and improved accessibility of reactive sites.

At higher pH values, aromatic diazonium groups become less stable in aqueous media and may undergo hydrolysis, reducing the effective density of reactive sites. Thus, although alkaline conditions increase reaction rate, they compromise reproducibility. pH 8 therefore represents a compromise between protein nucleophilicity and diazonium stability, providing robust sensor performance.

Mass transport within the polymer matrix facilitates the reaction but does not appear to be the dominant factor governing the signal. Diazotisation introduces ionic groups that hydrate the network and allow diffusion of soluble whey proteins toward the reactive sites. The higher response observed at pH 8 is therefore mainly attributed to the increased nucleophilicity of aromatic residues and faster azo coupling. Because calibration and sample measurements were performed under identical pH conditions, these effects do not bias quantification but rather improve reaction efficiency and reproducibility.

3.3. Linearity calibration with β-Lg using F_{RGB}, Bradford, and A280 methods

β-Lg is the predominant protein in whey, representing approximately 55–65% of its total protein content and its concentration is about 3–4 mg mL^{-1} (Gołębiowski et al., 2020; Le Maux et al., 2014; Pires et al., 2021). The isolated protein was used to validate the protein-specific response of the sensor. This validation supports the use of the method for analysing whey and concentrated whey protein samples, ensuring that the sensor response is due to protein interactions. β-Lg was diluted in Milli-Q water to concentrations ranging from 1 to 8 mg mL^{-1} for the A280 and F_{RGB} methods, and from 0 to 1.2 mg mL^{-1} for the Bradford

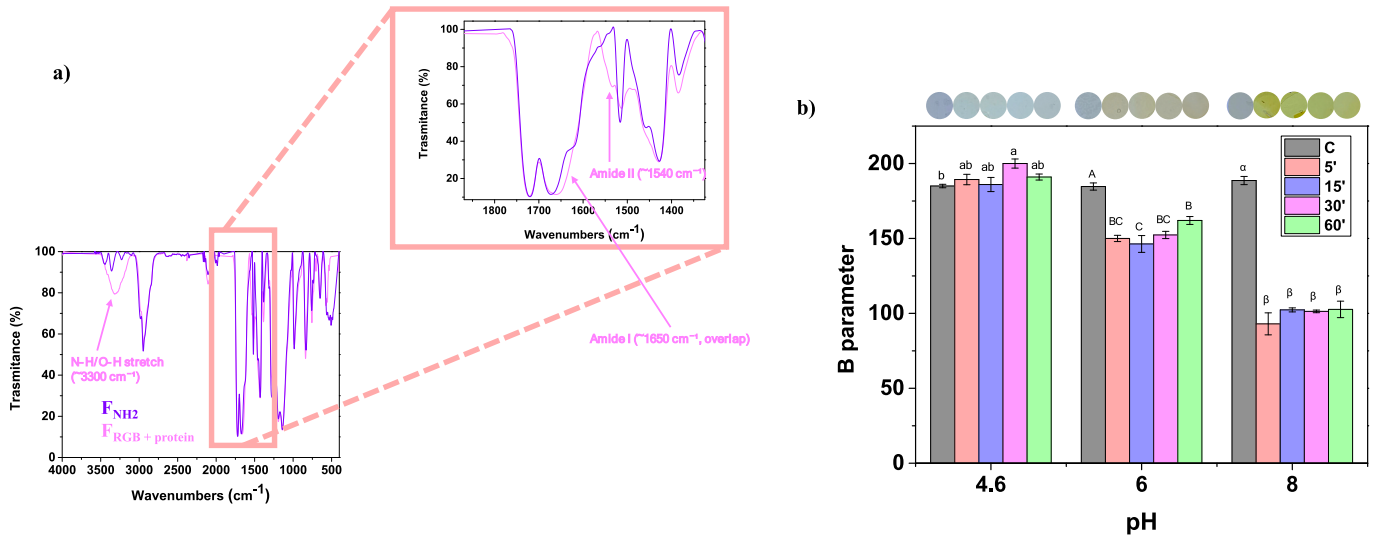

Fig. 2. a) ATR-FTIR spectra showing protein incorporation. After incubation, the film exhibits the appearance of an amide II band (~1540 cm^{-1}) and a band broadening in the 1640–1660 cm^{-1} region associated with the amide I contribution, partially overlapped with the vinylpyrrolidone carbonyl vibration. These spectral changes confirm protein incorporation into the polymer matrix. b) Diazotisation time and pH optimisation. The Y-axis represents the B parameter of the F_{RGB} disks diazotised for different times (5, 15, 30, and 60 min) and subsequently incubated with acid whey adjusted to three different pH values (4.6, 6, and 8) using 0.1 M NaOH. C corresponds to the control films incubated with distilled water adjusted to each pH. Real film images at each time and pH are shown. Bars represent the mean of three replicates ± SE. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. Different letters above the bars indicate statistically significant differences among treatments within the same pH group ($p < 0.05$).

method due to its limited linearity above this concentration. Dilutions were then measured using the Bradford protocol, A280, and F_{RGB} method.

As expected, a linear trend was observed in the calibration curves of β-Lg measured by A280 and the Bradford assay (A595) (Fig. 3a and b, respectively), both of which are widely established methods for protein quantification (Bradford, 1976; Ennis, 1957; Simonian, 2002). Notably, the RGB-based method also showed a strong linear correlation with

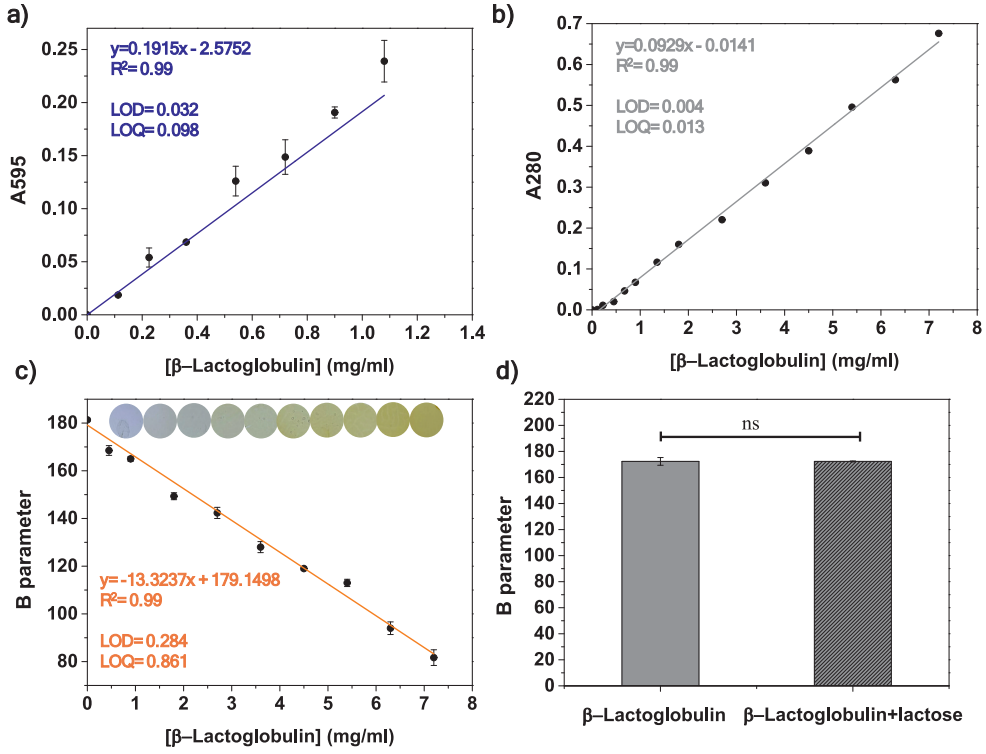

Fig. 3. a) Calibration line of β-lactoglobulin (β-Lg) (mg mL^{-1}) using the Bradford method. 200 μL Bio-Rad Protein Dye diluted 1:4 with 10 μL sample. b) Calibration line of β-Lg (mg mL^{-1}) using the A280 method with 0.5 mm path-length quartz cuvettes. c) Calibration line of β-Lg (mg mL^{-1}) using the F_{RGB} method; the Y-axis represents the B parameter, and real film images at each concentration are shown. d) Interference assay of the F_{RGB} method with lactose (50 mg mL^{-1}). The Limit of Detection (LOD) and Limit of Quantification (LOQ) values for each method are also displayed. Error bars represent the standard deviation of triplicate measurements ($n = 3$). In the A280 panel, the experimental variability is smaller than the marker size and therefore the error bars are not visually distinguishable. Statistical significance was assessed using Student's *t*-test ($p < 0.05$). "ns" denotes non-significant differences.

protein concentration, reaching an R^2 value of 0.99 for the B parameter (Fig. 3c). This behaviour can be attributed to the increasing formation of azo bonds between the diazotised polymer and aromatic amino acids (primarily tyrosine and histidine) present in the protein structure (Sahal et al., 2002). As the protein concentration increases, more reactive sites are available for the coupling reaction, leading to a more intense chromogenic response on the polymer surface, which is then effectively captured by digital colour analysis (Ma et al., 2023; Vallejo et al., 2025).

As shown in Fig. 3d, the potential interference of lactose was evaluated, given that it is one of the major components of whey, typically present at concentrations of approximately 50 mg mL^{-1} (Fox & McSweeney, 1997). As illustrated, the B parameter remained unchanged in the presence of lactose, indicating that even at high concentrations, lactose does not affect the colourimetric response of the sensor. Therefore, it can be considered that lactose does not interfere with protein quantification in complex matrices such as whey or commercial whey protein preparations.

3.4. Linearity in whey samples and interference study

After confirming linearity with β-lactoglobulin (β-Lg), the method was evaluated in a complex matrix using sweet whey (Section 2.3). The protein content of the whey was first determined by the Bradford assay and dilutions (0–5 mg mL^{-1}) were analysed following the F_{RGB} procedure. A strong correlation between the B parameter and protein concentration was obtained ($R^2 = 0.99$; Fig. 4a), similar to that observed with β-Lg. The response is mainly governed by β-Lg, the most abundant whey protein containing accessible aromatic residues (Kontopidis et al., 2004; Pires et al., 2021), although other whey proteins may also contribute through azo coupling. Additional structural and experimental evidence supporting the contribution of different whey proteins to the sensor response is provided in the Supplementary Information (SM-Section S2, Fig. S2). Because the reaction targets protein-bound aromatic residues, the method provides an estimate of total protein concentration rather than protein structure or conformation. As in other colorimetric protein assays, differences in individual protein response factors are averaged through calibration with a representative whey protein, enabling reliable estimation of overall protein concentration (Besediuk et al., 2024; Fraczkiewicz & Braun, 1998; Hendrix et al., 1996).

Although β-lactoglobulin dominates the response, the sensing mechanism is not protein-specific. Other whey proteins such as α-lactalbumin and bovine serum albumin also undergo azo coupling, with lower response factors due to differences in aromatic residue accessibility. Accordingly, the F_{RGB} film operates as a total protein assay in which proteins contribute proportionally to their reactivity, and calibration with a representative whey protein enables reliable estimation of overall protein concentration rather than selective detection of a single species.

The applicability of the F_{RGB} method depends on the accessibility of

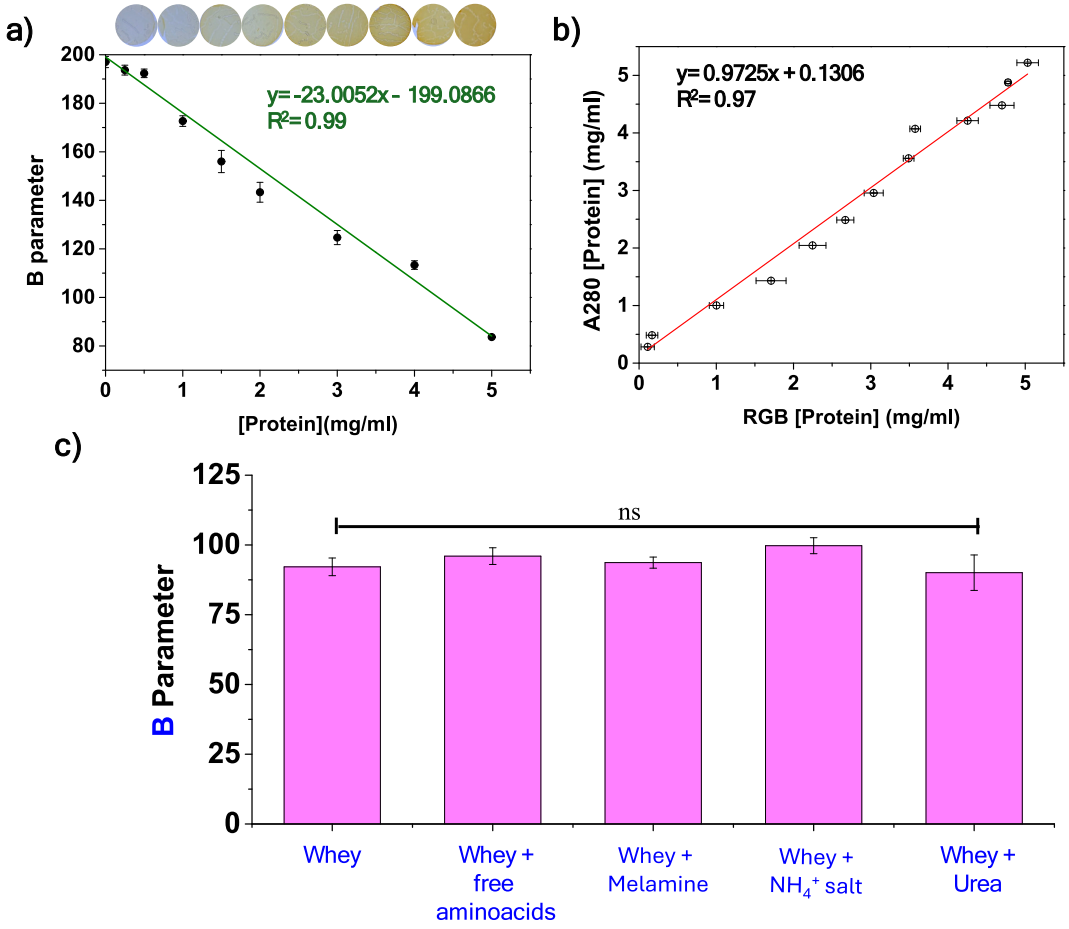

Fig. 4. a) Calibration curve of the F_{RGB} method using sweet whey. Representative film images after incubation at each protein concentration (mg mL^{-1}) are shown. b) Comparison of protein concentration (mg mL^{-1}) in whey determined by the F_{RGB} and A280 methods. c) Adulteration scenario: effect of non-protein nitrogen (NPN) compounds on the F_{RGB} response. Sweet whey (5 mg mL^{-1} protein) was supplemented with free amino acids (glycine, leucine and glutamine, 2 mg mL^{-1} each; total 6 mg mL^{-1}), urea (5 mg mL^{-1}), ammonium salt (5 mg mL^{-1}) and melamine (5 mg mL^{-1}). No significant variation in the B parameter was observed, demonstrating selectivity of the sensing mechanism toward protein-bound aromatic residues. Bars represent mean values ± SE ($n = 3$); "ns" indicates non-significant differences (Student's *t*-test, $p > 0.05$).

soluble proteins to the diazotised surface. In whey and whey-derived concentrates, proteins remain dissolved and readily interact with the film, enabling direct measurement. In contrast, complex dairy matrices such as milk, yogurt or cheese may limit diffusion and would likely require specific pretreatment and independent validation. Therefore, the present study is intentionally restricted to whey and whey-derived protein concentrates, which correspond to the intended analytical application of the method.

To further validate the method, 13 whey samples with known protein concentrations (previously quantified by the Bradford assay) were analysed using both the A280 absorbance and RGB-based approaches (SM-Section S3, Fig. S3). A linear correlation was obtained between both measurements ($R^2 = 0.97$; Fig. 4b), demonstrating good agreement with the reference method and confirming accurate protein quantification across different samples.

To evaluate performance under realistic fraud scenarios, whey samples (5 mg mL^{-1} protein) were supplemented with representative non-protein nitrogen (NPN) sources, including free amino acids (glycine, leucine and glutamine), urea and an ammonium salt, while maintaining constant pH. Free amino acids were added simultaneously (2 mg mL^{-1} each; total 6 mg mL^{-1}), exceeding the native protein concentration (~120%), whereas urea and ammonium salt were individually added at concentrations equivalent to the protein content (5 mg mL^{-1}). Despite these severe adulteration conditions, the F_{RGB} response did not change (Fig. 4c), demonstrating that the signal depends on protein-bound aromatic residues rather than total nitrogen. In contrast, nitrogen-based methods may overestimate protein content in the presence of NPN compounds (Hayes, 2020). Therefore, the F_{RGB} method can serve as a rapid preliminary authenticity screening prior to confirmatory laboratory analysis.

Unlike nitrogen-based methods such as Kjeldahl, which quantify total nitrogen regardless of its chemical form, the F_{RGB} response remained unchanged in the presence of non-protein nitrogen compounds. This confirms that the sensing mechanism is selective for protein-bound aromatic residues, enabling discrimination between true protein content and nitrogen-based adulteration.

3.5. Linearity calibration with commercial WPC using the F_{RGB} method

To evaluate applicability under real-use conditions, the F_{RGB} sensor was tested with two commercial whey protein concentrate (WPC) formulations (Evowhey Protein 2.0, biscuit and coffee-with-milk flavours; HSN). Based on the labelled protein contents (78 and 76 g/100 g, respectively), aqueous solutions covering 0–120 mg mL^{-1} were prepared and analysed using the F_{RGB} procedure. RGB parameters obtained from film images were plotted against protein concentration to generate calibration curves for both products. Because commercial WPC supplements contain non-protein additives (sweeteners, flavourings and stabilisers), calibration was performed using the real formulation rather than purified protein solutions to ensure quantitative accuracy under practical conditions.

Fig. 5 shows the response of the coffee-with-milk WPC sample. As previously observed for β-lactoglobulin and whey, the B parameter was linear only at low concentrations (0–≈16 mg mL^{-1}) and reached a plateau at higher concentrations (Fig. 5a), whereas the G parameter remained linear across the full evaluated range (0–120 mg mL^{-1}) (Fig. 5b).

To further validate the method, an independent set of 14 WPC samples was analysed using both A280 spectrophotometry and the F_{RGB} method. Protein concentrations determined by both approaches showed good agreement, yielding a linear correlation ($R^2 = 0.97$; Fig. 6c; SM-Section S3, Fig. S4). These results confirm that the polymeric film provides reliable quantitative estimation of protein concentration in commercial formulations.

Analytical performance was further assessed in terms of precision, accuracy and reproducibility. Repeatability evaluated using three independent F_{RGB} discs ($n = 3$) yielded relative standard deviation (RSD) values below 5% for the B parameter. Accuracy was determined by spike–recovery experiments performed in a commercial WPC matrix, providing a recovery of 94 ± 1.46%. Films synthesised on different days showed consistent calibration slopes and measured concentrations, indicating low inter-batch variability. Additional data are provided in SM-Section S4.

The biscuit-flavoured WPC exhibited comparable calibration behaviour (Fig. 6), with early saturation of the B parameter and sustained linearity of the G parameter. The similarity of calibration profiles for both formulations indicates that flavouring additives do not interfere with the sensing response.

The F_{RGB} sensing mechanism is governed by a heterogeneous covalent azo-coupling reaction rather than by adsorption equilibria. Proteins diffuse through the aqueous phase to the polymer interface and react irreversibly with diazonium groups, whereas low-molecular-weight additives present in commercial WPC (sweeteners, flavourings or surfactants) lack activated aromatic residues and therefore cannot

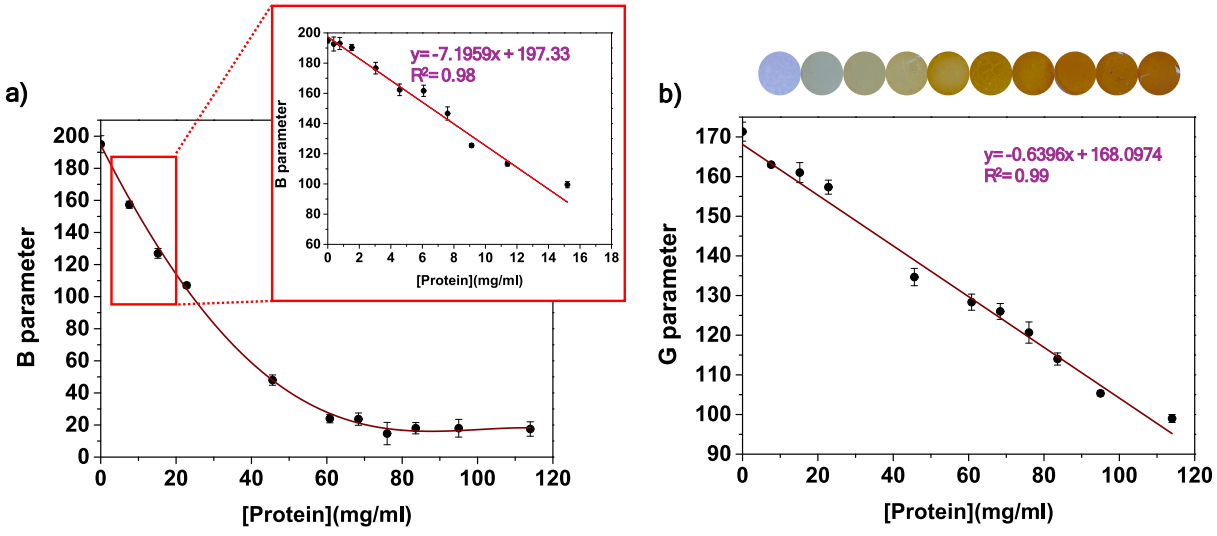

Fig. 5. a) Calibration curve of Evowhey Protein 2.0 HSN (coffee with milk flavour) using the F_{RGB} method, plotting the B parameter from image analysis. b) Calibration line of Evowhey Protein 2.0 HSN (coffee with milk flavour) using the F_{RGB} method, plotting the G parameter. Real film images at each concentration of the commercial formula are also presented.

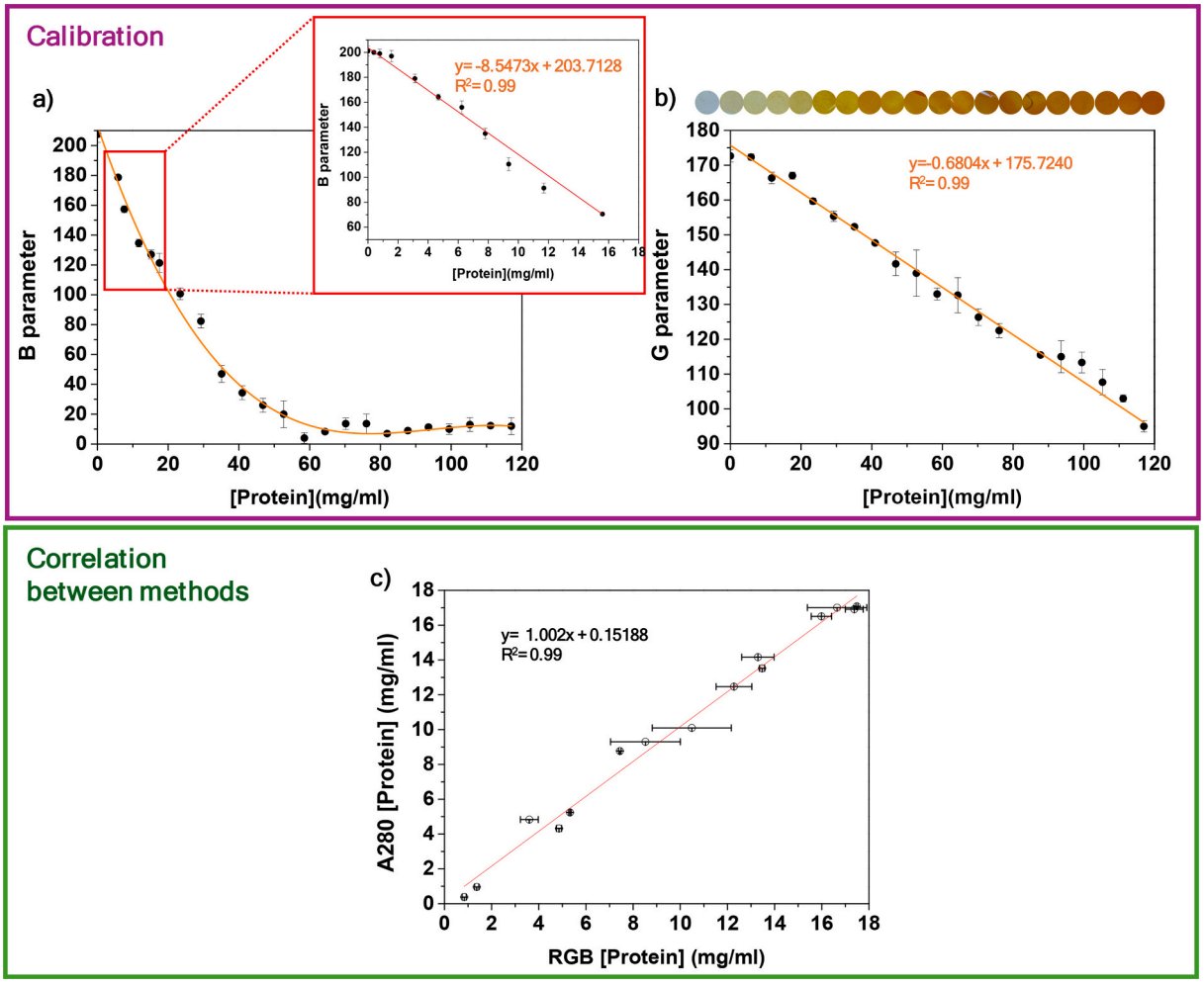

Fig. 6. a) Calibration curve of Evowhey Protein 2.0 HSN (biscuit flavour) using the F_{RGB} method, plotting the B parameter from image analysis. b) Calibration line of Evowhey Protein 2.0 HSN (biscuit flavour) using the F_{RGB} method, plotting the G parameter. Real film images at each concentration of the commercial formula are also shown. c) Protein concentration (mg mL^{-1}) determined by the F_{RGB} and A280 methods in Evowhey Protein 2.0 HSN (biscuit flavour).

participate in the reaction. Consequently, these components do not chemically compete with proteins for sensing sites, explaining the comparable calibration behaviour observed across formulations.

As the azo-coupling reaction progresses, the film colour evolves from bluish to red tones. The absorption band of the azo chromophore first develops in the blue region of the visible spectrum (~450 nm), producing a rapid decrease and early saturation of the B channel. At higher protein concentrations the absorption extends toward longer wavelengths (~500–550 nm), while the G channel remains responsive. Consequently, the analytical response shifts from the high-sensitivity B channel at low concentrations to the G channel at higher concentrations, effectively extending the dynamic range of the method. Absorbance spectra of the reacted films (SM-Section S1, Fig. S1a) confirm this behaviour and demonstrate that the B-channel plateau originates from optical saturation rather than depletion of reactive diazonium sites.

The F_{RGB} material therefore exhibits a dual-range analytical behaviour in which two colour channels provide linear responses in complementary concentration domains. The B channel enables sensitive quantification of dilute protein solutions, whereas the G channel allows direct measurement of concentrated WPC without dilution, expanding the working range of the method for on-site quality control.

Conventional colorimetric assays rely on a single analytical signal and therefore require dilution when analysing concentrated samples due to photometric saturation. In contrast, the F_{RGB} approach exploits two optical channels derived from the same chromogenic reaction. This strategy converts a saturable response into a multi-range quantitative measurement and enables direct analysis of both dilute whey streams and highly concentrated WPC solutions.

The method can also be positioned relative to other portable protein detection approaches. Simple colorimetric strips are rapid but typically semi-quantitative, whereas portable spectrometers and electrochemical sensors offer higher sensitivity at the cost of dedicated instrumentation and operational complexity. The F_{RGB} method provides quantitative analysis using only a polymeric film and a smartphone image, with minimal sample preparation and very low cost per test. Although its detection limit (~0.284 mg mL^{-1}) is higher than laboratory reference techniques, it matches the concentration range encountered in whey and WPC and is therefore intended as a rapid screening and process-control tool rather than a replacement for laboratory methods.

Finally, the operational characteristics of the material were considered. The precursor films are stable during storage at room temperature when protected from light, and diazotisation is performed immediately before use to ensure reactivity of the diazonium groups. Because the sensing reaction is irreversible, the films are single-use; however, the material cost is low (~0.05–0.10 € per test) and no specialised instrumentation is required beyond image acquisition, supporting routine on-site screening applications.

4. Conclusions

A polymeric film sensor based on diazonium–azo coupling (F_{RGB}) was developed for rapid protein quantification in whey and whey protein concentrates. The method operates as a solid-phase colorimetric assay read by smartphone imaging and is intended as a screening and process-control tool rather than a replacement for official laboratory methods.

The sensor showed consistent performance in real whey and commercial WPC samples and was not affected by lactose or non-protein nitrogen compounds, enabling detection of adulteration scenarios that may compromise nitrogen-based assays.

A key feature of the system is its dual-range analytical behaviour: the blue channel provides high sensitivity at low protein concentrations, while the green channel remains linear at high concentrations. This complementary response allows direct measurement of both dilute whey and highly concentrated WPC without dilution using a single sensor and a single image acquisition.

Consequently, the F_{RGB} method enables rapid, low-cost and equipment-free estimation of protein concentration directly at the point of use. The approach is particularly suited to reception control, formulation adjustment and authenticity screening in whey-derived protein products, simplifying routine analysis under real production conditions. Because the sensing mechanism relies on covalent reaction with aromatic residues common to many proteins, the methodology could potentially be extended to other protein-containing matrices through matrix-specific calibration and optimisation of film composition and accessibility.

CRediT authorship contribution statement

J. Lucas Vallejo-García: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Miriam Trigo-López:** Writing – review & editing, Writing – original draft, Supervision. **Saturnino Ibeas:** Resources, Formal analysis, Data curation. **Alba Torija-López:** Methodology, Investigation, Formal analysis. **María D. Busto:** Writing – review & editing, Resources, Funding acquisition. **María C. Pilar-Izquierdo:** Writing – review & editing, Resources, Conceptualization. **Gloria López:** Writing – review & editing, Conceptualization. **Carlos Sanchez:** Writing – review & editing, Conceptualization. **Saúl Vallejos:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2026.149004.

Data availability

Open Data is available at https://riubu.ubu.es/handle/10259/5684 under the name "UBU-Polymers Research Group 05122025".

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