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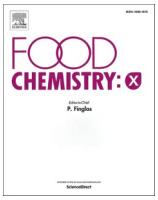

Unveiling the nutritional spectrum: A comprehensive analysis of protein quality and antinutritional factors in three varieties of quinoa (*Chenopodium quinoa* Wild)

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ABSTRACT

Quinoa (*Chenopodium quinoa*) is renowned for its high protein content and balanced amino acid profile. Despite promising protein characteristics, plant-based sources usually possess antinutritional factors (ANFs). This study aimed to analyze the nutritional and ANFs composition of three quinoa varieties (Black, Yellow, and Red), and assessed the protein quality. Among these varieties, Black quinoa showed the highest protein content (20.90 g/100 g) and total dietary fiber (TDF) (22.97 g/100 g). In contrast, Red quinoa exhibited the highest concentration of phenolic compounds (338.9 mg/100 g). The predominant ANFs identified included oxalates (ranging from 396.9 to 715.2 mg/100 g), saponins (83.27–96.82 g/100 g), and trypsin inhibitors (0.35–0.46 TUI/100 g). All three varieties showed similar *in vitro* protein digestibility (IVPD) (> 76.9 %), while Black quinoa exhibited the highest protein quality. In conclusion to ensure reduction of ANFs, processing methods are necessary in order to fully benefit from the high protein and nutritional value of quinoa.

1. Introduction

Chenopodium quinoa, also known as quinoa or quinua, is a native plant originating to the Andean Altiplano (South America) region in Peru. Quinoa can adapt to different climate conditions such as heat stress, drought, and cold weather. The main quinoa variety is White/Yellow, with other varieties such as Black, Red and Rainbow, demonstrating different levels of pigmentation (Aloisi et al., 2016; Chen et al., 2023; Sánchez-Velázquez et al., 2022). In 2013, the FAO declared quinoa as the seed of the future, based on its high nutritional value and genetic diversity (Ren et al., 2023). Quinoa is considered a pseudocereal, which are starch-rich seeds, typically with a higher protein content than cereals, and a low presence of gliadins (0.5–7 %) (Ren et al., 2023) thus being an option for celiac individuals (Capriotti et al., 2015; Morales et al., 2021; Silva et al., 2020).

Quinoa has a high protein content (15–19 %), which is higher than rice (6.6–8.4 %), maize (8.8–11.9 %), barley (7–14.6 %), sorghum (7–15 %) and millet (8.3–13.3 %) and contains all essential amino acids (EAA) (Balzotti et al., 2008; Brinegar & Goundan, 1993; Guerrieri & Cavaletto, 2017). Albumins and globulins represent the main storage proteins in quinoa with 35 % and 37 %, respectively (Sánchez-Velázquez et al., 2022; Zheng et al., 2019). Moreover, quinoa has a higher content of lipids, total fiber, and EAA compared to cereals such as rice, wheat, oat, and maize (Garutti et al., 2022; Hu et al., 2017; McKevith, 2004).

At present, quinoa is grown in different countries, with Peru being the largest producer and exporter of quinoa seeds (Ren et al., 2023). In addition, it has been shown that local growing conditions such as temperature, soil salinity, altitude, excess/lack of water and weather conditions (drought and cold) affect primary and secondary metabolism in quinoa plants (Burrieza et al., 2019; Cao et al., 2020) which could

Abbreviations: AAS, Amino acid score; BAPA, Na-benzoyl-L-arginine-p-nitroanilide hydrochloride; BV, Biological value; CE, Catechin equivalents; CGE, cyaniding-3-glucoside equivalent; EAA, Essential amino acid; EPC, Extractable phenolic compounds; IDF, Insoluble dietary fiber; IVPD, *In vitro* protein digestibility; IVPDCAAS, *In vitro* protein digestibility corrected amino acid score; NRS, Non-resistant starch; PER, Protein efficiency ratio; RS, Resistant starch; SDF, Soluble dietary fiber; SPE, Solid phase extraction; TAA, Total amino acid; TDF, Total dietary fiber.

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impact its nutritional composition. Another factor to take into consideration is the presence of antinutritional factors (ANFs), which comprise of a diverse group of compounds such as protease inhibitors *i.e.* trypsin inhibitors, hemagglutinins, and toxic amino acids such as dihydroxyphenyl alanine and canavanine, as well as glycosides (saponins, cyanogenic, oestrogens and goitrogens), phenolics (tannins and anthocyanins), phytic acid and oxalates (Thakur et al., 2019; Thakur et al., 2021). Some of these ANFs can reduce nutrient availability, through reduced protein digestibility and mineral absorption, thus, leading to promotion of kidney stone formation and respiratory system malfunction (Maradini Filho et al., 2017). Apart from the negative effects of ANFs, some members of these group are in fact also associated with beneficial biological effects, *i.e.* anthocyanins and lectins which have shown hypolipidemic, hypoglycaemic, cancer-preventative and antioxidant properties (Paśko et al., 2008; Thakur et al., 2019; Waszkowiak et al., 2015). The most studied ANFs in quinoa are saponins, which are located in the outer layer of quinoa seeds. Saponins have a bitter taste, and have demonstrated toxic effects, hence making their removal necessary (El Hazzam et al., 2020; Mora-Ocación et al., 2022). Besides saponins, other ANFs that have been identified in quinoa seeds, are tannins, phytic acid, trypsin inhibitors and anthocyanins (Paśko et al., 2008; Tang et al., 2015). However, information regarding the varietal differences of some ANFs in quinoa seeds is scarce.

Protein quality depends on the amino acid composition and the protein digestibility (Cavada et al., 2023), reflecting if a food product will meet protein and EAA requirements (FAO/WHO/UNU, 2007). The Protein Digestibility Corrected Amino Acid Score (PDCAAS) is determined by the amino acid composition and the protein digestibility from a food source, when values are lower than 1.0, this reflects the limiting amino acids present in the protein source (Nosworthy, Neufeld, et al., 2017). Nosworthy, Hernandez-Alvarez, et al. (2023) reported a positive correlation between PDCAAS and the *in-vitro* protein digestibility corrected amino acid score (IVPDCAAS) across different samples tested. Raw and cooked soy, wheat, and oat showed a strong correlation (R^2 = 0.9647) between these two assays. Thus, IVPDCAAS could be used as a rapid technique to assess protein quality. There are other metrics used to evaluate the protein quality, such as the Protein Efficiency Ratio (PER). PER assesses the efficacy of consumed protein by measuring growth over a four-week period using murine models (Hoffman & Falvo, 2004; Nosworthy, Franczyk, et al., 2017) although a theoretical PER could also be determined (Sánchez-Velázquez, Cuevas-Rodríguez, et al., 2021). Another measurement for protein quality is Biological Value (BV), this measures the nitrogen used for tissue formation and is divided by the nitrogen in the evaluated food (Hoffman & Falvo, 2004), and thus a high BV correlates with a high supply of EAA. PDCAAS and PER values are mandatory requirements for protein quality in USA and Canada, respectively (Nosworthy, Medina, et al., 2023).

The aims of this research were: 1) to determine the nutritional composition, 2) to analyze a broad spectrum of ANFs (including anthocyanins, oxalates, phytic acid, saponins, trypsin inhibitors, phenolics, tannins, cyanogenic glycosides, and lectins) in three commercially grown and commonly consumed quinoa varieties (Black, Yellow, and Red), and 3) to assess the protein quality through *in vitro* protein digestibility, amino acid composition, and protein quality indices (AAS, EAAI, BV, PER, and IVPDCAAS). To the best of our knowledge, this study is the first to provide a comprehensive overview and comparative analysis of a broad range of ANFs and protein quality parameters. Through this research, we aim to enhance the comprehensive understanding of quinoa proteins by elucidating the influence of varietal differences on protein quality and nutritional value. This study seeks to provide insights into the complex interactions between quinoa proteins and the presence or absence of specific antinutrients, thereby advancing the broader understanding of the applicability, protein quality, and digestibility of quinoa proteins.

2. Materials and methods

2.1. Reagents

Kits for dietary fiber, available carbohydrates (K-ACHDF) and resistant starch (K-RAPRS) were purchased from Megazyme® (Bray, Ireland), the test for cyanogenic glucosides (MQuant®) was from Merck (Gillingham, UK) and for sheep hemagglutination kit from Rockland Immunochemicals (Pennsylvania, USA). Antinutritional reagents and enzymes: BAPA (benzylsulfonyl-D-arginyl-prolyl-4-amidinobenzylamide), calcium chloride, catechin, chymotrypsin (EC 3.4.21.1), diosgenin, ethylenediaminetetraacetic acid (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 for lectin extraction were purchased from Thermo Scientific (Loughborough, UK). Acetone, acetic acid, ethanol, methanol, hexane, sulfuric acid, hydrochloric acid, formic acid, and petroleum ether, all HPLC grade, were obtained from Merck.

2.2. Quinoa flour sample preparation

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

The quinoa flour samples were defatted for ANF assessment following the procedure by Sánchez-Velázquez, Ribéreau, 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 magnetic stirrer. Afterwards, the slurry was centrifuged at 5000*g* for 30 min at 4 °C, the resultant supernatant discarded, and the pellet re-extracted twice under the same conditions. The defatted quinoa flour samples were dried under the fume hood overnight to remove remnant solvent. The defatted quinoa flour was then stored in a plastic antistatic bag at RT for further analyses.

2.3. Proximal composition

The nutritional composition of raw and defatted quinoa flour was determined using the official AACC methods (American Association of Cereal Chemistis-ACCC 2000). The standard methodology was followed for ash content (AACC 08-16.01) and lipid content (AACC 30-25.01). The protein content was measured by the Kjeldahl method (AACC 46-30.01), and crude protein was calculated using a nitrogen conversion factor of 6.25 (Nitrogen to Protein conversion factor). All analyses were carried out in quintuples, and average values were calculated.

2.3.1. Total dietary fiber (soluble and insoluble), available carbohydrates and resistant starch

Quantification of available carbohydrates and dietary fiber were conducted with Megazyme® kit (K-ACHDF 06/18) based on AOAC (Official Method 991.43) and AACC methods (Method 32-07.01), according to the manufacturer's instructions. The calculations were performed by using Megazyme Mega-Calc™ (K-TDFR), downloaded from www.megazyme.com.

2.3.2. Available carbohydrates

Available carbohydrates (D-glucose and D-fructose) were determined in aliquots from the above-mentioned dietary fiber sample preparation. Analysis was done following according to the manufacturer's

instructions. Finally, results were calculated using Megazyme Mega-Calc™ spreadsheet (K-ACHDF), downloaded from www.megazyme.com.

2.3.3. Resistant starch

Analysis of resistant and non-resistant starch (NRS) was performed using Megazyme® kit (K-RAPRS 11/19), based on the methods of AOAC (Official Method 2002.02), and AACC (Method 32-40), with slight modifications. RS and NRS were calculated using Megazyme Mega-Calc™ (K-RAPRS), downloaded from www.megazyme.com.

2.4. Mineral and trace element analysis

The content of K^{+}, Mg^{2+}, Na^{+}, P^{3-}, Cu^{+}, Fe^{2+}, Mn^{2+}, Se, Zn^{2+} and Ca^{2+} was quantified after ash determination (2 g of sample in a Phoenix microwave furnace overnight at 780 °C). Samples were dissolved in concentrated nitric acid (1 mL), incubated for 10 min, and filled up to 25 mL with ultrapure water. The samples were filtered (0.45 μm nylon syringe filter) and analysed by ICP-OES iCAP 7600 DUO, Serial number: IC76DC151510; Model: Cetac ASX-520 auto-sampler.

2.5. Analysis of antinutritional factors (ANFs)

2.5.1. 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 measured for each sample at 520 and 700 nm. The total anthocyanin content was calculated as cyanidin-3-glucoside equivalents (mg CGE /L).

2.5.2. Cyanogenic glycosides

Cyanogenic glycosides were determined in a semi-quantitative approach as cyanides following solvent extraction (Hartanti & Cahyani, 2020). 2 g defatted quinoa flour samples were mixed with 20 mL 60 % ethanol, sonicated for 30 min, and centrifuged at 4150*g* for 10 min. The cyanide content was determined with Cyanide Test Kit, colorimetric (MQuant®) in the supernatant using a colorimetric cyanide kit based on the reaction of cyanide ions with chlorinating agent to form cyanogenic chloride.

2.5.3. Oxalates

The oxalate content was determined according to Adeniyi et al. (2009) with some modifications. Briefly, 2 g of sample were digested for 1 h with 10 mL of 6 M HCl at 95 °C and adjusted to a total volume of 250 mL. After filtration, the solution was titrated with NH_4OH until colour change (salmon pink to faded yellow) and filtered again. Insoluble oxalate was precipitated by heating to 90 °C and addition of $CaCl_2$ (5 %). Following centrifugation (1800 *g*, 20 min), the pellet was mixed with H_2SO_4, filtered, and made up to 300 mL. Then, the solution was heated near boiling point and titrated against 0.05 M $KMnO_4$ to a faint pink colour. Oxalates were calculated in mg/100 g sample according to the following equation where 16.012 mg represents the weight of CaC_2O_4 in 100 g, 2.4 and 2 representing the dilution factor and weight of sample, respectively.

$$Oxalate = \left(\frac{Volume\ titration\ (ml)\ x\ 16.012\ mg\ x\ 2.4}{2}\right) x\ 100$$

2.5.4. Phytic acid

Phytic acid extraction was performed following the method of Young (1936), with slight modifications. Briefly, 0.5 g defatted quinoa was 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. In a 96 well plate, 150 μL of sample was added, followed by addition of 50 μL Wade reagent, and incubation at RT before absorbance measurement at 500 nm.

2.5.5. Saponins

Saponins were extracted in defatted quinoa samples in a ratio of 1:20 in 80 % methanol for 16 h. The samples were then centrifuged at 4150*g* for 10 min, and the supernatant collected (Hiai et al., 1976). 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 spectrophotometer. Diosgenin was used as standard (0.5–0.1 mg/mL), and the saponins content was expressed as mg of diosgenin per 100 g of sample (mg/100 g).

2.5.6. Lectins

The lectin content was determined using Gonzalez De Mejia et al. (2005) protocol extraction, and then a semi-quantitative hemagglutination assay was used. Lectins were extracted by mixing 1 g of flour sample with 10 mL of 10 mM PBS, pH 7.4 for 12 h at 4 °C. The supernatant resulting 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.

2.5.7. Tannins

Tannins were extracted by mixing 0.5 g of defatted sample and 5 mL 4 % HCl in methanol for 18 h (Gonzalez De Mejia et al., 2005). The samples were then centrifuged at 4500*g* for 10 min and the supernatants collected. In a 96-well plate, 50 μL of sample extract, 100 μL of 1 % vanillin in methanol, 100 μL of 10 % HCl in methanol, were added, mixed, and incubated for 10 min at RT. The absorbance was determined at 500 nm using a plate reader. Catechin was used as standard (1.0–0.25 mg/mL), and the tannin content was expressed as mg of catechin equivalent per gram of sample (mg CE/g).

2.5.8. Trypsin inhibitors

Trypsin extraction was carried out by mixing 0.5 g defatted sample 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 (TUI/mg sample).

2.6. Total phenolic content by Folin-Ciocalteu and fast blue BB assays

The total phenolic content (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 determine the true content of polyphenols. The approach of Pico et al. (2020) was followed with slight modifications. Thus, EPC (extractable phenolic compounds) and SPE (solid phase extraction) fractions were analysed using FC and FBBB assays. For the FC assay, 10 μL sample/standard and 40 μL FC reagent (25 %) were added to wells in a 96 well-plate, followed by addition of 150 μL 4 % Na_2CO_3. After a 30 min incubation at RT in the dark, the absorbance was determined at 765 nm with gallic acid used as

standard (Fernando et al., 2022).

The FBBB reaction is based on its coupling specificity to the diazonium group of the FBBB reagent to an aromatic ring with activating hydroxyl group (Pico et al., 2020). For the assay, 200 µL of sample/standard and 20 µL of FBBB reagent (0.1 %) were added to a 96 well-plate, shaken for 5 s and incubated for 1 min. After addition of 20 µL 5 % NaOH, the plate was incubated at RT for 120 min in the dark, followed by absorbance recording at 420 nm, against gallic acid as standard.

2.7. Amino acid analysis

The amino acid profiles of samples were analysed according to Carrasco-Castilla et al. (2012). 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 by HPLC after basic hydrolysis according to Yust et al. (2004). Amino acids were determined by HPLC, after derivatization with diethyl ethoxymethylenemalonate. D,L-aminobutyric acid was used as an internal standard and a 300 mm × 3.9 mm i.d. reversed-phase column (Novapack C_{18} 4 µm; Waters, Milford, MA. USA).

2.8. Protein quality parameters

2.8.1. In vitro protein digestibility (IVPD)

For measuring the IVPD of quinoa flours, samples were weight as 62.5 mg equivalents in protein, combined with 10 mL miliQ water and equilibrated at 37 °C at 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 s for 10 min (Nosworthy et al., 2018). The IVPD was calculated using the change in pH value over the 10 min period (Δ pH_{10min}) from the initial value of around pH 8.0 as follows:

$$IVPD = 65.66 + 18.10\ (\Delta\ pH_{10min})$$

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

2.8.2. AAS

The AAS of raw quinoa flours (Black, Yellow, and Red) was calculated using the amino acid composition and the FAO/OMS/UNU (1985) requirement pattern.

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

2.8.3. Essential amino acid index (EAAI) on total amino acids (TAA)

The EAAI (%) was calculated according to Pastor-Cavada et al. (2010) by dividing EAA by TAA and multiplication by 100.

2.8.4. Biological value (BV)

The BV (%) of quinoa protein was calculated according to Amza et al. (2013) by the following formula:

$$\mathrm{BV}\ (\%) = 1.09^{*}(EAAI) - 11.73$$

2.8.5. Protein efficiency ratio (PER)

The theoretical PER value of raw quinoa flours was calculated according to Amza et al. (2013) based on the following five equations.

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

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

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

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

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

2.9. Statistical analysis

All data were processed with Minitab and GraphPad Prism 10 software. One-way ANOVA with Tukey post-hoc test was used to compare the data from the three quinoa samples ($p < 0.05$). Data from the nutritional and antinutritional analysis is presented as mean ± standard deviation (SD) of quintuple measurements. A principal component analysis (PCA) and correlation analysis (Pearson) was performed for protein content, IVPD, ANFs, and phenolic compounds using GraphPad Prism 10 software.

3. Results and discussion

The consumption of plant-based foods has gained importance over recent years due to their environmental sustainability, ethical considerations, dietary preferences, and health benefits, such as hypoglycaemic, hypotensive, antioxidant, hypolipidemic, and anticancer properties (Manzanilla-Valdez et al., 2024; Nosworthy, Hernandez-Alvarez, et al., 2023). Plant protein sources contain varying amounts of ANFs, which are known to have adverse effects on human health. These effects include reduced protein digestibility, impaired mineral absorption, hemagglutination, and decreased amino acid bioavailability (Manzanilla-Valdez et al., 2024). Currently, there is a significant information gap regarding the presence or absence of ANFs in plant-based sources, particularly quinoa. Therefore, it is crucial to quantify the antinutrients present in various commercially available quinoa varieties.

Nutritional composition and protein quality parameters of Black, Yellow, and Red quinoa were analysed in raw flours, while ANFs analysis including anthocyanins, cyanogenic glucosides, oxalates, trypsin inhibitors, saponins, phenolic compounds, tannins and phytic acid were assessed in defatted quinoa samples to avoid lipid interferences.

3.1. Nutritional assessment

The nutritional composition of the three quinoa varieties is shown in Table 1. The protein content of raw quinoa flour ranged between 19.34 and 20.90 g/100 g, with the Yellow quinoa sample being lower compared to the average of the other two samples. The results are aligned with other authors, reporting protein contents between 13.7 and 23 g/100 g (Abugoch James, 2009; Aloisi et al., 2016; Balzotti et al., 2008). In general, quinoa has a higher protein content, compared with cereals such as barley (10.8–11.0 g/100 g), rice (7.5–9.1 g/100 g), oat (11.6 g/100 g), cowpea (8.8–12.1 g/100 g), ayocote bean (18.82 g/100 g) and corn (10.2–13.4 g/100 g), thus emphasizing quinoa is a rich source of protein (Elsohaimy et al., 2015; Graf et al., 2015; Maradini Filho et al., 2017; Osuna-Gallardo et al., 2023).

The lipid content in Black, Yellow, and Red raw quinoa was consistent with no difference, ranging from 3.89 to 5.15 g/100 g. Similarly, Rodríguez Gómez et al. (2021) reported lipid values of 3.90–5.21 g/100 g in six different varieties of quinoa, although others have found higher lipid contents (5.5–14.5 g/100 g) (Maradini Filho et al., 2017; Nowak et al., 2016; Präger et al., 2018). It has been reported that both genotype and environmental conditions, such as soil pH, soil salinity, water availability, temperature and altitude, can influence the proximate composition of quinoa seeds. These factors may have contributed to the observed variations in lipid content (Elsohaimy et al., 2015; Nowak et al., 2016; Präger et al., 2018; Rodrigues et al., 2020). Ash content, representing the total mineral composition following combustion (Table 1), showed minimal variation among the quinoa varieties, ranging from 2.32 to 2.58 g/100 g. These findings align with recent

Table 1
Nutritional composition of Black, Yellow, and Red quinoa raw flours (dry basis).

	Black	Yellow	Red
Moisture (g/100 g)	11.53 ± 0.07^{ab}	12.04 ± 0.12^{a}	10.90 ± 0.13^{b}
Ash (g/100 g)	2.57 ± 0.10^{a}	2.32 ± 0.11^{a}	2.58 ± 0.74^{a}
Lipid (g/100 g)	5.15 ± 0.14^{a}	3.89 ± 0.28^{a}	4.86 ± 0.68^{a}
Protein (g/100 g)	20.90 ± 0.17^{a}	19.34 ± 0.52^{b}	20.25 ± 0.20^{a}
TDF (% *w*/w)	22.97 ± 0.20^{a}	19.43 ± 0.45^{b}	16.15 ± 0.90^{c}
IDF (% w/w)	22.65 ± 0.10^{a}	16.83 ± 0.14^{b}	14.57 ± 0.06^{c}
SDF (% w/w)	0.32 ± 0.06^{c}	2.40 ± 0.31^{a}	1.58 ± 0.8^{b}
D-glucose (g/100 g)	56.07 ± 2.45^{b}	64.36 ± 2.63^{a}	66.4 ± 2.63^{a}
D-fructose (g/100 g)	4.04 ± 0.71^{a}	2.55 ± 0.63^{ab}	1.70 ± 0.30^{c}
Total available carbohydrates (g/100 g)	60.12 ± 3.16^{c}	66.9 ± 3.26^{a}	68.1 ± 2.93^{b}
RS (g/100 g)	9.05 ± 0.98^{b}	8.90 ± 0.75^{b}	10.29 ± 0.79^{a}
NRS (g/100 g)	44.36 ± 2.62^{b}	45.90 ± 3.07^{a}	44.28 ± 3.07^{b}
TS (g/100 g)	53.41 ± 3.6^{b}	54.8 ± 3.75^{a}	54.57 ± 3.79^{a}

Different superscript letters in the same row indicate statistical differences between quinoa flours by ANOVA and Tukey's multiple range test ($p < 0.05$). Data are expressed as mean ± SD, $n = 5$. IDF = insoluble dietary fiber, SDF = soluble dietary fiber, TDF = total dietary fiber, RS = resistant starch, NRS = non-resistant starch and TS = total starch.

literature, which reports average ash values between 2.01 and 2.97 g/100 g (Cavada et al., 2023; Elsohaimy et al., 2015). Furthermore, these findings are consistent with the Food Composition Database for Biodiversity (FAO/INFOODS), which lists a range of 2.3 to 4.8 g/100 g for quinoa (Angeli et al., 2020; Burrieza et al., 2019). Notably, quinoa seeds exhibit higher ash content compared to other common grains, such as rice (0.2 g/100 g), wheat (1.1 g/100 g) and corn (0.7 g/100 g) (De Bock et al., 2021; De Bock et al., 2022).

3.2. Carbohydrate analysis

3.2.1. Dietary fiber and available carbohydrates

In the current quinoa samples, the total available carbohydrate content ranged from 60.12 to 68.12 g/100 g, which is lower compared to other starch-rich crops such as wheat (86.3 g/100 g), maize starch (83.7 g/100 g), ripe banana (69.2 g/100 g), and commercial cereals (78.9 g/100 g) (McCleary et al., 2020). Quinoa has been recognized for its comparatively lower glycaemic index (GI), making it a favourable option for individuals with hyperglycaemia and impaired glucose tolerance. Based on the D-glucose content, which is indicative of the glycaemic index in foods (Priyanka et al., 2018), the Black quinoa variety particularly stands out with a low GI value of 50.28 g/100 g. This is in contrast to the Yellow and Red varieties, which contain 61.6 and 59.5 g glucose per 100 g, respectively, classifying them as medium GI foods. The lower GI of Black quinoa reinforces its suitability as a low GI alternative. While previous studies have identified sucrose as the principal sugar in quinoa, other sugars such as galacturonic acid, arabinose, xylose, glucose, galactose, arabinose, and fructose have also been reported (Lamothe et al., 2015; J. Liu et al., 2020; Pereira et al., 2019). However, the current methodology was limited to assessing D-fructose content, which ranged from 1.70 to 4.04 among the three quinoa varieties.

The consumption of dietary fiber is strongly recommended for human health, as it promotes gastrointestinal motility, lowers blood glucose and cholesterol levels, and is beneficial in the management of cardiovascular disease, type 2 diabetes mellitus, and cancer (Kurek et al., 2018; Lamothe et al., 2015; Liu et al., 2020). Total dietary fiber (TDF) can be categorized into soluble dietary fiber (SDF) and insoluble dietary fiber (IDF). Previous studies have demonstrated that SDF possess superior physicochemical properties, such as water and oil holding capacities, compared to IDF (J. Liu et al., 2020). Consequently, cereals and pseudocereals with a significant SDF content (> 6 g/100 g) are promising candidates for the formulation of various food products, including bread, confectionery and healthy snacks (Dhingra et al., 2012). According to existing literature, quinoa samples typically exhibit TDF values ranging from 7.0 to 16.43 g/100 g, with SDF ranging from 1.3 to 6.1 g/100 g, and IDF ranging from 4.9 to 14.39 g/100 g (Kurek et al., 2018; Miranda-Ramos & Haros, 2020; Repo-Carrasco-Valencia & Serna, 2011; Ruales et al., 2002). In contrast, in the current study, both TDF and IDF were found to be higher than those reported in the literature, with the highest levels observed in Black quinoa (22.97, 22.65 g/100 g), followed by Yellow (19.43, 16.83 g/100 g) and Red quinoa varieties (16.15, 14.57 g/100 g). These discrepancies may be attributed to varietal differences or the effects of processing, such as grinding, heating, soaking or exposure to solvents, which are known to influence the composition and availability of TDF (Dhingra et al., 2012). Moreover, when compared to cereals, quinoa generally exhibits a markedly higher TDF content than wheat (10.7 g/100 g), rice (2.8 g/100 g), white bread (5.8 g/100 g) and corn (7.3 g/100 g) (McCleary, 2023). Interestingly, in this study, Black quinoa exhibited the lowest soluble SDF content (0.32 %) compared to Red (1.58 %) and Yellow (2.40 %) quinoa varieties. Despite having the highest TDF content, the differences in SDF suggest that Black quinoa may have distinct techno-functional properties, potentially influencing its applications in food formulations compared to the other quinoa varieties.

3.2.2. Resistant starch (RS) and non-resistant starch (NRS)

RS is defined as the portion of starch that resists hydrolysis by human enzymes in the small intestine (McCleary et al., 2020). RS can be categorized into five subtypes based on its origin and structure (McCleary, 2023; McCleary et al., 2020). Starch is the major component in quinoa, with total starch content reported to range from 53.4 to 91.9 g/100 g (Dong et al., 2021; Junejo et al., 2022; G. Li & Zhu, 2017; Peng et al., 2022). The primary structure of quinoa starch is predominantly amylopectin, which imparts unique physicochemical properties, such as low gelatinization temperatures and slow retrogradation, to quinoa (Dong et al., 2021; G. Li & Zhu, 2018). While the total starch content reported in this study aligns with the literature, there is relatively limited information on the resistant and digestible starch fractions in quinoa. The RS values observed in this study, ranging from 8.9 to 10.3 g/100 g, are consistent with the findings of Peng et al. (2022), who reported RS values ranging from 5.94 to 11.71 g/100 g in native quinoa. However, RS content in quinoa is generally lower than that found in maize (41.7 g/100 g), green banana (38 g/100 g), pinto beans (35.6 g/100 g), and potato starch (56.8 g/100 g) (McCleary, 2007, 2023).

RS is fermented in the colon, leading to the production of short-chain fatty acids such as butyrate, acetate and propionate, which play a key role in stimulating enteroendocrine hormones such as glucagon-like peptide-1 (GLP-1) and peptide YY. These hormones have beneficial effects on carbohydrate metabolism (Bojarczuk et al., 2022). Therefore, a higher RS content in foods can positively influence gut microbial fermentation, leading to both local and systemic beneficial effects (Ashwar et al., 2016; Bojarczuk et al., 2022). Overall, the research suggests that the digestion of carbohydrates in quinoa seeds is slowed, primarily due to the high TDF and starch content, making quinoa a valuable contributor to reducing the risk of hypoglycaemia.

3.3. Mineral composition

The mineral content of quinoa flour is notably higher compared to

other cereals such as maize, rice and wheat (Angeli et al., 2020), a finding that is supported by the comparatively higher ash content observed in this study. The current mineral and trace element concentrations (Table 2) align with previously reported values, including Ca^{2+} (514–1360 mg/Kg), and Zn^{2+} (21.5–48 mg/Kg) as documented by Angeli et al. (2020), as well as Na^{+} (57–160 mg/Kg) and P (4322–5716 mg/Kg) reported by De Bock et al. (2021). Among the quinoa varieties analysed, Red quinoa showed higher concentrations of K^{+}, Mg^{2+}, Na^{+}, P^{3-}, Cu^{2+}, Se and Zn^{2+}, compared to Black and Yellow quinoa. Notably, there is a lack of data regarding trace elements such as Cu^{2+}, Mn^{2+} and Se content in quinoa seeds. Vega-Gálvez et al. (2010) indicated that soil type and fertilizer application can significantly influence the mineral composition of quinoa, thus, potentially leading to variability in the concentration and availability of specific elements. Additionally, studies by Präger et al. (2018) and De Bock et al. (2021) have demonstrated considerable variability in mineral content among different quinoa sources, with varieties from Peru, for instance, showing higher quantities of Mg^{2+}, Fe^{2+}, Ca^{2+} (Reguera et al., 2018).

3.4. ANFs

In order to minimize interferences during the assessment of ANFs, a defatting process was implemented on all quinoa flours, using hexane as solvent (Huang et al., 2021). This defatting procedure effectively reduced total lipid content by 93 % across all three samples, demonstrating its efficacy. Previous studies have quantified various ANFs in quinoa (Maradini Filho et al., 2017), however, certain ANFs such as cyanogenic glycosides and lectins have not been extensively quantified to date.

Polyphenols are known to interact with proteins, forming "protein-phenolic" complexes, that can alter the secondary and tertiary structure of proteins, affecting their thermal stability and techno-functional properties (Günal-Köroğlu et al., 2023). Moreover, these interactions may also result in reduced EAA and decreased protein digestibility (Manzanilla-Valdez et al., 2024). In the present study, polyphenols quantification was performed using both the widely used FC assay and the more recently developed FBBB assay. The FC assay is known to interact with several compounds, such as monosaccharides, aromatic amines, sulfur dioxide, ascorbic acid, vitamins, ketones, thiols and organic acids (Ravindranath et al., 2021), potentially leading to an overestimation of the total polyphenol content. In contrast, the FBBB assay has been proposed for its specificity in coupling the diazonium group of the reagent to aromatic rings (Medina, 2011). As shown in Table 3, the TPC was significantly higher across all quinoa varieties when measured with the FBBB assay compared to the FC assay, with Black and Yellow quinoa displaying a 2.7-fold increase and Red quinoa a1.5-fold increase. Pico et al. (2020) reported that the FBBB assay is more sensitive than the FC assay for various samples, including quinoa, red kidney bean, brown rice, lentils, chickpea, and yellow corn. The observed differences in fold change between the FC and FBBB assays for different quinoa varieties highlight a key limitation of the FC assay: its differential responsiveness to individual polyphenols, which can obscure accurate detection of varietal differences in polyphenol composition (Medina, 2011; Pico et al., 2020).

To address potential interferences, present in the extracts, this study employed SPE to remove interfering compounds such as reducing sugars, proteins, ascorbic acid and enediols prior to measuring the polyphenol content (Pico et al., 2020). This approach improved the accuracy of the FC/FBBB assays in detecting the "true" polyphenol content in quinoa extracts. Notably, Yellow and Red quinoa varieties showed a 6.38 and 5.26 % reduction in polyphenol content with the FC assay after SPE, indicating the presence of interfering compounds in the initial extract (EPC), whereas no significant reduction was observed in the Black quinoa sample (Table 3). Furthermore, no marked reduction was detected in FBBB assay results post-SPE, confirming the specificity of the FBBB method. These findings are consistent with those reported by Pico et al. (2020), who reported SPE-processed quinoa extracts yielding 113.67 and 315.93 mg GAE/100 g when measured by FC and FBBB assays, respectively.

Meanwhile, Nickel et al. (2016) reported TPC values of 97.60 and 116.77 mg GAE/100 g in natural quinoa measured by FC. Miranda et al. (2010) reported a lower value of 28.41 mg GAE/100 g in quinoa seeds, while L. Li et al. (2021), assessed thirteen quinoa varieties, yielding values that ranged between 89 and 213 mg GAE/100 g (FC). These results are comparable, though slightly lower than those values reported in this study, likely due to differences in the extraction methods and conditions. Overall, the TPC reported in this study exceeds those of cereals such as wheat (56 mg GAE/100 g), rye (103 mg GAE/100 g), millet (139 mg GAE/100 g) and barley (88 mg GAE/100 g) (Li et al., 2021).

Anthocyanins, a subgroup of flavonoids, are water-soluble pigments responsible for the red, purple, and blue hues in many fruits and vegetables (Constantin & Istrati, 2022; Mattioli et al., 2020). Anthocyanins stability is influenced by factors such as pH, light, temperature, and molecular structure (De Pascual-Teresa & Sanchez-Ballesta, 2008). Anthocyanins have demonstrated antioxidant and anti-inflammatory properties (Farzaneh & Carvalho, 2017; Kanha et al., 2019; Pastor-Cavada et al., 2010), but they can also reduce protein digestibility by forming complexes with proteins, thus decreasing their bioavailability (Ayvaz et al., 2023; Garutti et al., 2022). In this study, Black quinoa showed the highest anthocyanin concentration (19.3 mg CGE/L), with significantly lower concentrations in Yellow (16.9 mg CGE/L), and Red quinoa (15.8 mg CGE/L), (Fig. 1a). Gorinstein et al. (2007) reported higher anthocyanin values in quinoa (96.4 mg CGE/100 g) and in three different varieties of amaranth (83.0–94.6 mg CGE/100 g). Paśko et al. (2010) also reported higher anthocyanin values in pseudocereals, with

Table 2
Mineral content of Black, Yellow, and Red quinoa raw flours (ppm).

Minerals	Black	Yellow	Red
Ca^{2+}	466.9 ± 1.14^{a}	478.7 ± 1.15^{a}	366.2 ± 0.36^{b}
K^{+}	5141.5 ± 16.3^{ab}	4552.1 ± 9.7^{b}	6322.5 ± 0.3^{a}
Mg^{2+}	1451.2 ± 6.9^{a}	1550.3 ± 3.0^{a}	1523.2 ± 6.1^{a}
Na^{+}	31.8 ± 0.8^{b}	114.7 ± 3.0^{b}	294.5 ± 6.5^{a}
P^{3-}	4061.8 ± 15.1^{b}	4190.9 ± 11.9^{b}	4723.9 ± 13.7^{a}
Fe^{2+}	38.5 ± 0.12^{a}	31.8 ± 0.07^{b}	41.4 ± 0.09^{a}
Cu^{2+}	5.8 ± 0.0^{a}	3.8 ± 0.01^{b}	5.9 ± 0.03^{a}
Mn^{2+}	36.6 ± 0.14^{a}	35.8 ± 0.04^{a}	18.8 ± 0.02^{b}
Se	0.028 ± 0.0^{a}	0.045 ± 0.0^{a}	0.027 ± 0.0^{a}
Zn^{2+}	27.8 ± 0.04^{c}	21.3 ± 0.05^{b}	35.0 ± 0.11^{a}

Different superscript letters in the same row indicate statistical differences between quinoa flours by one-way ANOVA and Tukey's multiple range test ($p < 0.05$). Data are expressed as mean ± SD, $n = 5$.

Table 3
Total polyphenols content in extracts measured by Folin–Ciocalteu and FBBB reactions (expressed in mg/100 g of gallic acid equivalents) without the removal of interferences (Control, EPC) and after the removal of soluble interferences by solid phase extraction (SPE). The percentage of decrease/increase in the content of phenolics compounds value from the EPC to the SPE is indicated as ↑↓ (%).

Sample	FC			FBBB		
	Control (EPC)	SPE	↑↓ (%)	Control (EPC)	SPE	↑↓ (%)
Black	112.5 ± 4.5^{b}	111.9 ± 3.5^{b}	↓ 0.53	305.3 ± 9.1^{bc}	317.1 ± 18.2^{ab}	↑ 3.72
Yellow	126.8 ± 3.5^{b}	118.7 ± 0.5^{b}	↓ 6.38	338.9 ± 31.8^{a}	334.7 ± 14.7^{a}	↓ 1.23
Red	182.5 ± 8.5^{a}	172.9 ± 4.7^{a}	↓ 5.26	279.4 ± 20.8^{c}	287.6 ± 11.5^{c}	↑ 2.85

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.

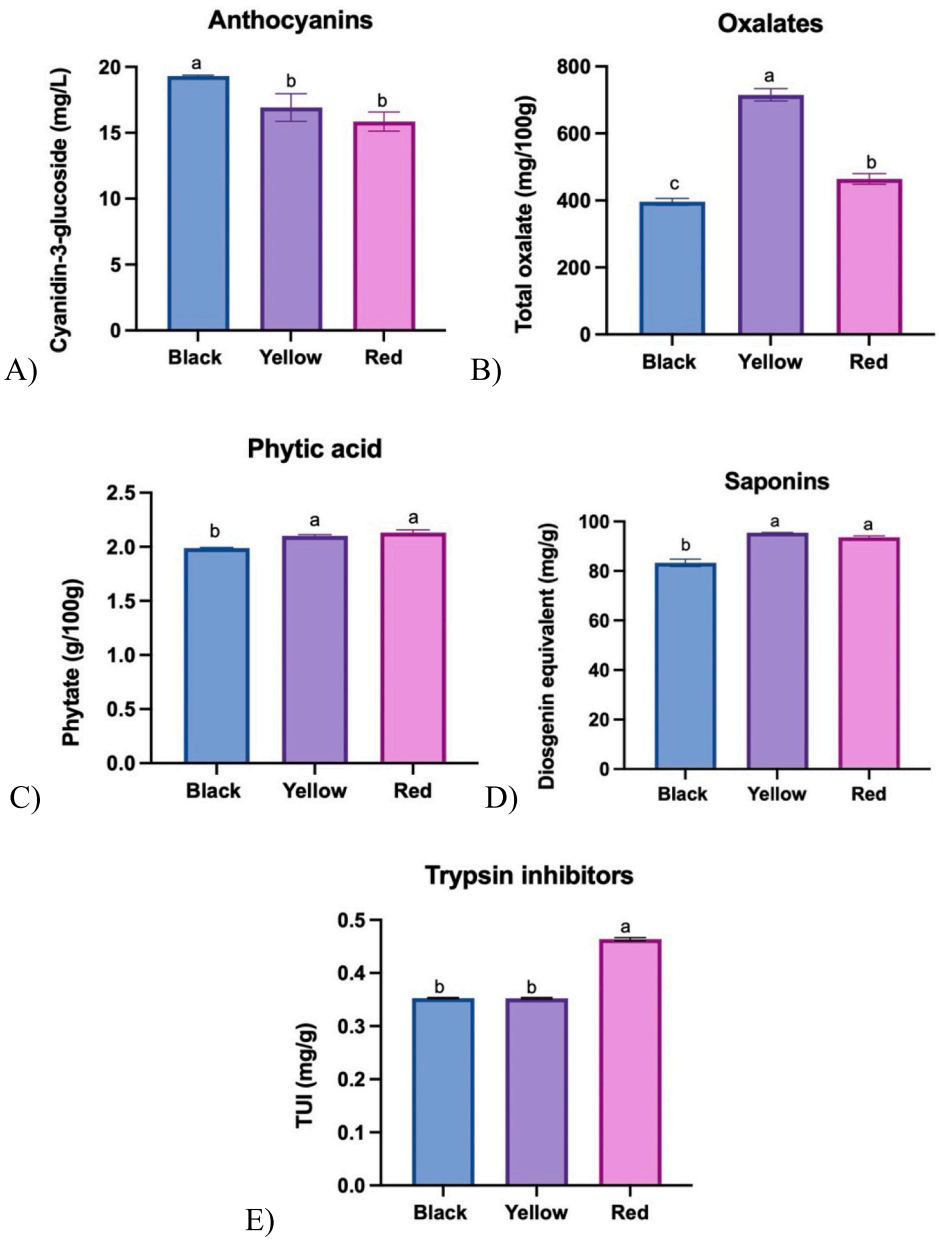

Fig. 1. Antinutritional factors of Black, Yellow, and Red quinoa defatted flours. a) anthocyanins, b) oxalates, c) phytic acid, d) saponins, e) trypsin inhibitors. Different superscript letters between bars indicate statistical analysis differences between quinoa flours by One-way ANOVA and Tukey's multiple range test. Data are expressed as mean ± SD, $n = 5$, ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

120 mg CGE/100 g in quinoa, 103.6 mg CGE/100 g in Amaranthus Aztek, and 90.83 mg CGE/100 g in Amaranthus Rawa. These values suggest that anthocyanin content in quinoa can increase due to factors such as germination and light exposure, a trend also observed in buckwheat. Anthocyanin values in quinoa reported by different authors (Gorinstein et al., 2007; Paśko et al., 2010) are higher than those reported in *Amaranthus hybridus* (83.5 mg CGE/100 g), *Amaranth hypochondriacus* (91.0 mg CGE/100 g), *Amaranth cruentus* (94.6 mg CGE/100 g), jasmine rice (83.0 mg CGE/100 g) (Gorinstein et al., 2007), and Black sorghum (11.0 mg CGE/100 g) (Awika et al., 2005).

Cyanogenic glycosides are naturally occurring nitrogenous plant secondary metabolites that are highly toxic due to their ability to inhibit the electron transport system by binding to cytochromes, releasing hydrogen cyanide (HCN) upon tissue disruption and enzymatic hydrolysis during digestion (Bolarinwa et al., 2016; Cowan et al., 2021; P. Thakur et al., 2021). Given the amino acid profiles in quinoa flours, it was hypothesized that Yellow quinoa would contain lower levels of cyanogenic glycosides compared to Black and Red quinoa (Supplementary Material a). Indeed, Black and Red quinoa exhibited similar HCN levels (0.004–0.007 mg/L CN^-), which are substantially lower than those found in soy protein (0.07–0.3 mg HCN per kg), soybean hulls (1.24 mg HCN per kg), apricot pits (89–2170 mg HCN per kg), cassava leaves (451 mg HCN per kg), sorghum (2400 mg HCN per kg), and lima beans (2000–3000 mg HCN per kg) (Simeonova & Fishbein, 2004). Human toxicity occurs at doses between 0.5 and 3.5 mg HCN per kg of body weight (Bolarinwa et al., 2016), while the FAO/WHO limit is 10 mg HCN per kg. Based on the results obtained in this study, quinoa poses not toxicity risk for human consumption. Cyanogenic compounds can be decreased by 25–98 % through cooking, soaking and fermentation (Salim-ur-Rehman et al., 2014).

Oxalates or oxalic acid are organic acids capable of forming water-soluble salts when binding to Na^+, K^+ and NH^{4+} and water-insoluble

complexes when bound to Ca^{2+}, Fe^{2+} and Zn^{2+} (Lo et al., 2018; López-Moreno et al., 2022). In plants, oxalate synthesis is essential for calcium regulation, plant defence mechanisms and heavy metal detoxification (Petroski & Minich, 2020). The binding of oxalates to calcium and other minerals in the stomach and small intestine can significantly reduce mineral absorption, which is the primary reason oxalates are considered as ANFs (Lo et al., 2018). Additionally, oxalates pose other health risks, such as the formation of kidney stones (Maradini Filho et al., 2017). High oxalate contents are commonly found in plants belonging to the Amaranthaceae, Chenopodiaceae, and Polygonaceae families (Li & Savage, 2015; Lo et al., 2018; López-Moreno et al., 2022). According to these authors, a normal diet should not exceed 50–200 mg oxalates per day to avoid potential adverse effects. The current study found high oxalates levels in Yellow (715.2 mg/100 g), Red (464.3 mg/100 g), and Black (396.0 mg/100 g) quinoa varieties (Fig. 1b). These findings are consistent with existing literature, where oxalate concentrations in quinoa seeds, have been reported in the range of 131-184 mg/100 g (Lo et al. (2018); Siener et al., 2006). Although, cultivation conditions may influence oxalic acid accumulation, processing methods such as boiling in water, cooking, and soaking have been shown to effectively reduce oxalate content by 30-76.9 % (Franceschi & Nakata, 2005). It is noteworthy that the seeds used in this study were not washed or processed, aside from milling and sieving.

Phytic acid, also known as phytate or myo-inositol hexaphosphate (IP6), is a major phosphorus storage form in plants, synthesized during seed development. This compound can form insoluble complexes with Zn^{2+}, Fe^{2+}, Mg^{2+} and Ca^{2+} in the gastrointestinal tract, reducing their bioavailability (López-Moreno et al., 2022; A. Thakur et al., 2019). The current study observed phytic acid concentrations of 1.97, 2.13 and 2.21 g/100 g in Black, Yellow, and Red quinoa, respectively (Fig. 1c). These values are slightly higher than those reported by Maldonado-Alvarado et al. (2023), which ranged from 1.07 to 1.22 g/100 g for White, Red and Black quinoa, respectively, possibly due to differences in quinoa varieties and growing conditions, as their study involved quinoa grown in Latin America. Rosero et al. (2013) reported lower phytic acid levels (0.97 and 1.94 mg/ g) in four varieties of quinoa, Nariño variety from Colombia, commercial quinoa “Anapqui’s” and -IICA- 020–Oruro from Bolivia and quinoa Huancavelica from Peru, likely attributable to prior desaponification and phytase activity (Rosero et al., 2013). Germination and fermentation processes can further reduce phytic acid content, due to the activation of intrinsic phytase (Maldonado-Alvarado et al., 2023; Maradini Filho et al., 2017). Additionally, the high presence of Zn, Fe and Mg in this study’s Red quinoa may contribute to its elevated phytic acid levels (Table 2) (Lo et al., 2018). Nonetheless, the high TDF content in quinoa may mitigate the negative effects of phytic acid (López-Moreno et al., 2022). Overall, soaking, germination, and fermentation are affective methods for reducing phytate levels (Egli et al., 2002; Maradini Filho et al., 2017; Starzyńska-Janiszewska et al., 2023).

Saponins are triterpene glycosides, characterized by their amphipathic structure, consisting of hydrophobic aglycones and hydrophilic carbohydrate chains (Lo et al., 2018). These compounds are present in several plant families, including Amaranthacea, Apiacea, Curcubitaceae, Lamiaceae and Polygalaceae, as well as in quinoa seeds, ginseng root, soybean, chickpeas, alfalfa, and licorice (Lo et al., 2018; Navarro del Hierro et al., 2018). Saponins are water and ethanol-soluble and can have toxic effects, such as haemolysis of erythrocytes, and can also form complexes with Fe, and Zn, reducing their bioavailability (Maradini Filho et al., 2017). Despite their potential toxicity, saponins exhibit various biological activities, including antimicrobial, antiviral, anticancer, antidiabetic, antithrombotic and anti-inflammatory effects (Rodríguez Gómez et al., 2021; A. Thakur et al., 2019). Quinoa contains several saponins structures including oleanolic acid, hederagenin, phytolaccagenic acid and 30-*O*-methyl-espergulagenate (Maradini Filho et al., 2017). Depending on the saponins content in plants, these can be classified in 2 groups: sweet (20–40 mg/ g^{-1}) and bitter (>470 mg/g^{-1}) (Angeli et al., 2020). Hernández-Ledesma (2019) reported the saponin content in quinoa seeds from different genotypes, this varied among 140 to 2300 mg/100 g in bitter genotypes and 20 to 40 mg/100 g in sweet genotypes. The current study found saponin concentrations of 83.27, 95.51 and 96.82 mg/g in Black, Yellow, and Red, respectively (Fig. 1d). These values are higher compared to Starzyńska-Janiszewska et al. (2023), who reported a saponins content of 11.67 mg/g in high-saponin quinoa seeds, which was composed of different types of saponins such as hederagenin (47.2 %), oleanolic acid (30.5 %), phytolaccagenic acid (20.2 %) and serjanic acid (2.1 %). The extraction solvent plays a crucial role in determining the amount and types of saponins extracted, with ethanol yielding higher recovery compared to water (ethanol 5.51 g/100 g; ethanol: water 4.43 g/100 g; and water 0.26 g/100 g) (Navarro del Hierro et al., 2018). Positive biological effects of saponins in quinoa have been demonstrated, with Zhang et al. (2022) showing that saponin doses up to 50 mg/kg body weight are nontoxic in rats, while higher amounts may affect gut microbiota composition, liver, and kidney cells. Saponins content can be decreased by soaking in cold water, washing and cooking, with an approximate reduction of 15 % (Lo et al., 2018; Vega-Gálvez et al., 2010).

Trypsin inhibitors, part of protease inhibitor family, are widely distributed in the plant kingdom, particularly in legumes (K. Liu, 2021; A. Thakur et al., 2019). These inhibitors reduce protein digestibility and can cause gastrointestinal discomfort, including diarrhoea and gas (Miranda et al., 2010). Quinoa contains low levels of trypsin inhibitors, which are not considered harmful to human health (Vega-Gálvez et al., 2010). The current study found trypsin inhibitor levels of 0.36 TUI in Black, 0.35 TUI in Yellow, and 0.46 TUI Red quinoa **(**Fig. 1e**).** These values fall within the range reported for different quinoa varieties (0.17–15.09 TUI/mg protein) (Maradini Filho et al., 2017; Pesoti et al., 2015; Tavano et al., 2022), which are considerably lower than those found in soybean (24.5–41.5 TUI/mg), beans (12.9–42.8 TUI/mg) and lentils (17.8 TUI/mg) (Liu, 2021). Trypsin inhibitors can be reduced through heat treatments such as boiling, roasting, autoclaving and microwave cooking, as well as by soaking in water for 18 to 22 h (Maradini Filho et al., 2017; Osuna-Gallardo et al., 2023).

Lectins are carbohydrate-binding glycoproteins with key roles in nitrogen storage for plant development and protection against predators (De Coninck & Van Damme, 2022). Lectins are typically found in nuts, cereals, and legumes, lectins are resistant to gastrointestinal enzymes, allowing them to interact with intestinal epithelial cells, alter cell permeability, and reduce nutrient absorption, including protein utilization (Hartanti & Cahyani, 2020; López-Moreno et al., 2022). Despite these potential adverse effects, lectins have shown clinical benefits, including antitumoral and anticancer activity in different cell lines (Cavada et al., 2023; Konozy et al., 2022). The current study’s qualitative hemagglutination assay indicated that lectins were not detectable below concentrations of 1.1 mg/mL in Black, 0.94 mg/mL in Yellow, and 1.39 mg/mL in Red quinoa varieties (Fig. 2). Adamcová et al. (2021) reported higher lectin concentrations in *Phaseolus vulgaris*, *Glycine* max and *Lycopersicon esculentum*, with hemagglutination occurring at much lower concentrations (0.1 mg/mL, 0.2 μg/mL and 1.0 μg/mL) compared to the quinoa varieties examined in this study.

Tannins, a group of high molecular weight phenolic compounds (ranging from 500 to 3000 Da), exhibit heat stability and have a specific affinity for binding to proteins and starches, consequently leading to a reduced nutritional value and availability (Maradini Filho et al., 2017; Petroski & Minich, 2020). Tannins are known to inhibit amylase activity and interfere with iron absorption (Lo et al., 2018; A. Thakur et al., 2019). Furthermore, tannins can form complexes with proteins between the hydroxyl group of tannins and the carboxyl group of proteins, thus leading to a decrease in EAA availability and protein digestibility (Samtiya et al., 2020). In quinoa seeds, tannin content has been reported to be around 0.53 % (Maradini Filho et al., 2017). However, at these low concentrations, tannins are not considered to pose significant health risks for quinoa consumption. In the present study, only the Black

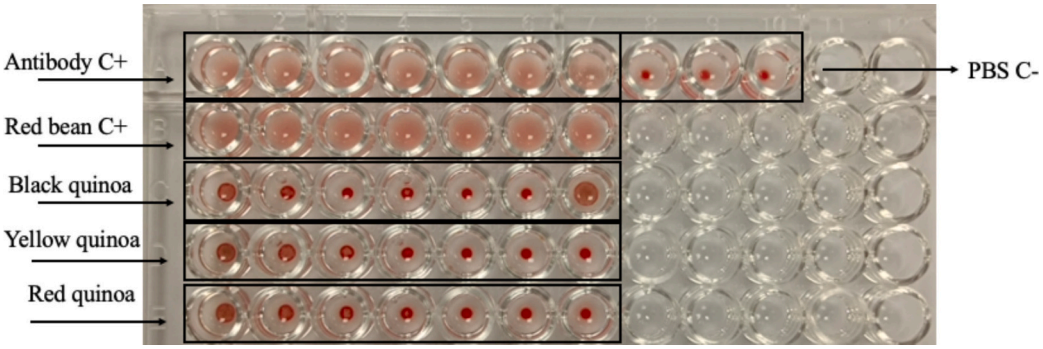

Fig. 2. Hemagglutination assay.
Positive control (C+) Antibody: 0.8, 0.4, 0.2, 0.1, 0.05 and 0.025 mg/mL. Red bean lectin (C+): 2.9, 0.8, 0.4, 0.2, 0.1, 0.05 and 0.025 mg/mL. Negative control (C-) PBS: 25 mL per well, Black quinoa: 2.21, 1.1, 0.55, 0.27, 0.13, 0.69 and 4.42 mg/mL. Yellow quinoa: 3.79, 1.89, 0.94, 0.47, 0.23, 0.12 and 0.059 mg/mL. Red quinoa: 5.6, 2.79, 1.39, 0.69, 0.35, 0.17 and 0.087 mg/mL. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

quinoa exhibited a detectable tannin content of 2 mg/100 g, whereas no tannins were detected in the Yellow and Red quinoa varieties, suggesting that tannins are either absent or present in negligible amounts in these varieties. The tannin content in Black quinoa is significantly lower than that reported for other grains such as amaranth (51.6–82.7 mg/100 g) (Jo et al., 2015), sorghum liberty (874 mg/100 g), sorghum alpha (36 mg/100 g) and sorghum IS8237C (265 mg/100 g) (Wu et al., 2018). Processing methods, such as washing and cooking, have been shown to reduce tannin content and enhance digestibility (Petroski & Minich, 2020). For instance, Wu et al. (2018) reported that steaming, flaking and toasting of sorghum grains can result in a substantial reduction in tanning content, ranging from 70 to 94 %. These findings suggest that appropriate processing techniques could further mitigate the potential negative effects of tannins, thereby improving the nutritional quality of quinoa.

3.5. Amino acid profile

EAA such as lys, leu, trp, val, ile, his, met, phe and thr are critically needed for human growth and metabolic functions (Dakhili et al., 2019). However, not all plant-based protein sources are equally rich in EAA. For example, cereals are typically deficient in lys, while legumes often lack sufficient met and cys (De Bock et al., 2021; De Bock et al., 2022). Over the years quinoa has gained attention due to its high protein content and balanced amino acid profile, making it an excellent option for human nutrition (De Bock et al., 2021; Elsohaimy et al., 2015). As shown in Fig. 3, amino acid profile compared to FAO/WHO guidelines revealed that the Black, Yellow and Red quinoa varieties presented higher values for ser, his, tyr, arg, val, ile, leu, lys, tyr, and phe than those previously reported by Elsohaimy et al. (2015), and Repo-Carrasco et al. (2003). However, there were no significant differences in gly, thr, pro, val, and trp. These observed differences between quinoa varieties, could be attributed to different factors such as soil salinity, pH, weather conditions, and water availability during growth, all of which can impact the macro- and micronutrient composition, as well as ANFs and amino acid profile (Chen et al., 2023). Only met, cys and gly showed lower values compared to previous reports (De Bock et al., 2021). Despite this, the quinoa flours studied met and exceed the FAO/WHO requirements for infants (FAO/WHO/UNU, 2007), thus fulfilling the necessary EAA requirements (Mota et al., 2016).

3.6. In vitro protein digestibility and protein quality

As presented in Table 4, protein quality parameters such as IVPD, AAS, EAAI, BV, IVPDCAAS and $PER_{1\text{-}5}$ were calculated. Quinoa flours showed an IVPD of 77.69 %, 77.61 % and 76.90 % for Black, Yellow, and Red, respectively. These findings are similar to those reported by Repo-Carrasco-Valencia and Serna (2011) for four different quinoa varieties (76.32–80.54 %), and closely align with the results of Elsohaimy et al. (2015) (78.37 %). The IVPD values reported in this study, are also comparable to those for amaranth seeds (76.03 %) (Najdi Hejazi et al., 2016), and raw pulses (75.01–84.85 %) (Sánchez-Velázquez, Ribéreau, et al., 2021), and are notably higher than those for wheat (47–59 %) (Elsohaimy et al., 2015). In terms of AAS, Red quinoa exhibited the lowest value (0.32 SAA), while Yellow quinoa had highest (0.45 SAA). An AAS score closer to or exceeding 1 indicates the absence of limiting

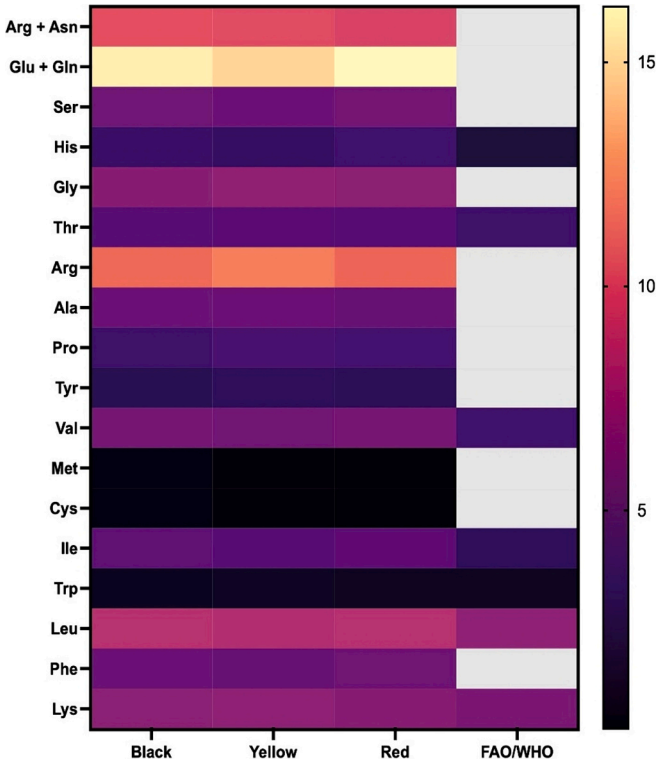

Fig. 3. Heat map of amino acid profile (amino acid g/100 $g_{protein}$) of quinoa flours and FAO/WHO infant pattern (1985). The change in colour of the scale from black to yellow indicate the amino acid content from low to high. Gly; glycine, Lys; lysine, Gln; glutamine, Glu; glutamic acid, Ser; serine, 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. Data presented as mean, $n = 5$, ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
In vitro protein digestibility and protein quality of Black, Yellow, and Red raw quinoa.

Quinoa	IVPD (%)	AAS[a]	EAAI[b] (%)	BV[c] (%)	PER 1[d]	PER2[d]	PER3[d]	PER4[d]	PER5[d]	IVPDCAAS[e] (%)
Black	77.69 ± 0.65[a]	0.44 (SAA)	240.87	250.81	2.90	3.00	3.18	2.75	3.14	34.18
Yellow	77.61 ± 0.54[a]	0.45 (SAA)	91.80	88.33	2.77	2.86	2.53	2.67	3.14	34.92
Red	76.90 ± 1.17[a]	0.32 (SAA)	57.35	50.79	2.87	2.95	2.75	2.69	3.12	23.96

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.

amino acids in the food being analysed (Nosworthy, Medina, et al., 2023). The AAS scores for Black and Yellow quinoa were higher than those reported for millet (0.38 Lys) (Culetu et al., 2021). The use of raw quinoa flours in this study likely explains the relatively low AAS scores. Nosworthy et al. (2018), demonstrated that cooking and extrusion can significantly increase the AAS of beans (*Phaseolus vulgaris* and *Vicia faba*).

Regarding IVPDCAAS, which used infant amino acid pattern requirements (FAO/OMS/UNU, 1985) as reference, due to their higher demands compared to adults and adolescents, the highest IVPDCAAS value was observed for Yellow quinoa (34.92 %), while Red quinoa had lowest (23.96 %) (Table 4). Although these IVPDCAAS values are lower compared to other studies, it should be noted that the quinoa samples in this study were not subjected to any processing. Previous research has shown that cooking and extrusion can significantly increase the IVPDCAAS in oat and bean products (Nosworthy et al., 2018 and Sánchez-Velázquez, Cuevas-Rodríguez, et al., 2021).

Black quinoa showed the highest EAAI (240.87 %), while Red quinoa had the lowest (53.35 %). The significantly higher EAAI in Black quinoa ($p < 0.05$) is attributable to the greater presence of EAA as met, ile, and leu, which increase the EAAI score (Fig. 3). The EAAI values for Yellow and Black quinoa are similar to those reported by Motta et al. (2019) for quinoa (50.4 %), amaranth (45.5 %) and buckwheat (52.1 %), and are slightly lower than those reported by Skrobot et al. (2019) for quinoa (46.3 %), amaranth (59.76 %), buckwheat (68.36 %) and wheat (56.07 %). Quinoa's EAAI was higher than that of oat flour (44.55 %), oat protein concentrate (45.92 %) (Sánchez-Velázquez, Ribéreau, et al., 2021), *Fabeae* beans (39.90–41.75 %) (Pastor-Cavada et al., 2010), and *Vicia faba* (69.95–87.11 %) (Samaei et al., 2020). An EAAI above 90 % typically indicates a protein with superior nutritional quality (Amza et al., 2013), which is expected for quinoa due to its high EAA content, making it well-suited to meet human metabolic needs (De Bock et al., 2021). BV of proteins is closely correlated with their EAA content (Hoffman & Falvo, 2004; Sánchez-Velázquez, Cuevas-Rodríguez, et al., 2021). The BV values were 250.81 %, 88.33 % and 50.79 % for Black, Yellow, and Red quinoa, respectively. These results are similar to, or even higher than those reported for raw pulses (35.22–106.87 %), extruded pulses (16.79–115.84 %) (Sánchez-Velázquez, Ribéreau, et al., 2021), cooked oat protein concentrate (84.37 %) (Sánchez-Velázquez, Cuevas-Rodríguez, et al., 2021), beef (80 %), casein (77 %), milk (91 %), soy protein (74 %) and whey protein (104 %) (Hoffman & Falvo, 2004). A BV greater than 70 % is indicative of high nutritional quality (Amza et al., 2013). However, it is important to note that BV does not account for interactions between proteins and other food components such as carbohydrates or fiber, nor does it consider the presence of ANFs, the complex digestion process, bioavailability or bioaccessibility (Sánchez-Velázquez, Ribéreau, et al., 2021).

The Theoretical PER is another useful measure of protein quality, particularly when the EAA profile is known (Sánchez-Velázquez, Ribéreau, et al., 2021). PER values can range from 0 to 2.7, with casein (2.7) serving as the standard reference protein. PER values higher than 2.7 indicate an excellent protein source (Hoffman & Falvo, 2004; Sánchez-Velázquez, Cuevas-Rodríguez, et al., 2021; Wang et al., 2023). In this study, quinoa PER values ranged from 2.53 to 3.18 (Table 4), with Black quinoa exhibiting the highest value (3.10). Malik and Singh (2022) reported PER values for quinoa (< 2.0), amaranth (1.52–2.57), buckwheat (2.55) and chia (1.82) based on murine assays. The PER values reported in this study are higher than those for beef (2.9), milk (2.5), peanuts (1.8), soy protein (2.2) and wheat gluten (0.8) (Hoffman & Falvo, 2004), as well as for red kidney bean (1.55), navy beans (1.51), lentils (0.86–1.42), chickpeas (2.32), pinto beans (1.64) (Nosworthy, Franczyk, et al., 2017). These previous studies used *in vivo* animal models, whereas PER values from oat flour (0.97–2.37), and oat protein concentrate (1.72–2.89) (Sánchez-Velázquez, Cuevas-Rodríguez, et al., 2021), were derived from theoretical PER calculations. It is important to note that theoretical and *in vivo* PER values can differ, as the *in vivo* PER directly measures how proteins impact growth in rats (Nosworthy et al., 2018), while theoretical PER relies on equations that account for twelve different amino acids, this approach is more conservative, yet it can give a glance of protein efficiency (Amza et al., 2013).

3.7. Principal component analysis

As presented in Fig. 4, the PCA statistical analysis was used to identify the most significant features between quinoa samples, particularly ANFs and IVPD. The first two principal components had the major distribution of variance of PC1 = 34.25 % and PC2 = 25.25 %. They accounted for 60.11 % of the overall variance taken together. PC1 is influenced by IVPD, oxalates, phytic acid, saponins, phenolic compounds and trypsin inhibitors, while PC2 is influenced by protein content and anthocyanins. These results showed that data was grouped into various clusters, for PC1 oxalates and IVPD were clustered and in the same area protein was found, while for PC2 phenolic compounds, phytic acid and saponins were clustered simultaneously. Furthermore, a negative correlation between IVPD and saponins (r = -0.7007) was found. This might be because saponins can form complexes and inhibit the absorption of iron, zinc, and proteins, thus modifying the permeability of the small intestine (Maradini Filho et al., 2017). Therefore, while the saponins content increases, the IVPD will decrease. This effect was observed in Red quinoa which has the highest saponins content and the lowest IVPD.

4. Conclusions

To the best of our knowledge, this study is the first to conduct a comprehensive comparative analysis of a wide spectrum of ANFs and protein quality across three different quinoa varieties. Notably, black quinoa exhibited the highest levels of protein, TDF, and IVPDCAAS, while also presenting the lowest concentrations of saponins, oxalates, and phytic acid. However, the IVPDCAAS and AAS values across all varieties were lower compared to other food sources. This can be attributed to the fact that the quinoa flours were not subjected to any

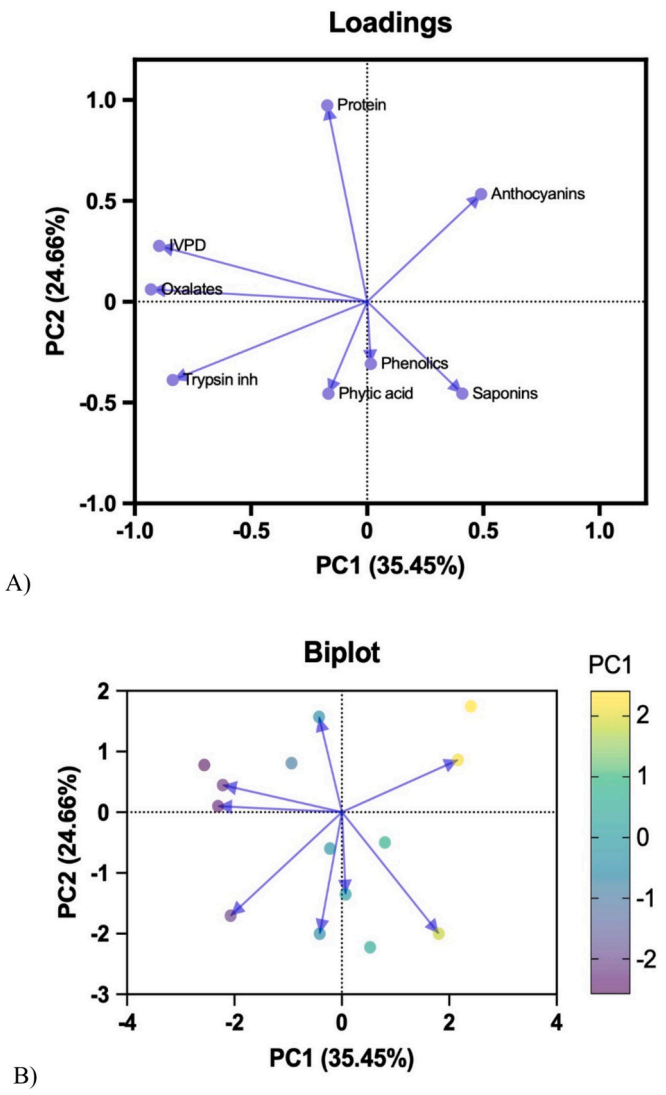

Fig. 4. Loading plot (A) and Biplot (B) of principal component analysis (PCA) for quinoa samples, ANFs and IVPD.

pre-treatment processes such as cooking, baking, extrusion, or fermentation, which are known to significantly enhance the overall protein digestibility of plant proteins.

Therefore, it is evident that processing methods are essential to reduce ANFs and fully leverage the high protein content and nutritional value of quinoa. Moreover, there remains a significant gap in the literature regarding the comprehensive assessment of ANFs in plant-protein sources. Addressing this gap is crucial, particularly in light of the growing interest in plant-based food ingredients. Future research should focus on developing tailored processing techniques that not only improve protein digestibility but also optimize the health benefits of plant-based products.

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CRediT authorship contribution statement

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

Data availability

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101814.

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