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"Enhancing quinoa (*Chenopodium quinoa* Willd) protein extraction: Alkaline solubilization coupled to isoelectric precipitation effects on structure, digestibility and antinutrients"

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ABSTRACT

Quinoa (*Chenopodium quinoa* Willd) has gained popularity as a plant-based protein source due to its high protein content and complete amino acid profile. However, protein extraction methods such as alkaline solubilization coupled to isoelectric precipitation (ASIP), can affect protein structure, digestibility, nutritional quality, and the composition of antinutritional factors. This study aimed to assess the effects of ASIP on the secondary structure, protein quality and antinutritional factors (ANFs) composition from three quinoa varieties. The results showed that quinoa protein isolates exhibited a decrease in random coil structures, while β-turns and β-sheets increased, as indicated by FTIR analysis. *In vitro* protein digestibility improved after protein extraction, ranging from 82.12% to 84.50%. The amino acid score ranged from 0.67 – 0.88, with Yellow quinoa protein concentrate exhibiting the highest value. Black quinoa protein isolate showed the lowest total oxalate content (105.00 mg/100g), while Red quinoa protein concentrate presented higher levels of phytic acid (2.0 g/100 g), saponins (150.0 mg/g), and total phenolic compounds (161.5 mg GAE/100g). Notably, gluten content decreased in all samples following protein extraction. Despite the presence of certain ANFs in quinoa protein isolates/concentrates, the protein quality of quinoa isolates and concentrates was not adversely affected. In conclusion, the extraction process reduced several ANFs, including lectins, oxalates, and gluten, while enhancing the overall protein quality.

1. Introduction

Plant-derived proteins have gained popularity, as a viable option for vegan, vegetarian and/or celiac individuals. Understanding the physical properties and processing behavior of plant proteins is crucial for developing new food products (Mäkinen et al., 2016; Manzanilla-Valdez, Ma et al., 2024; Nosworthy et al., 2023). Furthermore, knowledge of food digestibility and protein quality can provide insights into its behavior during human digestion.

In 2014, the FAO established that quinoa could be the crop of the future, due to its rich amino acid profile and the ability to grow in different climatic conditions. *Chenopodium quinoa* Willd. commonly known as quinoa or quinua is a pseudocereal that has been cultivated since the Inca era in the Peruvian region of South America. Quinoa has

Abbreviations: AAS, amino acid score; ASIP, alkaline solubilization and isoelectric precipitation; BV, biological value; CD, circular dichroism; DBQ, defatted black quinoa; DLS, dynamic light scattering; DRQ, defatted red quinoa; DSC, differential scanning calorimetry; DYQ, defatted yellow quinoa; EAA, essential amino acid; EPC, extractable phenolic compounds; FPLC, fast protein liquid chromatography; FTIR, fourier-transform infrared spectroscopy; IVPD, *in vitro* protein digestibility; IVPDCAAS, *in vitro* protein digestibility corrected amino acid score; PCRQ, protein concentrate red quinoa; PCYQ, protein concentrate yellow quinoa; PiBQ, protein isolate black quinoa; PER, protein efficiency ratio; RBQ, raw black quinoa; RRQ, raw red quinoa; RYQ, raw yellow quinoa; SPE, solid phase extraction; TAA, total amino acids.

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high protein content (17.7 – 20.9%) compared to cereals (6.6 – 15%), oat flour (11.5 – 15.83%), and similar to pea flour (18.27%), yet lower than soy flour (38.0%) (Pokharel et al., 2023; Ren et al., 2023; Sánchez-Velázquez et al., 2022; Sánchez-Velázquez et al., 2021; Tanger et al., 2020) and contains all EAA (essential amino acids), especially lysine and arginine (Ren et al., 2023), that are deficient in wheat and oats (Nosworthy & House, 2017; Nosworthy et al., 2017). Nowadays, quinoa is mainly cultivated in Latin America, in countries such as Argentina, Bolivia, Chile, Colombia, Ecuador, and Peru, being this last one the highest producer of quinoa with an approximate annual yield of 106,756 tons (Bazile, 2023; Dakhili et al., 2019). Moreover, the highest exporter of quinoa seeds is Bolivia, while United States of America the highest exporter (Bazile, 2023). Quinoa can endure different abiotic stresses such as drought, high salinity, and extreme rain (Dakhili et al., 2019; Manzanilla-Valdez et al., 2024). Therefore, quinoa production holds a significant potential due to its low production cost, adaptability to various climatic conditions, and high nutritional value (Angeli et al., 2020).

Research has shown that quinoa is rich in bioactive compounds, including proteins, phenolic compounds, saponins and oxalates (Maradini Filho et al., 2017; Manzanilla-Valdez, Boesch et al., 2024). The latter are also considered antinutritional factors (ANFs) along with trypsin inhibitors, tannins, lectins and phytic acid. These ANFs can negatively impact human health by inhibiting protein absorption, decreasing mineral (Mg^{2+} and Zn^{2+}) absorption, and causing bloating and diarrhea (Nosworthy & House, 2017; Manzanilla-Valdez, Ma et al., 2024). ANFs can be mitigated or eliminated by different techniques such as soaking, heating, fermentation, or germination (Nosworthy & House, 2017; Sánchez-Velázquez et al., 2021). Despite these challenges, the protein content, and the favorable amino acid profile of quinoa, make it a valuable source for protein isolation. Protein extraction involves different methods such as alkaline solubilization coupled to isoelectric precipitation (ASIP), ultrasound assisted extraction, enzymatic hydrolysis, salting in/out, ultrafiltration, among others (Mondor & Hernández-Álvarez, 2022). These methods aim to remove partially or completely undesirable compounds (Sánchez-Velázquez et al., 2021). Quinoa major proteins are 11S globulin, 7S globulin and 2S albumin, representing between 72–77% of its total protein content (Dakhili et al., 2019; Opazo-Navarrete et al., 2019).

Furthermore, quinoa grains are considered as a gluten-free option, as the percentage of glutelins is relatively low, at less than 5.0% (López et al., 2018; Martínez-Villaluenga et al., 2020). However, there is a lack of information regarding gluten content in quinoa protein isolates and concentrates, and the impact of protein extraction on glutelins (Alvarez-Jubete et al., 2009; Martínez-Villaluenga et al., 2020). Overall, different extraction methods can affect the proteiń secondary structure, functionality and overall nutritional and ANFs composition highlighting the importance of assessing the modifications of quinoa protein ingredients.

Moreover, assessing the protein digestibility of plant-based ingredients is another critical factor for protein quality evaluation. It refers to how proteases in the gastrointestinal tract can breakdown into amino acids (Shaghaghian et al., 2022). *In vitro* protein digestibility (IVPD) provides an estimate of the digestive process in humans by measuring the percentage of proteins hydrolyzed by proteolytic enzymes. This method is simple, cost-effective and offers an alternative to *in vivo* models (Rodríguez-Rodríguez et al., 2022). Additionally, theoretical calculations such as *in vitro* protein digestibility corrected amino acid score (IVPDCAAS), measures the protein quality and the amino acid composition, identifying limiting amino acids in food matrices. Furthermore, the five different theoretical protein efficiency ratio (PER) calculations assess the EAA ratio in a sample (Amza et al., 2013; Nosworthy et al., 2017, 2023).

In a previous study, the nutritional composition, ANFs and protein quality of three quinoa varieties (Black, Yellow, and Red) were evaluated (Manzanilla-Valdez et al., 2024). The analysis of ANFs revealed that oxalates (396.9–715.2 mg/100g), saponins (83.27–96.82 g/100g) and trypsin inhibitors (0.35–0.46 TUI/100 g) were the most prominent ANFs identified. Furthermore, Black quinoa exhibited the highest IVPDCAAS (34.18%) and the lowest concentrations of saponins, oxalates and phytic acid, making it the most favorable variety from a nutritional perspective. This study aimed to evaluate the impact of ASIP on quinoa proteins by examining their structural properties (using techniques such as circular dichroism, differential scanning calorimetry, Fourier-transform infrared spectroscopy, surface hydrophobicity, fast protein liquid chromatography, and SDS-PAGE), antinutritional composition (including phytic acid, anthocyanins, tannins, lectins, oxalates, saponins, trypsin inhibitors, and total phenolic content), and protein quality (assessing IVPD, amino acid profile, IVPDCAAS, PER, AAS, BV, and EAA/TAA ratios). Additionally, the study explored the presence of gluten in three quinoa varieties (*Chenopodium quinoa Willd*). By investigating how ASIP affects these parameters, the research aims to inform the development of quinoa protein ingredients with enhanced functional and nutritional qualities.

2. Material and methods

2.1. Reagents

Kit for cyanogenic glycosides was purchased from Merck MQuant® 114,417 (Gillingham, UK), and for sheep hemagglutination from Rockland Immunochemicals, Inc (USA). Antinutritional reagents and enzymes: BAPA (N-α-bemzoyl-L-arginine-4-nitroanilide), calcium chloride, catechin, chymotrypsin (EC 3.4.21.1), diosgenin, EDTA, fast blue reagent, Folin-Ciocalteu reagent, gallic acid, methyl red, potassium permanganate, trizma base, trypsin from porcine pancreas (EC 3.4.21.4) and vanillin, were purchased from Sigma-Aldrich (Dorset, UK). Dialysis cassettes were purchased from Thermo Scientific (Loughborough, UK). Acetone, acetic acid, ethanol, methanol, hexane, sulphuric acid, hydrochloric acid, formic acid, and petroleum ether all HPLC grade were obtained from Merck.

2.2. Plant materials

Black, and yellow quinoa seeds were purchased from Whole Foods Online (https://www.buywholefoodsonline.co.uk/), which were grown in Peru and Bolivia, respectively. Red quinoa seeds were obtained from Hodmedod́s British Pulses & Grains (https://hodmedods.co.uk) and were grown in the UK. Seeds were kept in original packaging at room temperature. All grains were ground into fine quinoa powder using an 8-inch laboratory hammer mill (Christy Turner, UK) and passed through a 500 nm sieve. The flour was stored in resealable antistatic plastic bags (Ant003 PAB) at room temperature (RT) (18 – 21 °C), away from the light for further use.

2.3. Defatting of quinoa flour

Quinoa flour samples were defatted for ANFs assessment following the procedure by (Sánchez-Velázquez et al., 2021) with slight modifications. Firstly, the flour was mixed with hexane in a 1:4 ratio (w/v) and stirred continuously for 60 min using a magnetic stirrer. Afterwards, the slurry was centrifuged at 5000 g for 30 min at 4 °C, the resultant supernatant was discarded, and the pellet was re-extracted twice under the same conditions (three extractions in total). Finally, the defatted quinoa flour was placed evenly on a tray and left under the fume hood overnight to remove the remnant solvent. The defatted quinoa flour was then stored in a plastic antistatic bag at room temperature for further analysis.

2.4. Quinoa protein isolation by alkaline solubilization coupled to isoelectric precipitation (ASIP)

Defatted quinoa samples were used for protein isolation (for each quinoa variety) following the method by Dakhili et al. (2019), with slightly modifications. Each sample was dispersed in NaOH (0.015 M) for a pH 9.5 in a ratio of 1:10 (quinoa g/ mL NaOH) and stirred for 1 h at RT (18 – 21 °C). Afterwards, the slurry was centrifuged at 15,000 g for 15 min at 4 °C. The supernatant was collected and adjusted to pH 4.5, using HCl 1 M for 1 h. Then, the supernatant was centrifuged following the same conditions. Finally, the pellet was recovered, frozen at -20 °C for 24 h and then freeze-dried for further analysis.

2.5. Protein determination

Total nitrogen content of quinoa protein isolates/concentrates were measured using an Elementar Vario Max Cube (Elementar-Straße 1, Germany) following the Dumas combustion method (AOAC International, 1995). Crude protein content of samples was calculated as total nitrogen multiplied by a conversion factor of 6.25. Results were expressed as g protein/100 g dw. For samples with low protein content, rice flour ($N = 1.35 \pm 0.04$) was used as the standard, while ethylenediaminetetraacetic acid (EDTA, $N = 9.58 \pm 0.04$) served as the standard for samples with high protein content.

2.6. Protein characterization

2.6.1. Circular dichroism (CD)

Quinoa raw flours and freeze-dried quinoa protein concentrates/ isolates were dissolved in DNA free Milli-Q-water, under stirring for 1 h at RT (18 – 21 °C) and centrifuged 10,000 g for 10 min at 4 °C. Then, soluble protein content was assessed using the Pierce™ BCA Protein Assay Kit (Thermo Scientific™, Catalog #23,225), afterwards quinoa protein extracts were adjusted at 0.2 mg protein/mL with DNA free Milli-Q-water. Afterwards, particle size was determined according to Sahin et al. (2024), by dynamic light scattering (DLS) with non-invasive back scattering (DLS-NIBS) using a Zetasizer Nano ZSU5700 (Malvern Panalytical Ltd., UK) and DTS0012 disposable cuvettes (PMMA, Wertheim, Germany). Measurements were done at 21 °C for 30 sec with a red laser output (10 mW, $\lambda = 632.8$ nm) and a detection angle of 173° backscattered light. Finally, the correlograms decay functions were processed by the Zetasizer XPLORER software v.3.2.1.

A circular dichroism (CD) spectrometer (Chirascan VX, Photphysics, United Kingdom) was used to generate secondary structure information for the samples in a liquid form. The background noise was first excluded by running the equipment with a blank cuvette, then the standard solution (dissolving media for proteins) was run to avoid the interference of the dissolving media. Afterwards, measurements were carried out with 220 μL solutions (0.2 mg/mL protein) at 20°C under constant nitrogen purge over 180–260 nm of far-UV in a 1.0 mm pathlength cuvette. The parameters were set to light bandwidth of 2 nm, step 1.0 and the scan were repeated three times by Pro-Data Chirascan Plus (Version: V4.4.2.0 Applied Photophysics Ltd., Leatherhead, Surrey, UK). The secondary structure compositions were analyzed using CDNN software (Version:2.1.0.223 Applied Photophysics Ltd., Leatherhead, Surrey, UK) (Ma et al., 2024). Finally, for interpretation BeStSel web server (https://bestsel.elte.hu/index.php, accessed in September 2024) was used for fold recognition (Micsonai et al., 2018). Chirascan Plus CD machine is funded by "Welcome-Trust, grant code: 094232″.

2.6.2. Fourier transform infrared spectra (FTIR)

Quinoa samples were analyzed on an Alpha II Bruker (Ettlingen, Baden-Wurttemberg, Germany) FTIR spectrometer system, coupled to an attenuated total reflectance (ATR) were performed between 400 and 4000 cm^{-1}, with laser and diamond crystal. Measurements were performed using ~100 mg of quinoa samples, which was placed on the surface of the ATR, and pressed with a diamond tip plunger. All measurements were performed in triplicates at RT (18 – 21 °C). Results were analyzed using OriginPro 2021 (9.8.0.200) (OriginLab Corporation, Northampton, MA, USA), and Amide I area (1700 to 1600 cm^{-1}) was analyzed by Gaussian-Lorenzian deconvolution peak (Ma et al., 2024).

2.6.3. Differential scanning calorimetry (DSC)

A DSC Q1000 TA Instruments Q Series™ (New Castle, UK) was used to determine the peak of degradation (Tp), initial peak (Ti), final peak (Tc), and the enthalpy of quinoa samples. Approximately 6 mg of each sample was accurately weighed into aluminium pans and 20 μL of distilled water was added for overnight hydration (12 h). The samples were then hermetically sealed using T-zero pans and lids (TA Instruments; New Castle, UK). A pan with distilled water was used as reference. The scanning temperature was raised from 20 to 140°C. Finally, the area underneath the peak from the endothermal curve (J/g) was calculated (Ma et al., 2024).

2.6.4. Fast protein liquid chromatography (FPLC)

A FPLC AKTA Purifier system (GE Healthcare, Uppsala, Sweden) equipped with a Superose 12 column (GE Healthcare) was used to analyse the molecular weight distribution of quinoa samples (Wang et al., 2023). Samples were extracted before gel filtration chromatography with 50 mM phosphate buffer (pH 7) containing 0.5 M NaCl in 1:10 (w/v) proportion for 30 min at RT (18 – 21 °C). The injection volume was 500 μL, and the elution buffer was 50 mM phosphate buffer (pH 7) containing 0.5 M NaCl with a flow rate of 0.5 mL/min. Elution of protein was monitored at 214 nm. Molecular masses were determined using blue dextran (2000 kDa), catalase (240 kDa), BSA (67 kDa), ovalbumin (43 kDa), ribonuclease (13.7 kDa) and cytochrome C (12.5 kDa) as molecular weight standards (Amersham Pharmacia LKB Biotechnology, Uppsala, Sweden).

2.6.7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Quinoa protein isolates were analyzed by gel electrophoresis and carried out according to the Laemmli method (Wang et al., 2023). Samples were solubilized in 1mL of Laemmli sample buffer (0.1 M Tris-Tricine, pH 6.8, 2% SDS, 5% β-mercaptoethanol and 0.025% bromophenol blue, 100 mM DTT) for reducing conditions and without DTT for non-reducing conditions (1,610,737, Bio-Rad, CA, USA), boiled for 10 min at 95°C and then centrifuged at 10,000 g for 10 min. Afterwards, samples were loaded onto a 12% CriterionTM XT Bis/Tris gel (20 μg protein per well) and run at 200 V with MES running buffer (Bio-Rad, CA, USA). Gel was stained using Bio-Safe Coomassie G-250 stain (Bio--Rad Catalogue #1,610,787, CA, USA). As a molecular marker, Precision Plus Protein™ standard (10–250 kDa, Bio-Rad Laboratories Inc., CA, USA) was used. Finally, image analysis was performed with ChemiDoc™ XRS system with Image Lab™ (Serial #721BR10638, USA).

2.6.8. Surface hydrophobicity (Ho)

Surface hydrophobicity (Ho) was measured following Mir et al. (2020) with slight modifications, using 1-anilino-8-naphthaleno-sulfonate (ANS) as fluorescence probe. Samples were adjusted to different concentrations (0.01, 0.02, 0.04, 0.06, 0.08, and 0.1 mg/mL) with PBS. Then, 5 μL of ANS was added to 1 mL of sample per concentration, and for blanks, the same concentrations were used without ANS. The fluorescence intensity was determined using a plate reader SPARK-10M® (TECAN, Switzerland), at 390 nm (excitation wavelength) and 460 nm (emission wavelength). After subtracting the blank from the samples containing ANS, the Ho was determined by calculating the initial slope of the fluorescence intensity as a function of protein concentration.

2.7. Amino acid analysis

The amino acid profiles of samples were analysed according to Wang

et al. (2023). Briefly, 2 mg of quinoa sample were hydrolysed in 6 N HCl (4 mL) at 110°C for 24 h in tubes sealed under nitrogen. Tryptophan was analysed after basic hydrolysis according to Elamine et al. (2022). Then, amino acids were determined in triplicates, after derivatization with diethyl ethoxymethylenemalonate by reverse phase-high performance liquid chromatography (RP-HPLC), using D,L-amino-butyric acid as internal standard. The RP-HPLC system (Beckman-Coulter, Inc, Fullerton, CA, ISA) consisted of a 126 solvent module, 166 detector, and IBM personal computer. Data acquisition and processing were carried out using 32 Karat 7.0 version (Beckman-Coulter). Samples (20 μL) were injected in a reversed-phase column (Novapack C_{18}, 300 mm x 3.9 mm i.d., 4 μm; Waters, Milford, MA. USA). A binary gradient was used for elution with a flow of 0.9 mL/min. The solvents used were a) sodium acetate (25 mM) containing sodium azide (0.2% w/v) pH 6.0 and b) acetonitrile. Elution was as follows: time 0.0 – 3.0 min, linear gradient from a/b (91:9) to a/b (86/14); 3.0 – 13.0 min, elution with a/b (86/14) to a/b (69:31); 30.0 – 35.0 min, elution with a/b (69:31), the column was maintained at 18 °C.

2.8. Protein quality

2.8.1. In vitro protein digestibility (IVPD)

For measuring the IVPD of quinoa protein isolates/concentrates, samples were weighed as 62.5 mg equivalents in protein, combined with 10 mL milli-Q-water and equilibrated at 37 °C pH 8.0. Then, 1 mL enzyme cocktail containing 31 mg chymotrypsin (40 units/mg protein), 16 mg trypsin (13,000 – 20,000 BAEE units/mg protein) and 13 mg protease (13 mg, > 3.5 units/mg protein) was added, and the pH recorded every 30 sec for 10 min (Nosworthy et al., 2018). The IVPD was calculated using the change in pH value over a 10 min period (delta pH_{10min}) from the initial value at pH 8.0 as follows (Eq. (1)):

$$IVPD = 65.66 + 18.10\ (d\ pH_{10min}) \tag{1}$$

Meanwhile the IVPDCAAS was calculated as a product of the amino acid score (AAS) and IVPD % (Nosworthy et al., 2018).

2.8.2. Amino acid score (AAS)

The AAS of quinoa protein isolates/concentrates (Black, Yellow, and Red) was calculated (Eq. (2)) using the amino acid results and the FAO/WHO (1985) requirement pattern.

$$\text{AAS} = \frac{mg\ of\ amino\ acid\ in\ 1g\ of\ total\ protein}{mg\ of\ amino\ acids\ in\ requirement\ pattern} \tag{2}$$

2.8.3. Biological value (BV)

The BV (%) of quinoa protein concentrates/isolates was calculated according to Sánchez-Velázquez, Ribéreau, et al. (2021) by the following formula (Eq. (3)):

$$\text{BV} = 1.09(\text{EAA index}) - 11.73 \tag{3}$$

2.8.4. Essential amino acid (EAA) on total amino acids (TAA)

The EAAI (%) was calculated according to Sánchez-Velázquez, Ribéreau, et al. (2021). This was done by dividing the EAA content by the TAA content and multiplying the result by 100.

2.8.5. Protein efficiency ratio (PER)

PER value of quinoa protein isolates/concentrates was calculated according to Sánchez-Velázquez, Ribéreau, et al. (2021), based on the following five equations (Eq. (4 -8).

$$\text{PER}_1 = -0.684 + 0.456(\text{Leu}) - 0.047\ (\text{Pro}) \tag{4}$$

$$\text{PER}_2 = -0.468 + 0.454(\text{Leu}) - 0.105(\text{Tyr}) \tag{5}$$

$$\text{PER}_3 = -1.816 + 0.435(\text{Met}) + 0.780(\text{Leu}) + 0.211(\text{His}) - 0.944(\text{Tyr}) \tag{6}$$

$$\text{PER}_4 = 0.08084(\text{Thr} + \text{Val} + \text{Met} + \text{Ile} + \text{Leu} + \text{Phe} + \text{Lys}) - 0.1094 \tag{7}$$

$$\text{PER}_5 = 0.06320(\text{Thr} + \text{Val} + \text{Met} + \text{Ile} + \text{Leu} + \text{Phe} + \text{Lys} + \text{His} + \text{Arg} + \text{Tyr}) - 0.1539 \tag{8}$$

2.9. Antinutritional assessment

2.9.1. Anthocyanins

The total content of monomeric anthocyanins was determined using the pH differential method (Zulfiqar et al., 2022). For extraction of anthocyanins, 1 g of each quinoa sample was mixed with 10 mL of 4% HCl in MeOH and left overnight with constant stirring, followed by centrifugation at 5000 g for 10 min. Samples were then diluted in 0.025 M potassium chloride (pH 1.0), and 0.4 M sodium acetate buffer (pH 4.5), and absorbance was measured for each sample at 520 and 700 nm. The total anthocyanin content was calculated as cyanidin-3-glucoside equivalents (mg CGE /L).

2.9.2. Lectins

The lectin content was determined following the extraction protocol by Manzanilla-Valdez et al. (2024), and then a semi-quantitative hemagglutination assay was used. Lectins were extracted by mixing 1 g of quinoa sample with 10 mL of 10 mM PBS, pH 7.4 for 12 h at 4 °C. The supernatant from centrifugation at 15,000 g for 30 min at 4 °C was brought to 80% ammonium salt saturation. The pellet was collected after a further centrifugation at 15,000 g for 30 min at 4 °C, resuspended with PBS (1:10, w/v) and dialyzed overnight using Slide-A-Lyzer™ G2 dialysis cassette (Thermo Scientific) against milli-Q-water and then lyophilized. The presence of lectins was analysed through their agglutination properties using a sheep hemagglutination kit (Rockland Immunochemicals, Pennsylvania, USA), according to the manufacturer's instructions.

2.9.3. Oxalates (soluble and total)

For soluble oxalates 1 g of sample was mixed with 10 mL of distilled water, while for total oxalates 1 g of sample was mixed with H_3PO_4, and incubated at 80°C for 30 min. Then, samples were cool down, and centrifuged at 14,190g for 10 min at room temperature. Finally, for soluble oxalates 1 mL was extracted and mixed with 1 mL of distilled water, while for total oxalates 1 mL was extracted and mixed with 1 mL of NaOH 3M, and pH was adjusted to 6–8. For oxalate determination the kit EnzyChrom™, EOXA-100 was used (Fukano, 2017), read at 595 nm, and calculated as follows (Eq. (9)):

$$Oxalates\ (\mu M) = \frac{ODsample - OD\ blank}{OD\ standard - ODsample}\ \text{x 500 x n} \tag{9}$$

Where:

500 is the effective concentration of the internal standard (μM) and, n is the dilution factor used.

2.9.4. Phytic acid

Phytic acid extraction was performed following the method of Manzanilla-Valdez et al. (2024), with slight modifications. Briefly, 0.5 g quinoa protein isolates/concentrates were mixed with 10 mL of 2.4% HCl and stirred for 16 h, followed by centrifugation at 4500 g. Then, the supernatant was mixed with 1 g of NaCl and stirred for 20 min, a 1 mL aliquot was taken and adjusted to a final volume of 25 mL. A 150 μL of sample was added per well to a 96 microplate well, followed by addition of 50 μL Wade reagent, and incubation at RT before absorbance measurement at 500 nm (Jenway, #6715 UV/Vis spectrophotometer). Sodium phytate was used as standard (0.03 – 0.6 μg/mL), and content was expressed as g of phytate per 100 grams of sample (g/100 g).

2.9.5. Saponins

Saponins were extracted in quinoa protein isolates/concentrates in a ratio of 1:20 in 80% methanol for 16 h (Manzanilla-Valdez et al., 2024). The samples were then centrifuged at 4150 g for 10 min, and the supernatant was collected. The pellet was resuspended with the same amount of 80% methanol and re-extracted as above, and both supernatants combined. For saponins measurement, aliquots of 200 μL were mixed with 50 μL of 80% methanol, 0.25 mL of vanillin and 2.5 mL of 72% sulfuric acid and read at 520 nm using a spectrophotometer (SPARK 10M, TECAN, Austria). Diosgenin was used as standard (0.1 – 0.5 mg/mL), and the saponins content was expressed as g of diosgenin per 100 grams of sample (mg/100 g).

2.9.6. Tannins

Tannins were extracted by mixing 0.5 g of quinoa protein isolates/concentrates and 5 mL of 4% HCl in methanol for 18 h (Osuna-Gallardo et al., 2023). The samples were then centrifuged at 4500 g for 10 min and the supernatants were collected. In wells of a 96-well plate, 50 μL of sample extract, 100 μL of 1% vanillin in methanol, and 100 μL of 10% HCl in methanol, were added and incubated for 10 min at RT. The absorbance was determined at 500 nm using a plate reader. Catechin was used as standard (0.25 – 1.0 mg/mL), and the tannin content was expressed as mg of catechin equivalent per gram of sample (mg CE/g).

2.9.7. Trypsin inhibitors

Trypsin extraction was carried out by mixing 0.5 g quinoa protein isolates/concentrates with 25 mL 0.01 M NaOH for 3 h, followed by centrifugation at 4150 g for 10 min (Liu, 2021). The supernatant was recovered and used for further analysis, and a blank (Tris-HCl buffer) was run for each sample. Trypsin inhibitory activity was measured as the residual activity using L-BAPA (Na-benzoyl-L-arginine-p-nitroanilide hydrochloride) as substrate. The absorbance was read at 410 nm and trypsin inhibition units (TIU/mg sample) were calculated (Eq. (10)) by dividing the absorbance difference of sample *versus* blank by the weight of each sample and multiplication by sample dilution (1:50).

$$Trypsin\ inhibitors\left(\frac{TIU}{mg}sample\right) = \frac{Abs\ sample - Abs\ blank}{Sample\ weight\ (mg)} * 50 \quad (10)$$

2.9.8. Phenolics quantification

TPC was determined using two assays, the commonly used Folin-Ciocalteu (FC) assay as well as the Fast Blue BB (FBBB) assay, which provides a higher accuracy to determining the actual content of polyphenols. The approach of (Pico et al., 2020) was followed with slight modifications.

For sample extraction, 1 g of quinoa protein isolates/concentrates was mixed with 8 mL acidified methanol with 0.1% formic acid (80% MeOH) for 15 min. Following sample centrifugation of 5000 g, the supernatants were removed, and the pellets mixed with 8 mL 0.1% formic acid in 70% acetone for 15 min. After centrifugation, the supernatants were combined and stored at – 80 °C, this solution was called EPC (extractable phenolic compounds). For the removal of polar interferences, SPE (solid phase extraction) cartridges from Oasis HLB 1cc (30 mg) were used. These were activated by adding 3 mL of 1% formic acid in methanol, followed by 3 mL of 1% formic acid in water. After that, 3 mL of EPC were eluted and collected in a new tube (fraction 1). Polar interferences were eluted with 1 mL of 0.05 M NaH_2PO_4 and discarded. Potentially remaining phenolic compounds that could be in the cartridges were eluted with 3 mL of 0.1% formic acid in methanol (fraction 2). Finally, fractions 1 and 2 were combined, this solution was called SPE.

2.9.8.1. Folin-Ciocalteu (FC). EPC and SPE were analyzed to determine any differences in both phenolics assays. 10 μL of sample/standard and 40 μL of Folin-Ciocalteu reagent (25%) were added to a 96 well-plate and shaken for 5 sec. Finally, 150 μL of 4% sodium carbonate was added, the solution was incubated at room temperature for 30 min in the dark. Gallic acid was used as standard (7.8 – 500 μg/mL), the plate was read at 765 nm (Fernando et al., 2022).

2.9.8.2. Fast blue BB (FBBB). FBBB reaction has been employed due to its coupling specificity to diazonium group of FBBB reagent to an aromatic ring with activating hydroxyl group (Pico et al., 2020). 200 μL of sample/standard and 20 μL of FBBB reagent (0.1%) were added to 96 well-plate and shaken for 5 sec and incubated for 1 min. Finally, 20 μL of 5% NaOH was added, the mixture was incubated at room temperature for 120 min in the dark. Gallic acid was used as standard (7.8 – 500 μg/mL), the plate was read at 420 nm.

2.9.9. Gluten analysis

Gluten quantification was carried out using a gluten Elisa kit (Morinaga, #M2103). For extraction 1 g of sample was mixed with 19 mL of sample extraction solution, and shaken overnight at room temperature, then samples were centrifuged for 20 min at 3000 g at 22 °C (Panda et al., 2015). Then samples were diluted by 20-fold with diluent I (provided by the kit). Wheat protein was used (0.78 – 25.0 ppb) as standard. Finally, the reaction was read at 450 nm, and at 600 nm. The gluten content in the samples was estimated with the following equation (Eq. (11)):

$$Gluten\ content\ (ppm) = \frac{OV * Dilution\ A * Dilution\ B * 0.85 * 1}{1000} \quad (11)$$

Where:

OV; observed value (ppb)

Dilution A; dilution for low range assay protocol.

Dilution B; dilution for low range assay protocol for overnight extraction method.

2.10. Statistical analysis

All analyses were processed using Minitab®, LLC (2024) and GraphPad Prism version 10.0.0 for windows (GraphPad Software, Boston, Massachusetts, USA). Data were evaluated using one-way ANOVA ($p < 0.05$) followed by Tukey post-hoc test. Results are presented as mean ± standard deviation, with all analyses conducted in quintuplicate.

4. Results and discussion

4.1. Protein extraction of quinoa samples

Quinoa protein isolates and concentrates were extracted from defatted quinoa flours (Black, Yellow, and Red) using the ASIP method reported by Dakhili et al. (2019). In a previous study conducted by Manzanilla-Valdez et al. (2024) the protein content of raw flours was assessed, showing values of 20.90, 19.34, and 20.25 g/100 g for RBQ (raw black quinoa), RYQ (raw yellow quinoa), and RRQ (raw red quinoa) flours, respectively. After ASIP extraction, PIBQ (black quinoa protein isolate), PCYQ (yellow quinoa protein concentrate), and PCRQ (red quinoa protein concentrate) exhibited protein contents of 94.03, 86.01, and 87.23 g/100 g, respectively, with a recovery yield of 48.24% for PCRQ, 49.23% for PCYQ, and 53.98% for PIBQ. These results align with those reported by Tavano et al. (2022), who demonstrated that using NaOH solution (0.1 mol/L) during quinoa protein extraction yielded an extraction efficiency of up to 85.7%. This method represents, one of the most effective and extensive protein extraction techniques when applied to defatted quinoa flour (Tavano et al., 2022). The terms "protein concentrates" and "protein isolates" refer to protein extracts obtained through specific extraction procedures, with their designation dependent on protein concentration on a dry basis (Cruz-Solis et al., 2023; Mondor & Hernández-Álvarez, 2022). Protein isolates are characterized by higher protein purity compared to concentrates. Protein

concentrates typically contain 35–89% protein, whereas protein isolates have a protein content exceeding 90%.

Under similar conditions, Aluko & Monu (2003) produced quinoa protein concentrates with a protein content of 65.52 g/100g. Similarly, Abugoch et al. (2008) reported quinoa protein isolates obtained at different solubilization pH with protein contents of 77.2 (pH 9) and 83.5 (pH 11) g/100 g, which align closely with the results obtained in this study. Variations in protein content among quinoa samples can be attributed to factors such as harvest time, environmental conditions, geographical location, and processing or extraction conditions, all of which influence the protein structure, quality, and proximate composition (Kaur et al., 2024; Wang et al., 2023). Therefore, a comprehensive characterization of quinoa raw flours, protein isolates and concentrates was performed to understand the effect of ASIP on the secondary structure, protein quality and ANFs.

4.2. Circular dichroism (CD) and fourier transformed infra-red (FTIR) analysis

The secondary structure of proteins can be assessed using CD spectroscopy within the far UV spectrum (190 – 260 nm and below). This method provides quantitative estimations of protein structures, which can be compared to other methods such as NMR or X-ray crystallography (Kelly et al., 2005; Micsonai et al., 2015, 2018). The CD spectrum in the far UV range is characterized by specific patterns corresponding to the percentages of α-helix, β-sheet, and random coils structure in the protein (Kelly et al., 2005; Micsonai et al., 2015, 2018), which are interpreted using a CD spectrum deconvolution software. Before CD analysis, particle size was measured by DLS to investigate if there was particle aggregation in quinoa flours and quinoa protein isolates or concentrates. The current study showed that only RYQ had Gaussian distribution plot (**Supplementary material A**). While RBQ, RRQ, PIBQ and PCRQ showed a bimodal distribution, with fraction of low size (<ca. 150 nm), this could be a representation of monomeric and quaternary structures (Sahin et al., 2024). Overall, all samples showed a correlogram <1.0.

All quinoa protein isolates, concentrates, and raw flours were analyzed using milli-Q-water as a buffer. For the final data interpretation, the Bestsel™ online software (https://bestsel.elte.hu/index.php, accessed September 2024) was employed for single spectrum analysis and fold recognition. The results shown in Fig. 1a-c indicate that all quinoa protein isolates/concentrates exhibited a strong positive peak at 190–195 nm, and a dominant negative peak before 210 nm, mainly attributed to the α-helix structure. Whereas dominant negative peaks at 215 nm and above correspond to the β-sheet conformation, which is less prominent in the structure of the isolates/concentrates (Mäkinen et al., 2016; Mir et al., 2021). The secondary structure composition of raw quinoa flours ranged from 36.1 – 50.9% α-helix, 1.6 – 6.6% β-sheet--parallel, and 10.3 – 10.7% β-turn. In contrast, PCYQ and PCRQ exhibited α-helix contents of 84.5 and 82.6%, respectively, with the remaining structure consisting of β-sheet-antiparallel, indicating that the ASIP extraction process unfolded the protein structure. Moreover, PIBQ showed 95.5% α-helix and 4.5% β-sheet-antiparallel, suggesting that the extraction method significantly altered the secondary structure of this sample.

These results are consistent with previous reports by Mir et al. (2021), and Mäkinen et al. (2016) which showed that the dominant positive and negative peaks in quinoa protein isolates did not change even when samples were heated to 40°C. This indicates that the temperature (40°C) applied during protein extraction does not affect the secondary structure of quinoa proteins. Furthermore, Li et al. (2023) reported the secondary structure of quinoa isolates, with and without ultrasound pretreatment. They found that the quinoa protein isolates produced were not affected by the pH of extraction (7.0 – 11.0) and observed a downward trend in β-sheet content between non-ultrasound (40.26%) and ultrasound-extracted quinoa (38.27%) isolates, while α-helix content showed no modifications (Kumar et al., 2022).

Navarro-Lisboa et al. (2017) examined the CD spectroscopy of quinoa protein concentrates prepared by alkaline solubilization (pH 9.5) and ultrafiltration (pH adjusted to 7.0 and 9.0). They found that quinoa concentrate at pH 9.0 contained highly structured α-helix domains, whereas the protein concentrates at pH 7.0 showed significant ($p > 0.05$) changes in protein spatial configuration. These results are similar

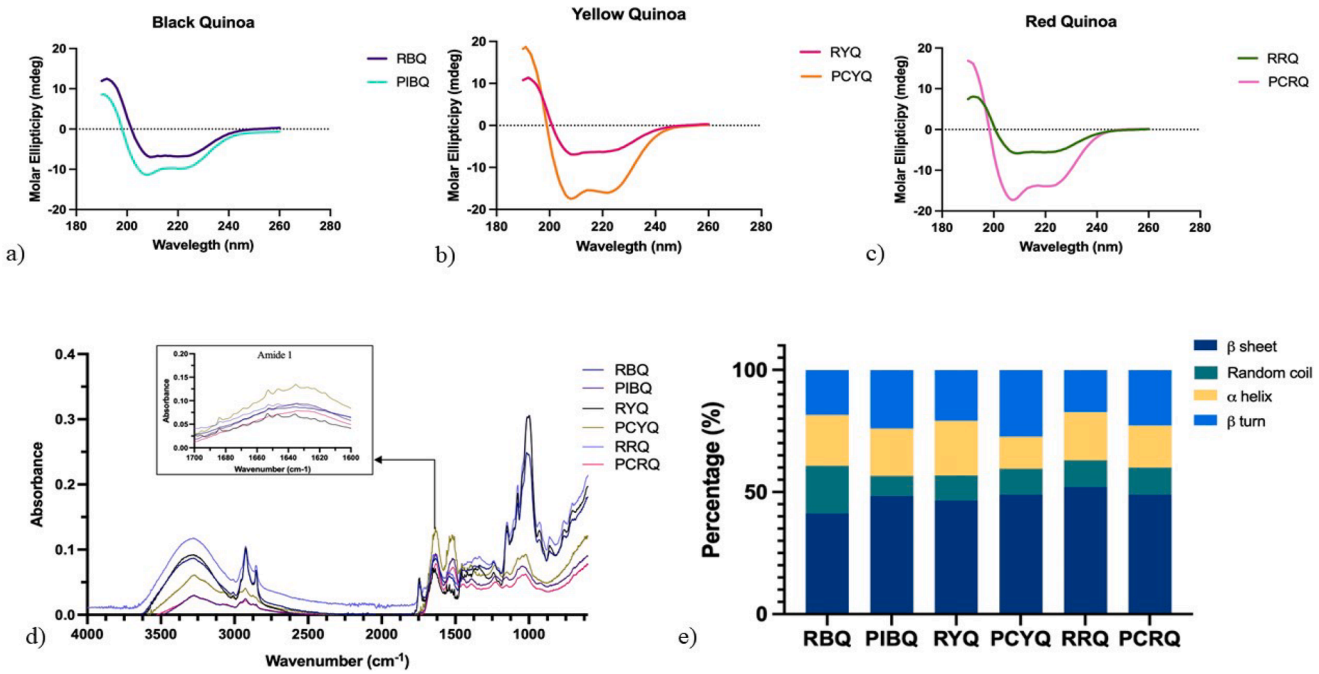

Fig. 1. Circular dichroism (CD) of quinoa samples: a) raw and black quinoa protein isolate, b) raw and yellow quinoa protein concentrate, and c) raw and red quinoa protein concentrate, and d) Fourier-transformed infrared spectroscopy (FTIR) spectrum of raw quinoa samples and protein isolates and concentrates, e) Secondary structure composition analyzed by secondary-derivative analysis of amide I. RBQ; raw black quinoa, PiBQ; Protein isolate black quinoa, RYQ; raw yellow quinoa, PCYQ; Protein concentrate yellow quinoa, RRQ; raw red quinoa, and PCRQ; Protein concentrate red quinoa). Data expressed as mean ± SD, n = 3, ($p < 0.05$).

to those observed for PIBQ in the current study. The characteristics of CD spectroscopy are influenced by amino acid profile, hydrogen bonding, polar groups, and protein polarizability (Kelly et al., 2005; Micsonai et al., 2015).

FTIR was carried out to analyze the secondary structure of quinoa proteins in the solid state and compare their native structures after protein extraction. In FTIR, protein structure analysis is divided into three regions: amide I (1600 – 1580 cm^{-1} corresponding to stretching vibration C = O), amide II (1580 – 1528 cm^{-1} corresponding to the bending vibration of -NH), and amide III (< 1400 cm^{-1}) (Vera et al., 2019; Bolje & Gobec, 2021; Zhao et al., 2022). Furthermore, characteristic frequency shifts caused by hydrogen bonding in amide bonds are interpreted as α-helix, β-sheets, and random coils (Vera et al., 2019).

The comparison of FTIR spectra between raw flours and protein isolates/concentrates of quinoa (Fig. 1d), revealed that RBQ and RRQ have similar β-sheet (46.2% RBQ, and 46.9% RRQ), and random coil (13.8% RBQ, and 12.3% RRQ) percentages. RYQ showed a lower percentage of β-sheets (43.8%) and higher random coil percentage (15.9%). Regarding protein extraction samples, PCYQ and PCRQ presented similar β-sheet content (51.5% and 50.6%, respectively) and random coil content (11.3% in PCYQ and 12.1% in PCRQ). Whereas PIBQ showed a lower percentage of β-sheet (47.9%) compared to PCYQ and PCRQ. Overall, in the protein isolates/concentrates, the α-helix content decreased, while the β-sheet content increased. These results are consistent with those of Vera et al. (2019), who reported that ultrasound extraction modified the secondary structure of quinoa proteins, increasing the β-sheet and β-turns content. Similarly, Mir et al. (2021) reported higher intensity peaks corresponding to β-turns in quinoa protein isolates obtained by alkaline solubilization and albumin isolated from quinoa. The main components of quinoa are 2S and 11S globulins, which contain α and β structures, indicating presence of reactive side chain groups (-NH, -OH, -SH) in quinoa (Zhao et al., 2022).

The FTIR results observed in this study align with the previously discussed CD data, indicating that black quinoa experienced minimal changes in secondary structure following protein extraction.

4.3. Differential scanning calorimetry (DSC)

The thermal characterization of quinoa was carried out by DSC, and Tp, To, Tf, and denaturation enthalpy (ΔH) were assessed (**Supplementary material B**). In DSC, a controlled increase in heat is applied to an enclosed pan, over a set period to detect changes in the physical properties of the sample (Gill et al., 2010). The results of the thermal characterization of raw flours and protein isolates/concentrates from quinoa samples are presented in Table 1. The lowest denaturation peaks were observed for PCYQ at 105.93 °C and PCRQ at 104.91 °C, respectively. Notably, only the yellow quinoa sample showed a statistically significant difference ($p < 0.05$) between the flour and the protein concentrate. In addition, there were no statistical differences ($p > 0.05$) in To and Tf among all quinoa flours and protein isolates/concentrates. The enthalpy energy of all samples ranged between 1901 – 2434 J/g. These findings are similar to those obtained by Abugoch (2008), reporting a Tp of 98.9 °C for quinoa flour and a denaturation range between 85.6 – 103.1 °C, using the same alkaline extraction conditions (pH 9) and similar pI (pH 5). This endotherm is attributed to the "chenopodin" protein. Furthermore, it was found that alkaline extraction at pH 11.0 is unsuitable for protein extraction as the protein becomes fully denatured due to extreme alkaline conditions (López et al., 2018). According to Navarro-Lisboa et al. (2017), some variations in the endothermal peaks of quinoa proteins can be attributed to the moisture content in the sample.

Ruiz et al. (2016) reported the thermal characterization of quinoa protein isolates obtained through alkaline extraction at varying pH levels (8 – 11). A single endothermic peak at 97.2 °C was observed for all samples, except those obtained at pH 11.0. These results are consistent with the findings of Abugoch James, (2009) and Dakhili et al. (2019), indicating that the denaturation of the globulin fraction occurs at temperatures above 97 °C (Gorinstein et al., 1996; Ruiz et al., 2016). The major proteins of quinoa are 2S albumin and 7S and 11S globulins (Dakhili et al., 2019; Navarro-Lisboa et al., 2017), which are expected to exhibit thermal denaturation peaks (Tp) at these temperatures. This Tp behavior has also been observed in other globulins sources such as amaranth (94 – 100 °C), soybean (92 °C), broad bean (94 °C), and sunflower (95 °C) (Ruiz et al., 2016).

Table 1
Differential scanning calorimetry (DSC) and Surface Hydrophobicity (H_0).

Sample	H_0	To (°C)	Tp (°C)	Tf (°C)	ΔH (J/g)
RBQ	42,755 ± 1533^{bc}	99.83 ± 0.18 ^{a}	106.43 ± 0.31^{ab}	118.91 ± 0.80^{a}	1901
RYQ	41,696 ± 1378^{c}	102.21 ± 3.1^{a}	112.09 ± 2.67^{a}	126.31 ± 2.84 ^{a}	2567
RRQ	27,360 ± 682^{d}	99.03 ± 1.65^{a}	107.37 ± 0.55^{ab}	120.89 ± 1.51^{a}	2214
PIBQ	44,123 ± 509^{bc}	102.24 ± 3.0^{a}	108.52 ± 3.03^{ab}	127.61 ± 0.99^{a}	2224
PCYQ	45,201 ± 863^{ab}	101.07 ± 1.70^{a}	105.93 ± 1.85^{b}	125.51 ± 3.49 ^{a}	2434
PCRQ	59,300 ± 1335^{a}	99.96 ± 0.49^{a}	104.91 ± 1.24^{b}	124.52 ± 2.38^{a}	2281

Ho; surface hydrophobicity, To; initial temperature, Tp; peak temperature, Tf; final temperature, ΔH; enthalpy. Different lower-case letters in the same column means significantly different ($p < 0.05$). RBQ; raw black quinoa, PIBQ; Protein isolate black quinoa, RYQ; raw yellow quinoa, PCYQ; Protein concentrate yellow quinoa, RRQ; raw red quinoa, and PCRQ; Protein concentrate red quinoa. Data expressed as mean ± SD, n = 3, ($p < 0.05$).

4.4. Fast Protein Liquid Chromatography (FPLC)

FPLC was used to analyze the molecular weight distribution of black, yellow, and red quinoa in raw, defatted and protein isolates/concentrates (Fig. 2). FPLC, a high-performance liquid chromatography method, offers high resolution and provides a detailed molecular weight (MW) protein profile (Wang et al., 2023). The MW protein profiles of RBQ and YBQ were similar, with RBQ showing the largest peak at 134.1 kDa. Interestingly, this pattern was also observed in defatted black (DBQ) and yellow (DYQ) quinoa samples, which presented four peaks with the presence of polypeptides (24.6 kDa in DBQ and 28.6 kDa in DYQ) and small peptides (2.8 and 0.22 kDa in DBQ, and 2.8 and 0.28 kDa in DYQ) (**Supplementary Material C**). These results indicate that the defatting process alters the molecular weight distribution of the proteins in black and yellow quinoa. Furthermore, PIBQ and PCYQ showed the highest peaks at low MW, 2.3 and 2.4 kDa, respectively. High MW proteins were still present in PIBQ and PCYQ, at 70.6 and 73.3 kDa, respectively, though they were less dominant compared to the raw and defatted samples. An interesting effect was observed in red quinoa samples. The defatting process increased the MW from 120.2 in RRQ to 132.9 kDa in DRQ. PCRQ showed a distinct protein distribution, with only two peaks at 101.3 and 6.0 kDa. Proteins with MW of 66, 52, 38, 16 kDa can be attributed to 7S globulin, while proteins < 9 kDa are likely 2S globulins (Ballegaard et al., 2023; Van de Vondel et al., 2022). During protein extraction, globulin polypeptides undergo aggregation and disaggregation due to temperature, alkaline pH, and freeze-drying processes, this results in the association and dissociation of the hexamer subunits of the protein fractions (Wang et al., 2023). Although 11S globulin, with a MW of 340 kDa, is reported as the major protein in quinoa, it was not detected in this study due to the FPLC limitations in accurately assessing such high MW proteins (Van de Vondel et al., 2022; Wang et al., 2023).

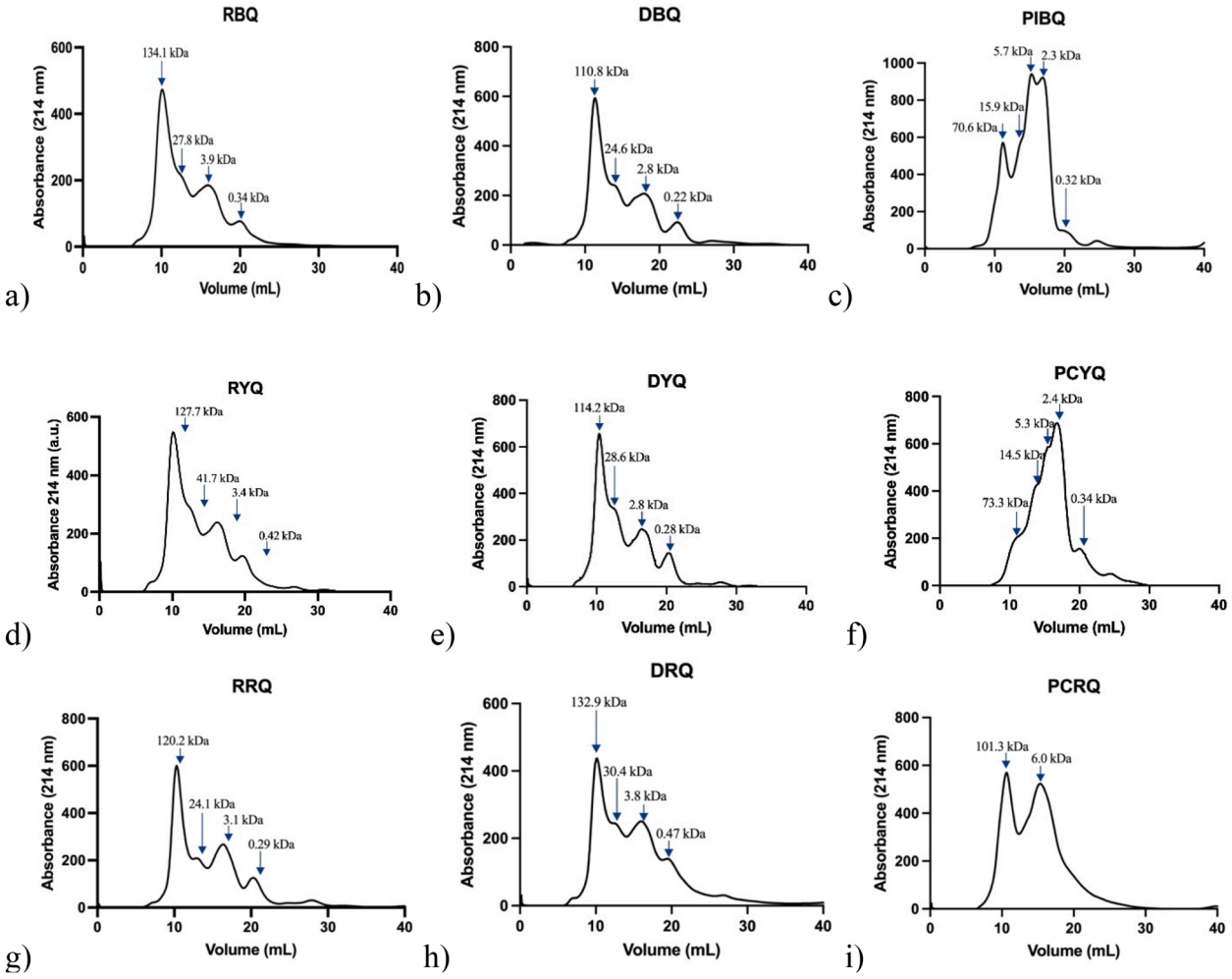

Fig. 2. Fast protein liquid chromatography (FPLC) analysis of quinoa samples a) raw black quinoa (RBQ), b) defatted black quinoa (DBQ), c) black quinoa protein isolate (PIBQ), d) raw yellow quinoa (RYQ), e) defatted yellow quinoa (DYQ), f) yellow quinoa protein concentrate (PCYQ), g) raw red quinoa (RRQ), h) defatted red quinoa (DRQ), and i) red quinoa protein concentrate (PCRQ).

4.6. SDS-PAGE

The electrophoretic profiles of raw flours and protein isolates/concentrates from quinoa varieties are shown in Fig. 3. Under reducing conditions, the main protein bands observed in RBQ, RYQ and RRQ correspond to 7S globulin (Fig. 3f), 11S globulin (both basic and acidic fractions, Fig. 3h and 3i), with less intensity observed for vacuolar-processing enzyme-like proteins (Fig. 3e) and oleosin 1 (Fig. 3j) (Shen et al., 2022; Poza-Viejo et al., 2023). Notably, RYQ lacks one band corresponding to the 11S globulin acidic fraction, which is present in RBQ and RRQ. Under non-reducing conditions, two high MW bands at 197.2 and 142 kDa (Fig. 3a and b), were detected but absent in the quinoa protein isolates and concentrates. The most intense bands were those for 7S globulin and the 11S "chenopodin" fraction, while 13S seed storage globulin showed less intensity, and a 6.5 kDa protein was undetected in RBQ (Abugoch et al., 2008; Abugoch, 2009; Abugoch et al., 2009;). These variations suggest that varietal differences impact quinoa's protein composition (Bock et al., 2021).

In PIBQ, PCYQ and PCRQ, under non-reducing conditions, higher intensity bands corresponding to 7S globulin (Fig. 3f) and 11S "chenopodin" globulin (Fig. 3g) were observed (Elsohaimy et al., 2015; Dakhili et al., 2019), alongside less intense bands for the 11S globulin acidic fraction (Fig. 3h) and 2S albumin (Fig. 3m) (Galindo-Luján et al., 2023; Poza-Viejo et al., 2023).

Furthermore, 13S globulin seed storage protein (Fig. 3c) was present in PIBQ and PCRQ, while 2S seed storage protein (Fig. 3k) was only present in PCYQ. Under reducing conditions, the 11S globulin acidic and basic subunits (Fig. 3h and i) appeared with increased intensity, and antimicrobial peptide 2-like bands (Fig. 3l) were also observed in PIBQ, PCYQ, and PCRQ. This aligns with Dakhili et al. (2019), who found that under reducing conditions, bands above 60 kDa were absent in quinoa protein concentrates. Conversely, when quinoa samples were treated with β-mercaptoethanol or DTT, disulfide bonds in 11S globulin were reduced, leading to the separation of acidic (30 – 40 kDa) and basic subunits (20 – 25 kDa). These results are consistent with findings from Valenzuela et al. (2013), Nongonierma et al. (2015), and Navarro-Lisboa et al. (2017), who identified molecular weight bands around 55 kDa corresponding to globulins, with polypeptides between 45 and 55 kDa corresponding to "chenopodin" subunits (a and b). Quinoa proteins are composed of approximately 37% 11S globulin and its subunits, 35% 7S globulin (~60 kDa), and 2S albumin (~10 kDa), with the remainder comprising glutelin fractions (Brinegar & Goundan, 1993; Navarro-Lisboa et al., 2017). Additionally, Shen et al. (2022), in an extensive proteomic analysis identified various quinoa proteins, including oleosin 1, oleosin 18.2 kDa, vicilin-like antimicrobial peptides (47 – 63.67 kDa), and vacuolar-processing enzyme-like proteins (64.61 kDa), all of which were present in this study's electrophoretic profiles. In summary, the raw flours and protein isolates/concentrates from quinoa samples exhibited a broad range of molecular weights and similar protein profiles. The protein extraction process (ASIP), effectively preserved the

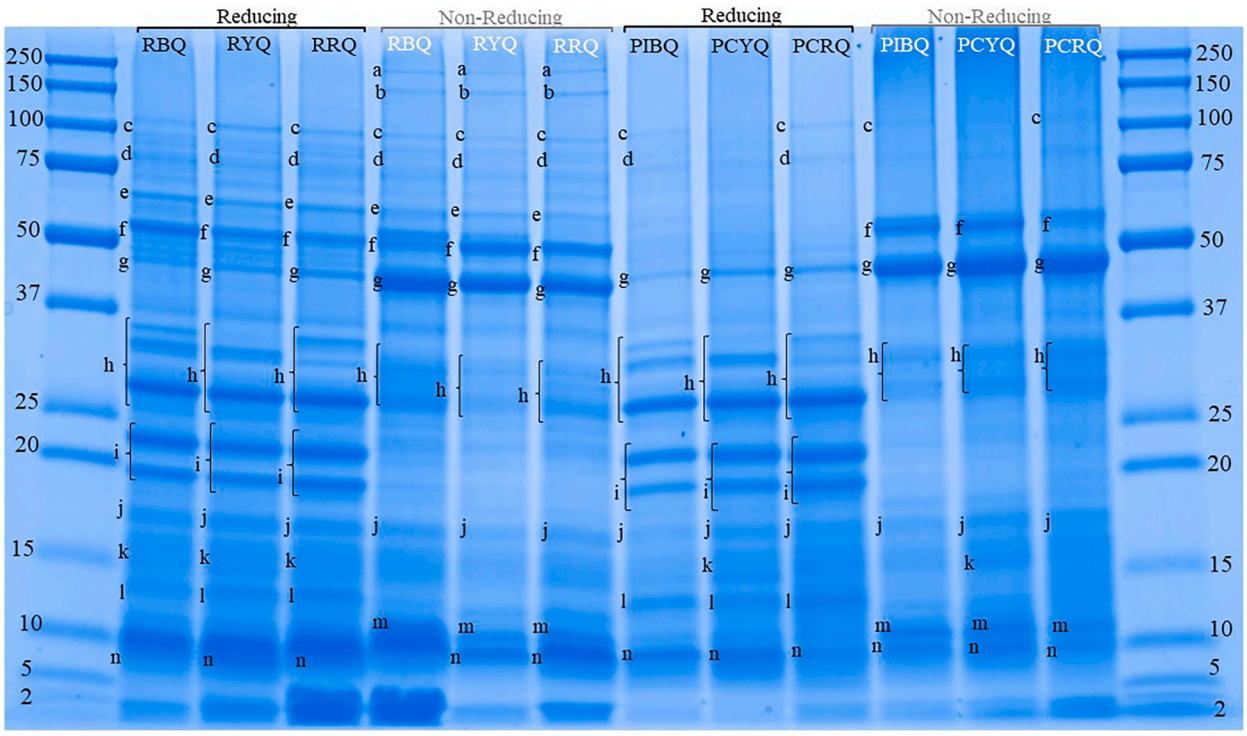

Fig. 3. SDS-PAGE of protein samples from raw flour and protein isolate/concentrates from quinoa. Lines: **1** and **14**) protein reference (kDa), RBQ; raw black quinoa, RYQ; raw yellow quinoa, RRQ; raw red quinoa, PIBQ; protein isolate black quinoa, PCYQ; protein concentrate yellow quinoa, and PCRQ; protein concentrate red quinoa. **a**; 197.2 kDa protein, **b**; 142 kDa protein, **c**;13S globulin seed storage protein 1 (109.47 kDa), **d**; 75.5 kDa protein, **e**; vacuolar-processing enzyme like (64.61 kDa), **f**;7S globulin (60 kDa), **g**; 11S "chenopodin" fraction (45 – 55 kDa), **h**; 11S globulin acidic subunit (30 – 40 kDa), **i**; 11S globulin basic subunit (20 -25 kDa), **j**; Oleosin 1 (18 kDa), **k**; 2S seed storage protein (15.55 kDa), **l**; antimicrobial peptide 2-like (12.9 kDa), **m**; 2S albumin (10 kDa), and **n**; 6.5 kDa protein.

main proteins bands corresponding to 11S globulin, indicating that the extraction steps, including pH adjustments, temperature variations, and centrifugation, did not significantly affect the molecular structure of quinoa proteins.

4.7. *Surface hydrophobicity (H_0)*

H_0 was assessed in quinoa flours and isolates/concentrates at different concentrations (0.01 – 0.1 mg/mL). The H_0 measurement uses a fluorescence probe, ANS, to detect the conformational structure changes in proteins and the interactions between ANS and hydrophobic binding sites (Vera et al., 2019). The ANS-binding assay is valuable for assessing protein aggregation, folding, and unfolding (López et al., 2019). The H_0 analysis results presented in Table 1 showed no statistical difference ($p > 0.05$) in H_0 between black quinoa samples (RBQ and PIBQ). However, yellow quinoa presented higher H_0 in PCYQ (45,201) than RYQ (41,696). While red quinoa showed the highest H_0 in PCRQ (59,300), and RRQ (27,360) showed the lowest H_0. In general, two main effects can be appreciated in H_0. Firstly, a decrease in H_0 could be attributed to protein aggregation, where higher concentration of proteins forms hydrophobic patches bind to ANS (López et al., 2018). Secondly, an increase in H_0 results from protein unfolding, which can be influenced by factors such as temperature or pH (Mäkinen et al., 2016). The higher H_0 observed in PCRQ suggests an increased exposure of hydrophobic groups following protein extraction, suggesting protein unfolding and enhanced interactions with the polar environment (Mäkinen et al., 2015; Mir et al., 2021). The amino acid profile of PCRQ demonstrated a notably higher content of hydrophobic amino acids such as Val (5.84 g/100g), Ile (5.17 g/100 g), Trp (1.03 g/100 g), Leu (9.03 g/100 g), and Ala (5.55 g/100 g) (Table 2), which likely accounts for the observed increase in H_0.

Mäkinen et al. (2015), reported a 2-fold times increase in H_0 of quinoa protein isolates, after heating and alkalinization pH (8.5 to 10.5),

Table 2
Amino acid profile of quinoa isolates/concentrates (g/100 g).

Amino acid	Black	Yellow	Red	Amino acid scoring patterns (FAO/WHO)	
				Children	Adults
Asp + Asn	9.62 ± 0.04^{b}	9.28 ± 0.06^{c}	10.13 ± 0.07^{a}	-	-
Glu + Gln	14.94 ± 0.01^{a}	15.02 ± 0.12^{a}	14.37 ± 0.0^{b}	-	-
Ser	5.93 ± 0.0^{a}	5.41 ±0.0^{c}	5.72 ± 0.0^{b}	-	-
His	**3.1 ± 0.0^{a}**	**3.1 ± 0.1^{a}**	**3.04 ± 0.0^{a}**	-	-
Gly	5.38 ± 0.2^{a}	5.56 ± 0.1^{a}	5.49 ± 0.1^{a}	-	-
Thr	**4.42 ± 0.0^{a}**	**4.46 ± 0.1^{a}**	**4.66 ± 0.6^{a}**	**3.4**	**0.9**
Arg	10.34 ± 0.2^{a}	10.62 ± 0.06^{a}	9.67 ± 0.02^{b}	-	-
Ala	5.24 ± 0.0^{b}	5.24 ± 0.1^{b}	5.55 ± 0.0^{a}	-	-
Pro	3.68 ± 0.0^{a}	3.48 ± 0.3^{a}	3.54 ± 0.2^{a}	-	-
Tyr	3.03 ± 0.0^{a}	3.01 ± 0.0^{c}	3.02 ± 0.0^{b}	-	-
Val	**5.74 ± 0.1^{a}**	**5.70 ± 0.0^{a}**	**5.84 ± 0.1^{a}**	**3.5**	**1.3**
Met	0.86 ± 0.0^{c}	1.60 ± 0.0^{a}	0.88 ± 0.0^{b}	-	-
Cys	0.87 ± 0.0^{b}	1.05 ± 0.0^{a}	0.79 ± 0.0^{c}	-	-
Ile	**5.22 ± 0.0^{a}**	**5.0 ± 0.0^{c}**	**5.17 ± 0.0^{b}**	**2.8**	**1.3**
Trp	**1.0 ± 0.0^{b}**	**0.97 ± 0.0^{c}**	**1.03 ± 0.0^{a}**	**0.8**	**0.5**
Leu	**8.87 ± 0.1^{b}**	**8.62 ± 0.0^{c}**	**9.03 ± 0.0^{a}**	**6.6**	**1.9**
Phe	5.69 ± 0.1^{a}	5.52 ± 0.0^{a}	5.64 ± 0.1^{a}	-	-
Lys	**6.07 ± 0.0^{c}**	**6.36 ± 0.0^{b}**	**6.43 ± 0.0^{a}**	**5.8**	**1.6**
Phe + Tyr	**8.72^{a}**	**8.53^{c}**	**8.66^{b}**	**6.3**	**1.9**
Met + Cys	**1.73^{b}**	**2.65^{a}**	**1.67^{c}**	**2.5**	**1.7**

Data are expressed as mean ± SD, $n = 3$, $p < 0.05$. Gly; glycine, Lys; lysine, Gln; glutamine, Glu; glutamic acid, Ser; serine, Ala; alanine, Leu; leucine, Met; methionine, Phe; phenylalanine, Trp; tryptophan, Pro; proline, Val; valine, Ile; isoleucine, Cys; cysteine, Tyr; tyrosine, His; histidine, Arg; arginine, Asn; asparagine, Asp; aspartic acid, Thr; threonine. Essential amino acids are presented in bold letters. FAO/WHO 1985 infant (2 – 6 months) amino acid requirements.

compared with quinoa flours. Furthermore, these results align with the CD and FTIR results, which showed no significant difference in the protein structure of black quinoa samples after protein extraction, while red quinoa samples displayed alterations in the secondary protein structure after extraction.

4.8. Amino acid profile

The amino acid profiles of PIBQ, PCYQ and PCRQ are presented in Table 2. In all quinoa protein samples, the predominant amino acids were Glu + Gln (14.4 – 15.02%), Arg (9.67 – 10.62%) and Asp + Asn (9.3 – 10.1%). In addition, EAA represented 40.97 - 41.72% of total amino acids, with the highest value observed in PCRQ. Quinoa isolates/concentrates meet the daily amino acid requirements for infants and children (FAO/WHO/UNU Expert Consultation, 2007). Thus, consumption of 100 g of either quinoa seeds or isolates meets the EAA requirements for children (1.38 – 1.40-fold) and adults (4.2 – 4.3-fold). The highest content of sulfur-containing amino acids (Met + Cys) was found in PCYQ (2.65 g/100 g), while the highest content of aromatic amino acids (Phe, Trp and Tyr) was exhibited by PIBQ (9.72 g/100g). In a previous study by Manzanilla-Valdez et al. (2024) quinoa flours (Black, Yellow, and Red) were found to have major amino acids Glu + Gln (14.86 – 16.24%), Asp + Asp (9.49 – 10.04%) and Arg (10.98 – 11.1%). Furthermore, an increase in sulfur-containing amino acids (Met and Cys) was observed after ASIP, with levels of 0.64%, 1.52%, and 0.88% for PIBQ, PCYQ and PCRQ, respectively. Mäkinen et al. (2015), reported a similar amino acid profile for quinoa protein isolates obtained through ASIP (solubilization at pH 9.0, and isoelectric precipitation at pH 5.0), aligning with findings in this study. Additionally, higher levels of Ala, Pro, Val, Gly, and Ser were found compared to our data, while Arg, His, Lys, Tyr and Glu were lower. These differences could be attributed to varietal differences, buffer composition, length of extraction or inclusively amino acid analysis methodology employed (Ravindran et al., 2024).

Overall, PIBQ, PCYQ and PCRQ showed an excellent amino acid composition compared to FAO/WHO requirements. It is essential to emphasize that the amino acid profile of a protein sample can significantly influence key parameters related to protein quality and IVPD (Wang et al., 2023).

4.9. Protein quality and In vitro protein digestibility (IVPD)

IVPD and protein quality were assessed in quinoa protein isolates/concentrates (Table 3). IVPD estimates the protein availability for intestinal uptake after digestion using different enzymes such as pepsin, chymotrypsin and pancreatin (Kumar et al., 2022). IVPD ranged from 82.12 to 84.50%, with PIBQ exhibiting the highest digestibility among all samples. IVPD from quinoa isolates was higher compared to other plant proteins such as chia albumin fractions (Mexican 67.65% and British 69.73%) (Wang et al., 2023), chia globulin fractions (Mexican 68.86% and British 67.83%) (Wang et al., 2023), soy isolate (55.2%) (Almeida et al., 2015), casein isolate (83.7%) (Almeida et al., 2015), rapeseed albumin (67.2%), rapeseed globulin (63.9%) and rapeseed glutelin (47.2%) (Joehnke et al., 2018). Due to the wide variety of IVPD methodologies used in the literature, data is difficult to compare and presents significant differences, as digestion conditions differ, particularly enzyme selection and activity are sensitive factors (Wang et al., 2023).

In the previous screening study on quinoa flours (Black, Yellow, and Red), the IVPD ranged between 76.90 to 77.69%, with no statistical difference between samples (Manzanilla-Valdez et al., 2024). Following protein extraction by ASIP, quinoa protein isolates/concentrates exhibited a notable increase in IVPD, showing an overall improvement of ~5.96%. Thus, the extraction process enhanced protein digestibility, as the absence or low content of carbohydrates and lipids facilitates digestion. Although IVPD is not a reflection of real protein digestibility, it provides useful information regarding the nutritional value of a protein source (Wang et al., 2023).

The amino acid score (AAS) is a ratio of the actual content of amino acids to the recommended values for metabolism (Almeida et al., 2015; FAO/WHO/UNU Expert Consultation, 2007). The AAS results were 0.69, 0.88 and 0.67 for PIBQ, PCYQ and PCRQ, respectively. These values are notably higher compared to quinoa flours (0.32 – 0.45) (Manzanilla-Valdez, Boesch et al., 2024), demonstrating the impact of ASIP on AAS. Moreover, increases in amino acids such as Ser, Tyr, Val, Met, Cys, Ile, Leu, and Phe were observed following protein extraction. However, the AAS values for quinoa isolates and concentrates remain lower than those for whey protein (> 1.0) (Almeida et al., 2022; Zhao et al., 2018).

The essential amino acid index (EAAI), ranged between 399.78 – 683.04%, compared to the standard (whole egg protein), indicating that quinoa protein isolates/concentrates have nearly or more than 3-fold times the EAAI of the reference protein source. Furthermore, the biological value (BV) ranged from 424.03 to 732.03, with PCYQ showing the highest BV among the samples. A BV higher than 100 is considered a good source of protein, highlighting the biological importance provided by the amino acid profile (Wang et al., 2023). These values are higher than those reported by Wang et al. (2023) for chia protein concentrates (Mexican 431.90, and British 189.40), chia albumin fraction (Mexican 383.56, and British 506.50) and globulin fraction (Mexican 311.98, and British 529.74). These differences may be attributed to the high content of certain amino acids such as His, Gly, Thr, Val, Ile, Trp, Leu, and Lys in quinoa, particularly in PCYQ. In the preceding study with quinoa flours (Black, Yellow, and Red), BV showed values ranging between 50.79 – 250.81, with black quinoa presenting the highest BV (Manzanilla-Valdez, Boesch et al., 2024). PIBQ, PCYQ and PCRQ exhibited higher BV compared to quinoa flours, which is attributed to their enhanced amino acid profiles. Notably, PCYQ and PCRQ showed significant increases in some amino acids such as Asp + Asn, Gln + Glu, His, Phe, Leu, Met and Cys. Moreover, a strong positive correlation between EAAI and BV was observed, indicating a direct proportionality. Both assessments revealed that PCYQ had the highest values, whereas

Table 3
In vitro protein digestibility and protein quality indexes of black, yellow, and red quinoa protein concentrates/isolates.

Quinoa	IVPD (%)	AAS[a]	EAAI[b] (%)	BV[c]	PER_1[d]	PER_2[d]	PER_3[d]	PER_4[d]	PER_5[d]	IVPDCAAS[e] (%)
Black	84.50 ± 1.32[a]	0.69 (SAA)	399.78	424.03	3.19	3.24	3.27	2.87	3.22	58.31
Yellow	82.12 ± 0.54[b]	0.88 (Trp)	683.04	732.78	3.09	3.13	3.42	2.90	3.26	71.39
Red	83.40 ± 0.75[ab]	0.67 (SAA)	467.90	498.28	3.27	3.31	3.40	2.93	3.22	55.88

Different letters in same column indicate statistical differences by Tukey post hoc ($p < 0.05$), data expressed as mean ±SD, n =5.
Note: EAAI (%), AAS, BV (%), PER1–5 and IVPDCAAS (%) are calculated values, no standard deviation is available.
[a] Amino acid score.
[b] Biological value.
[c] Protein efficiency ratio.
[d] *In vitro* protein digestibility.
[e] *In vitro* protein-digestibility corrected amino acid score.

PIBQ exhibited the lowest.

Protein efficiency ratio (PER) is also described in Table 3. Five different equations (PER_{1-5}) were used for PIBQ, PCYQ and PCRQ, with values estimated between 2.87 – 3.42. PER values are an estimation of theorical protein efficiency according to EAA (Amza et al., 2013; Nosworthy et al., 2017, 2023). PER values higher than the standard (casein) are considered excellent protein sources (Nosworthy et al., 2023). All quinoa samples had a PER higher than 2.5. PIBQ showed the lowest PER with 2.87 (PER_4) due to the low content of Thr (4.42 g/100 g), Met (0.86 g/100 g) and Lys (6.07 g/100 g), despite having higher content of Phe (5.69 g/100 g) and Ile (5.22 g/100g). Meanwhile, PCYQ had the highest PER (PER_3 3.42) due to a high content of Met (1.60 g/100 g) and His (3.10 g/100 g). These values are higher compared to biological PER such as milk (2.50), eggs (3.10), chicken (2.70), white rice (1.50), red kidney beans (1.55), black beans (1.61), split yellow peas (1.42), chickpeas (2.32) (Nosworthy et al., 2023), and theoretical PER values as chia protein concentrate (2.50 – 3.11), chia albumin fraction (2.06 – 2.97), chia globulin fraction (2.54 – 3.21) (Wang et al., 2023), mustard seeds protein isolate (2.57) (Alireza-Sadeghi et al., 2006), black quinoa flour (2.90 – 3.18), yellow quinoa flour (2.53 – 3.14), and red quinoa flour (2.69 – 3.12) (Manzanilla-Valdez et al., 2024).

In vitro protein digestibility corrected amino acid score (IVPDCAAS) was assessed for PIBQ, PCYQ and PCRQ. IVPDCAAS is a protein quality method that takes into consideration EAA requirements (FAO/WHO/UNU Expert Consultation, 2007). IVPDCAAS values in this study were 58.31%, 71.39% and 55.88%, for PIBQ, PCYQ and PCRQ, respectively. PIBQ and PCRQ values for IVPDCAAS are higher than those reported in the previous study by Manzanilla-Valdez et al. (2024), where quinoa flours exhibited an IVPDCAAS range of 23.96 – 34.92%. This increase highlights the impact of the ASIP extraction process on protein quality, demonstrating its effectiveness in enhancing these values (Sánchez-Velázquez et al., 2021). On the other hand, PCYQ showed higher IVPDCAAS compared to raw flours such as red lentil (62.07%), green lentil (58.15%), green split pea (59.53%), chickpea (58.16%), faba bean (52.79%), and black bean (43.98%) (Sánchez-Velázquez et al., 2021). Sánchez-Velázquez et al. (2021), and Nosworthy et al. (2018), demonstrated that cooking and extrusion increased the IVPDCAAS in oat and beans, respectively. It is important to emphasize the current lack of comprehensive data regarding the IVPDCAAS in protein isolates and concentrates. This gap in the literature highlights the need for further research to fully understand the protein quality and digestibility of these concentrated protein sources, especially given their growing relevance in both nutritional science and food industry applications.

4.10. Antinutrients (ANFs)

Antinutrients or antinutritional factors, such as lectins, phytates, oxalates, tannins, and saponins, are compounds produced by plants that have been considered harmful to human health (López-Moreno et al., 2022). While ANFs are well-documented in raw materials as flours and seeds, there is a lack of information regarding their presence or absence after protein extraction (Manzanilla-Valdez, Ma et al., 2024). This information is particularly crucial given the increasing use of plant-based ingredients in the food market. Understanding the impact of protein extraction processes on the levels of ANFs in quinoa protein isolates and concentrates is essential for developing safe and nutritious plant-based food products. Therefore, the final objective of this study was to evaluate the effect of ASIP on ANFs in quinoa protein isolates and concentrates.

4.10.1. Anthocyanins

Anthocyanins are water-soluble compounds responsible for natural color pigments such as red, blue, purple, and orange (Ayvaz et al., 2023). They are considered antioxidant agents due to their radical scavenging activity (Pastor-Cavada et al., 2010; Farzaneh & Carvalho, 2017). Despite this, anthocyanins can also negatively impact human health by impairing growth, reducing protein digestibility, and inhibiting the absorption of minerals such as Ca^{2+} and Mg^{2+} (Garutti et al., 2022; Ayvaz et al., 2023). For this reason, anthocyanins content was measured in PIBQ, PCYQ and PCRQ. However, anthocyanins were not detected in the samples (data not shown). In the previous study, the anthocyanins content in black, yellow, and red quinoa raw flours ranged from 15.8 – 19.3 mg CGE/L (Manzanilla-Valdez et al., 2024). The differences in anthocyanin levels could be attributed to the protein extraction method used (ASIP), as these are water-soluble compounds that may be washed out during the ASIP extraction process.

4.10.2. Lectins

Lectins are glycoproteins that can attach to erythrocytes and cause agglutination (Samtiya et al., 2020). Furthermore, lectins can cause growth impairment, damage the small intestine epithelium, and stimulate pancreatic hypertrophy and hyperplasia (Samtiya et al., 2020; López-Moreno et al., 2022). However, isolated lectins can be useful in breast cancer treatment due to their anti-proliferative effects (Cavada et al., 2023). Fig. 4 shows the results for hemagglutination in PIBQ, PCYQ, and PCRQ, at concentrations lower than 2.21, 3.79, and 5.6 mg/mL, respectively. Two positive controls were used, an antibody provided by the kit (KPA-3913) and a lectin isolated from red bean (*Phaseolus vulgaris*), which presented hemagglutination at $\leq$ 0.8 mg/mL, and $\leq$ 2.9 mg/mL, respectively. PIBQ, PCYQ, and PCRQ showed no hemagglutination at any of the concentrations assayed.

In the previous investigation on black, yellow, and red quinoa raw flours, hemagglutination activity was observed at concentrations ranging from 1.1 to 5.6 mg/mL (Manzanilla-Valdez et al., 2024). The absence of hemagglutination in PIBQ, PCYQ and PCRQ, suggests that lectins may have been removed during the protein extraction process or potentially affected by freeze-drying. Some studies have reported that protein extraction coupled to isoelectric precipitation, eliminates or reduces hemagglutination, as observed in pea (37.2 HU/g protein in seeds), faba bean (18.8 HU/g protein in seeds), and soybean (3.2 HU/g protein in seeds) (Fernández-Quintela et al., 1997), and faba bean protein isolate (1.0 HU/g) (Arntfield et al., 1985).

4.10.3. Oxalates

Oxalates are compounds that can form soluble (Na^{2+}, K^{2+}, and ammonium salts) and insoluble (Ca^{2+}, Fe^{2+}, and Mg^{2+} salts) complexes (Huynh et al., 2022). They are mostly toxic, forming kidney stones, acting as blood clotting agents and impairing the absorption of minerals such as Ca^{2+} and Mg^{2+} (Huynh et al., 2022; López-Moreno et al., 2022). The soluble and total oxalate content of RBQ, RYQ, RRQ, PIBQ, PCYQ, and PCRQ is presented in Fig. 5a. The current study found that RBQ had the highest total oxalate content (236.7 mg/100 g), while RRQ exhibited the highest soluble oxalate content (136.5 mg/100 g). Additionally, PCRQ showed the highest levels of both soluble (112.9 mg/100 g) and total oxalates (311.2 mg/100 g). In contrast, PIBQ presented the lowest amount of total (105.01 mg/ 100 g) and soluble oxalate content (69.32 mg/100 g). These findings suggest that the ASIP extraction process significantly reduced the oxalate content in quinoa samples. Previous research has shown that cooking and soaking processes can reduce the oxalate content by 30 – 76.9%. It is well known that oxalates primarily accumulate in leaves, steam, roots and hypocotyls seeds (Manzanilla-Valdez et al., 2024). However, an interesting phenomenon was observed in red quinoa, where total oxalate content increased in PCRQ. This suggest that insoluble complexes might have formed during the extraction process, and thus the soaking step during ASIP did not effectively reduce the oxalate content in this sample (Maradini Filho et al., 2017).

There is limited information in the literature regarding oxalate content in protein isolates/concentrates from different protein sources.

4.10.4. Trypsin inhibitors

Trypsin inhibitors are proteases that can be divided into Kunitz

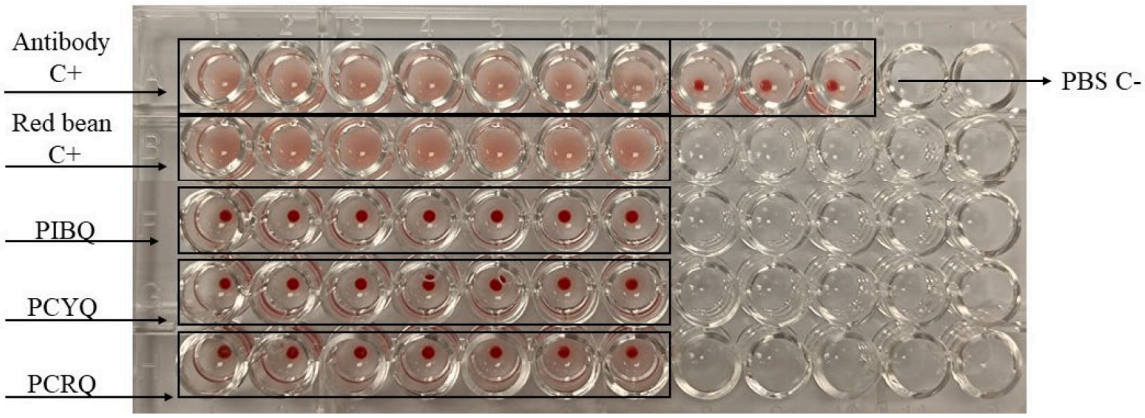

Fig. 4. Hemagglutination assay. Positive control (C+) Antibody: 0.8, 0.4, 0.2, 0.1, 0.05 and 0.025 mg/mL. Red bean lectin (C+): 2.9, 0.8, 0.4, 0.2, 0.1, 0.05 and 0.025 mg/mL. Negative control (C-) PBS: 25 mL per well, Black quinoa protein isolate (PIBQ); 2.21, 1.1, 0.55, 0.27, 0.13, 0.69 and 4.42 mg/mL. Yellow quinoa protein concentrate (PCYQ); 3.79, 1.89, 0.94, 0.47, 0.23, 0.12 and 0.059 mg/mL. Red quinoa protein concentrate (PCRQ); 5.6, 2.79, 1.39, 0.69, 0.35, 0.17 and 0.087 mg/mL. Different soluble protein concentrations were used for analysis as these depended on protein solubility and concentration for each sample.

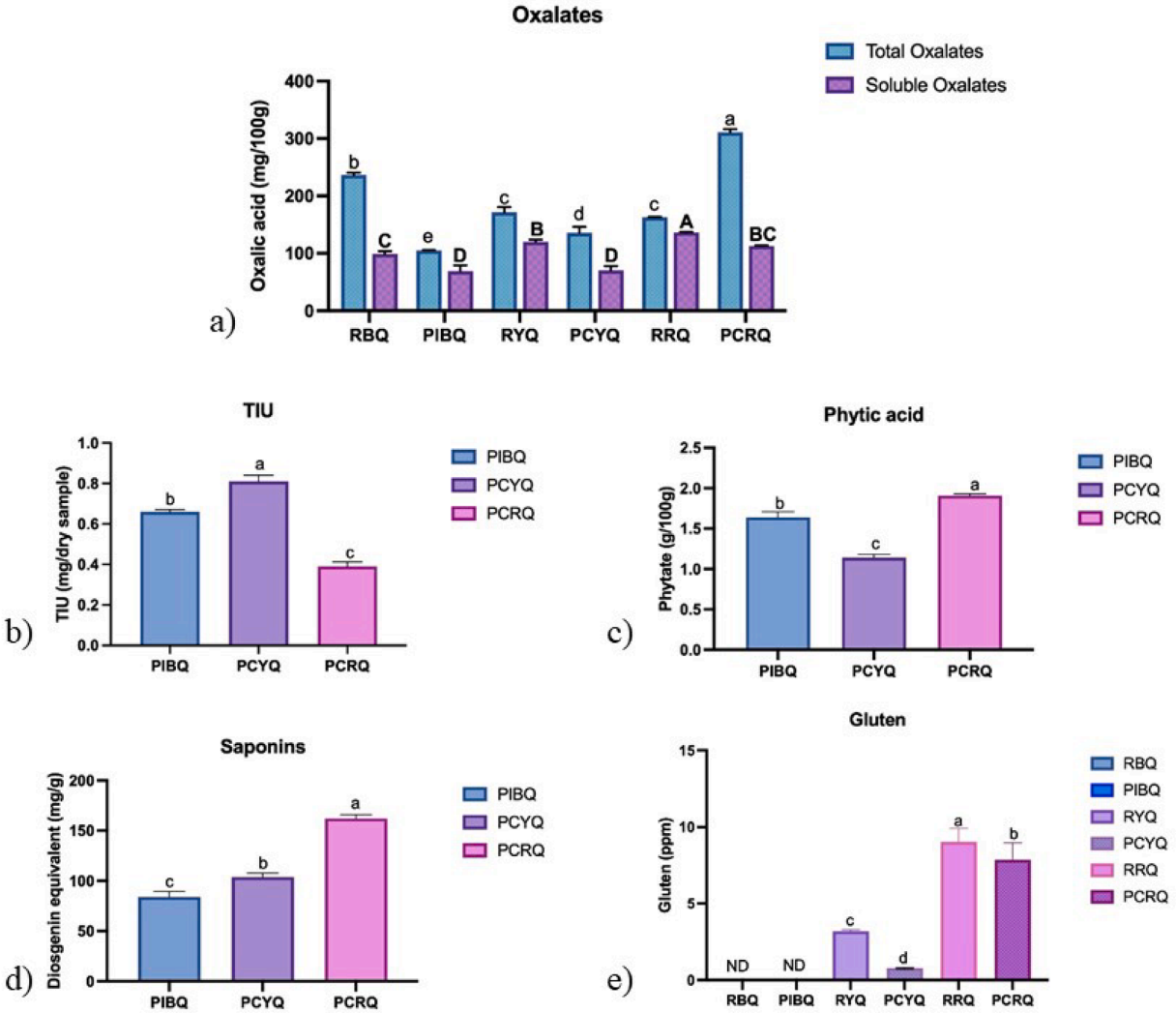

Fig. 5. Anti-nutritional assessment of black, yellow, and red quinoa protein isolates and concentrates. a) total and soluble oxalates, b) trypsin inhibitory units (TIU), c) phytic acid, d) saponins, and e) gluten content. Different superscript letters between bars indicate statistical analysis difference between quinoa flours by One-way ANOVA and Tukey`s multiple range test. Data expressed as mean ± SD, $n = 5$, ($p < 0.05$). RBQ; raw black quinoa, PIBQ; Protein isolate black quinoa, RYQ; raw yellow quinoa, PCYQ; Protein concentrate yellow quinoa, RRQ; raw red quinoa, and PCRQ; Protein concentrate red quinoa.

trypsin inhibitors and Bowman-Birk inhibitors (Condori & De Camargo, 2023). Kunitz inhibitors are responsible for trypsin inhibition, reducing protein digestibility and causing diarrhea and blotting (Miranda et al., 2010; Condori & De Camargo, 2023). The results for PIBQ, PCYQ, and PCRQ are presented in Fig. 5**b**, showing 0.66, 0.81, and 0.39 TIU/mg, respectively. Quinoa seeds have presented trypsin inhibitory activity (TIA) ranging from 0.17 to 15.1 TIU/mg (Pesoti et al., 2015; Maradini Filho et al., 2017; Tavano et al., 2022). Furthermore, in the prior work (Manzanilla-Valdez et al., 2024) quinoa flours exhibited TIU activity of 0.36 TIU/mg dry sample for black quinoa, 0.35 TIU/mg dry sample for yellow quinoa, and 0.46 TIU /mg dry sample for red quinoa. Following protein extraction using ASIP, an increase in TIU was observed in black and yellow quinoa protein isolate and concentrate, respectively. Trypsin inhibitors are water-soluble proteins belonging to the 2S albumin fraction (Moreno & Clemente, 2008; Katsube-Tanaka & Monshi, 2022), these can be solubilized in the medium (water) and precipitated during the extraction process at the selected pH (4.5), increasing their concentration in protein isolates/concentrates (Wang, 1971; Roychaudhuri et al., 2004). This effect has also been observed in chickpea Kabuli (from 20.89 to 21.16 TIU/mg) (Mondor et al., 2009), and faba bean (from 4.5 to 15.8 TIU/g) (Dumoulin et al., 2021). Despite this, trypsin inhibitors can be decreased or removed by heating processes such as boiling, roasting, autoclaving, microwave, and baking, as well as by long periods of water soaking (>18 h) (Maradini Filho et al., 2017).

4.10.5. Phytic acid

Due to the lack of phytase in the human digestion system, phytic acid, also known as myo-inositol hexaphosphate (IP6), cannot be absorbed in the intestine (Thakur et al., 2019). Phytic acid binds to minerals such as Zn^{2+}, Fe^{3+}, Mg^{2+} and Ca^{2+}, forming soluble complexes, that cannot be digested (Thakur et al., 2019; Thakur et al., 2021; López-Moreno et al., 2022). Additionally, phytic acid decreases the availability of proteins in the small intestine (Thakur et al., 2019; Thakur et al., 2021). Despite this, some studies have reported that phytic acid can offer cardiovascular protection, prevent kidney stone formation, and reduce the risk of colorectal cancer (Duraiswamy et al., 2023; Zhang et al., 2023). The results of phytic acid in this study were 1.69, 1.14, and 1.92 g/100 g in PIBQ, PCYQ, and PCRQ, respectively (Fig. 5c). These results are lower than those reported in the preceding study (Manzanilla-Valdez et al., 2024) for black (1.97 g/100 g), yellow (2.13 g/100 g), and red (2.21 g/100 g) quinoa flour, but higher compared to those reported by Maldonado-Alvarado et al. (2023) for germinated quinoa flours from black (1.22 g/100 g), white (1.07 g/100 g), and red (1.03 g/100 g) varieties. Germination and fermentation processes are known to reduce the phytic acid content in quinoa (Maradini Filho et al., 2017; Thakur et al., 2019). Furthermore, some authors have reported that after ASIP the phytic acid content in protein ingredients decreased in faba beans (from 107.6 to 70.5 mg/g protein), chickpeas (from 101.0 to 53.6 mg/g protein), and soybeans (from 89.6 to 62.7 mg/g) (Fernández-Quintela et al., 1997). Phytic acid content can be reduced during the soaking process prior to ASIP extraction. Additionally, during the solubilization step, phytic acid may be further removed through the slurry mixing (sample/water) and subsequent centrifugation steps. Soaking has been shown to effectively reduce phytic acid levels, thereby improving the nutritional quality of the final product (Manzanilla-Valdez, Ma et al., 2024).

4.10.6. Saponins

Saponins are glycosides that can be classified as steroidal or triterpenoid and are mostly soluble in water and ethanol solutions (Panigrahy et al., 2022; Song et al., 2024). Saponins can bind to Fe^{3+} and Zn^{2+}, inhibiting mineral absorption, causing hemolysis in red blood cells, and impairing lipid metabolism efficiency (Samtiya et al., 2020). However, triterpenoid saponins can act as antioxidants and have antimicrobial properties (Zhang et al., 2023). Saponin content in PIBQ, PCYQ and PCRQ was 84.0, 103.8, and 162.2 mg/g, respectively (Fig. 5d). Compared to the previously analyzed quinoa flours - black (83.7 mg/g), yellow (95.5 mg/g), and red (96.8 mg/g) varieties (Manzanilla-Valdez et al., 2024) – the saponins content in PCYQ and PCRQ was found to be higher. As saponins are water soluble compounds, they may become solubilized during the protein extraction process, increasing their concentration in the protein concentrates/isolates. Few studies have examined saponin content in protein isolates/concentrates. Garg et al. (2020), reported a 50% reduction in saponins in *Prosopis cineraria* seed protein concentrate by applying different temperatures (30 – 60 °C) and pH (8 – 10) during protein extraction. On the other hand, Illingworth et al. (2022), found no effect on saponins content in *Moringa oleifera* protein isolates produced by alkaline extraction and micellization. Consumption of saponins exceeding 50 mg/kg of body weight is considered toxic and can affect gut microbiota, liver, and kidney cells (Vega-Gálvez et al., 2010; Lo et al., 2018).

4.10.7. Tannins

Tannins are classified into three categories: hydrolysable, condensed, and complex tannins, and they are water-soluble compounds (Ren et al., 2023; Zhang et al., 2023). Tannins exhibit an affinity for proteins, they can form protein-tannin complexes and decrease their bioavailability (Gilani et al., 2005; Lo et al., 2018). Furthermore, they can also form complexes with carbohydrates and proteins, inhibiting amylase activity (Gilani et al., 2005; Lo et al., 2018). Alternatively, tannins can act as antioxidants in small amounts (Ren et al., 2023; Zhang et al., 2023). The results from this study, showed low tannins levels in PIBQ (0.009 mg/100 g) and PCRQ (0.004 mg/100 g), while PCYQ had no detectable tannins. The results of this study revealed low tannin levels in PIBQ (0.009 mg/100 g) and PCRQ (0.004 mg/100 g), while no tannins were detected in PCYQ. These values are slightly higher than those reported in the former research (Manzanilla-Valdez et al., 2024), where black quinoa flour exhibited a tannin content of 0.002 mg/100 g, and both yellow and red quinoa flours showed no detectable tannins. Garg et al. (2020), demonstrated a significant tannin reduction of 95% in protein concentrates from *Prosopis cinerari* seeds using ASIP. Furthermore, Illingworth et al. (2022) compared the effects of ASIP, and salt extraction/micellization on *Moringa oleifera* protein isolates and found a tannin reduction of 38.7% with the ASIP technique. Overall, tannins can be reduced or washed-out during protein extraction due to their water solubility. Furthermore, dehulling can also reduce the presence of these ANFs (Manzanilla-Valdez, Ma et al., 2024).

4.10.8. Phenolic compounds

Phenolic compounds are mainly known for their antioxidant capacity and can be divided into phenolic acids, tannins, and flavonoids (Yusoff et al., 2022). Due to their chemical structure of one or more aromatic rings coupled with one or more hydroxyl groups, phenolic compounds act as strong antioxidants (Yusoff et al., 2022). However, these can bind to proteins, forming protein-phenolic interactions that modify secondary or tertiary protein conformations (Gunawan et al., 2022). Therefore, in this study the phenolic content was assessed using two different methodologies: FBBB and FC, with a clean-up step involving SPE. The results are displayed in Table 4. SPE increased the polyphenol content (expressed in gallic acid equivalents) in both FC and FBBB assays. PIBQ, PCYQ, and PCRQ after SPE showed results 2-fold higher than the EPC fraction. In the FC assay, PCRQ (SPE) showed the highest concentration of 207.4 mg GAE/100 g, while PCYQ had the lowest concentration at 140.7 mg GAE/100 g. Similarly, PCRQ showed the highest concentration of 200.5 mg GAE/100 g in the FBBB assay, while PIBQ had the lowest concentration at 131.1 mg GAE/100 g. SPE removes interferences such as enediols, alcohols, sugars, and vitamin C (Pico et al., 2020). These results are consistent with Li et al. (2021), who reported a range of 89 to 213 mg GAE/100 g in thirteen quinoa varieties, and Pico et al. (2020), who reported FC (113.7 mg GAE/100 g) and FBBB (315.9 mg/100 g) after SPE in quinoa flour.

Furthermore, compared to the previously analyzed Black, Yellow,

Table 4
Comparison of extractable phenolic compounds (EPC) of quinoa protein concentrates/isolates measured by Folin–Ciocalteu and FBBB reactions (expressed in mg/100 g of gallic acid equivalents) without the removal of interferences (Control) and after the removal of soluble interferences by solid phase extraction (SPE). The percentage of decrease or increase is indicated as ↓ % or ↑ %, respectively.

Sample	Folin-Ciocalteu			Fast Blue BB		
	Control (EPC)	SPE	↑↓ (%)	Control (EPC)	SPE	↑↓ (%)
PIBQ	147.5 ± 7.8^{c}	155.8 ± 1.5^{b}	5.4 ↑	67.5 ± 1.0^{e}	131.1 ± 0.5^{c}	94.2 ↑
PCYQ	112.4 ± 6.6^{d}	140.7 ± 3.5^{c}	25.2 ↑	79.4 ± 2.5^{e}	149.5 ± 1.2^{b}	88.3 ↑
PCRQ	161.5 ± 3.4^{b}	207.4 ± 5.8^{a}	28.4 ↑	101.5 ± 2.4^{d}	200.5 ± 1.8^{a}	97.5 ↑

Different superscript letters in the same column indicate statistical differences between quinoa flours by ANOVA and Tukey`s multiple range test. Data are expressed as mean ± SD, $n = 5$, $p < 0.05$. EPC, extractable phenolic compounds; SPE, solid phase extraction. PIBQ; Protein isolate black quinoa, PCYQ; Protein concentrate yellow quinoa, and PCRQ; Protein concentrate red quinoa.

and Red quinoa flours (Manzanilla-Valdez et al., 2024) by FC and FBBB, Red quinoa had the highest phenolic content by FC (182.5 mg GAE/100 g), while Yellow quinoa exhibited the highest phenolic content by FBBB (334.7 mg/100 g). After ASIP, phenolic content was higher in FC, while lower in FBBB. It has been reported that FC has less specificity, in comparison with FBBB that has a specific reaction were fast blue diazonium salts with the active hydroxyl groups present in the phenolic compounds (Pico et al., 2020; Ravindranath et al., 2021).

Overall, the total phenolic content of PIBQ, PCYQ, and PCRQ presented in this study is higher compared to those of chickpea Desi protein isolate (134.0 – 164.0 mg GAE/100 g), chickpea Kabuli protein isolate (97.0 – 106.0 mg GAE/100 g) (Mondor et al., 2009), and mustard protein isolate (120.0 mg/100 g) (Alireza-Sadeghi et al., 2006).

4.10.9. Gluten analysis

Glutelins, or gluten, are classified into two main fractions: polymeric glutenins and monomeric gliadins (Shewry & Belton, 2024). Furthermore, these proteins can cause allergenicity in the gut, triggering a chronic autoimmune response known as celiac disease (Singla et al., 2024). This condition occurs in the duodenum and jejunum, where IgA activation leads to inflammation, diarrhea, and vomiting (Shewry & Belton, 2024; Singla et al., 2024). According to the Codex Alimentarius FAO/WHO, any food or product containing ≤ 20 mg/kg (20 ppm) of gluten can be labeled as "gluten free". In this study, gluten content in quinoa flours and protein isolates/concentrates was assessed. Gluten content was undetectable in RBQ and PIBQ (Fig. 5e). Notably, RRQ had the highest gluten content at 9.034 ppm, while RYQ showed 3.197 ppm. After protein extraction, PCYQ and PCBQ exhibited a decrease in gluten content to 0.775 and 7.807 ppm, respectively, representing a reduction of 13.55% to 75.76%. This reduction is likely to the solubility of gluten proteins in basic solutions, as the alkaline solubilization step (pH 9 -10) in the extraction process effectively removes this fraction (Haros & Schönlechner, 2017). Some authors have reported the percentage of glutelins in quinoa flours to be around 18.1% to 31.6% (Abugoch James, 2009; Haros & Schönlechner, 2017; Martínez-Villaluenga et al., 2020; Van de Vondel et al., 2022). To our knowledge, this is the first study assessing gluten content in quinoa protein isolates/concentrates.

Overall, there is a notable lack of information regarding ANFs in protein isolates and concentrates. As observed throughout this study, certain ANFs can increase during protein extraction, potentially decreasing protein digestibility and thereby affecting protein quality. Therefore, it is crucial to measure ANFs in protein isolates and concentrates to understand how these protein ingredients will interact with other compounds and impact functional, nutritional, and sensory characteristics of plant-based food formulations.

5. Conclusions

This study is the first, to our knowledge, to evaluate the effects of quinoa protein isolates and concentrates obtained by ASIP on a broad spectrum of ANFs and protein quality metrics. Our findings provide a comprehensive analysis of ASIP's impact on the structural, functional, and nutritional properties of quinoa protein isolates (PIBQ) and concentrates (PCYQ, PCRQ). ASIP significantly modified the secondary structure of PIBQ and PCYQ, increasing β-sheet and β-turn content while reducing α-helix structures. Despite these structural changes, thermal properties remained stable, indicating that ASIP preserves protein stability without inducing detectable denaturation during extraction.

ASIP effectively reduced tannins, gluten, and soluble oxalates across all samples, potentially enhancing palatability and digestibility. Notably, PCRQ showed a slight increase in saponins and oxalate content, warranting further investigation. Overall, all protein isolates and concentrates exhibited high IVPD, exceeding 82.12%, and a robust amino acid profile, with EAA constituting over 40% of the total amino acid content. The EAAI ranged from 399.78% to 683.04%, and PER values consistently exceeded 2.8.

In conclusion, ASIP presents a promising technique for producing quinoa protein isolates and concentrates with enhanced functional properties while preserving their nutritional value. Although some ANFs remain present, this research underscores ASIP's potential for producing tailored plant protein ingredients with improved nutritional profiles, functional properties, and sensory appeal. Further studies are recommended to explore both traditional and innovative protein extraction methods, aiming to optimize plant protein ingredients for the alternative protein and food science fields.

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CRediT authorship contribution statement

Maria Lilibeth Manzanilla-Valdez: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Christine Boesch:** Writing – review & editing, Validation, Supervision, Conceptualization. **Cristina Martinez-Villaluenga:** Writing – review & editing, Validation. **Sarita Montaño:** Writing – review & editing, Supervision. **Alan Javier Hernández-Álvarez:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fhfh.2024.100191.

Data availability

No data was used for the research described in the article.

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