Food Bioscience 56 (2023) 103238

Contents lists available at ScienceDirect

Food Bioscience

journal homepage: www.elsevier.com/locate/fbio

Effect of protein extraction and fractionation of chia seeds grown in different locations: Nutritional, antinutritional and protein quality assessment

Yan Wang [a], Oscar Abel Sánchez-Velázquez [b], Cristina Martínez-Villaluenga [c], Francisco M. Goycoolea [a], Alan Javier Hernández-Álvarez [a,*]

[a] School of Food Science & Nutrition, University of Leeds, LS2 9JT, Leeds, UK
[b] Food Technology, Centro de Investigación y Asistencia en Tecnología y Diseño Del Estado de Jalisco A. C., Guadalajara, 44270, Jalisco, Mexico
[c] Department of Technological Processes and Biotechnology, Institute of Food Science Technology and Nutrition (ICTAN-CSIC), José Antonio Novais 6, 28040, Madrid, Spain

ARTICLE INFO

Keywords:
Albumins
Amino acid score
Antinutrients
Chia
Degumming
Essential amino acids
Globulins
Protein extraction
Protein quality

ABSTRACT

Chia (*Salvia hispanica*) has become increasingly popular in recent years due to its high protein content, among other nutritional benefits. This study aims to evaluate the nutritional and antinutritional composition, protein profile and protein quality of two chia seeds grown in Mexico and the UK, as well as to assess the impact that occurs during protein extraction and fractionation. Protein content of chia samples showed an increase after degumming, defatting, and extraction, obtaining protein concentrates from Mexican (MPC) and British (BPC) chia seeds with 88.32 and 89.20 g/100 g dw, respectively. Main protein fractions found in both chia seeds were globulins (Glo) and albumins (Alb). Essential amino acid index (EAAI) of chia samples ranged between 189.40 and 496.73% showing a 2-fold increase in comparison to the reference protein. *In vitro* protein digestibility (IVPD) increased after protein extraction (91% for MPC and BPC) but decreased after fractionation (~68%). Trypsin inhibitors increased 78–82% after protein extraction, while total phenolics content (TPC) increased 7.77- and 5.76-fold for Mexican albumins (MAlb) and British albumins (BAlb), respectively. Phytic acid content showed a reduction of > 90% after extraction/fractionation. These findings showed that depending on the extraction and/or fractionation methods used the protein quality, digestibility and antinutrients will be highly influenced.

1. Introduction

Chia (*Salvia hispanica* L.) is an annual herbaceous plant native to Mexico and Guatemala (Kaur & Bains, 2020; Marineli et al., 2014). Chia is consumed around the world as whole seeds, flour, and oil, and has become increasingly popular in the food industry (Ali et al., 2012; Cisternas et al., 2022). Chia seeds have attracted growing attention owing to their remarkable nutritional composition, characterized by a wide variety of essential nutrients including protein, fat (abundant omega-3 fatty acids), carbohydrates, dietary fiber, vitamins, and minerals. These nutrients present in chia seeds confer numerous health benefits, thereby enhancing human well-being (Grancieri et al., 2019a; Martínez-Cruz & Paredes-López, 2014; Orona-Tamayo et al., 2015). Chia seeds also contain abundant dietary fibers which are mainly present on the outer surface of the seeds. The chia mucilage is composed mainly of carbohydrates that have shown great gelling, emulsifying, water-holding ability and shear-thinning properties, and have been commonly used in bakery products, beverages and frozen meats (Campos et al., 2016; Capitani et al., 2013; Felisberto et al., 2015). Many chia phytochemicals are considered to have beneficial effects on human health, for example, polyphenols, tocopherols, phytosterols and carotenoids in chia seeds have shown preventive effects against chronic diseases such as obesity, diabetes, and cancer (De Falco et al., 2017; Grancieri et al., 2019a, Grancieri et al., 2019b). These compounds act as antioxidants through several mechanisms including free radicals scavenging, hydrogens donation and metal ions chelation (De Falco et al., 2017).

With the increasing awareness regarding healthy diets and food

* Corresponding author.
E-mail address: a.j.hernandezalvarez@leeds.ac.uk (A.J. Hernández-Álvarez).

https://doi.org/10.1016/j.fbio.2023.103238
Received 2 August 2023; Received in revised form 2 September 2023; Accepted 2 October 2023
Available online 10 October 2023

sustainability in recent years, the concept of using protein-rich plant sources to replace animal proteins has become popular. Although plant proteins are considered to have relatively lower biological value compared to animal proteins, the demand for plant proteins is quite high since they are easier to produce and have lower environmental impacts (Timilsena et al., 2016a, 2016b). Moreover, the food industry is increasingly interested in the production of plant protein concentrates and isolates not only because they are used as food functional ingredients but also because they are able to improve the qualities of food products, such as consistency, texture, flavour, and nutritional value (Lqari et al., 2002). Due to the high protein content in chia seeds (~19%), improved digestibility (77–80%) and balanced amino acid profile (Valdivia-López and Tecante, 2015), the extraction of chia protein has been widely investigated in recent years. The most common method to extract chia proteins is alkaline solubilization coupled to isoelectric precipitation (Mondor and Hernández-Álvarez, 2022). However, this method still has some drawbacks as the alkaline and acid treatments may influence the functional properties and nutritional quality of protein ingredients, therefore, optimization of the extraction conditions is important to reduce the undesirable changes of chia protein during extraction (López et al., 2018a). The quality of dietary protein depends on multiple factors, prominently including the amino acids composition, protein digestibility, presence of antinutritional and other dietary compounds (Bos et al., 2000). *In vivo* models have demonstrated that chia seeds consumption presents a favorable protein digestibility and a well-balanced amino acid profile (Da Silva et al., 2016; Jood & Singh, 2001). Consequently, there is a need to further research the protein quality of chia seed and understand the effect of processing on chia proteins.

Chia proteins have shown valuable functional attributes that make them highly advantageous in food formulation. They have displayed remarkable capabilities for water and oil absorption, thereby contributing to notable improvements in texture and moisture retention within baked goods (López et al., 2019). Furthermore, chia proteins exerted significant emulsifying and foaming properties, facilitating their successful incorporation into diverse food applications such as bakery and desserts (Kotecka-Majchrzak et al., 2020; López et al., 2019; Segura-Campos, 2019; Vázquez-Ovando et al., 2013). Additionally, the nutritional composition of chia seeds is dependent to fluctuations driven by different factors such as climatic conditions, geographical location soil attributes, and cultivation year (Grancieri et al., 2019a). With both the functional properties and nutritional characteristics, chia protein has recently showed important research advances and raised its commercial value. Even though the high protein content of chia seeds made this source attractive to explore for their wider applications, the structure, function, and health benefits of chia proteins have not been fully understood yet. In this context, this study endeavors to evaluate the modifications produced by protein extraction and fractionation. The assessment spans the nutritional and antinutritional composition, protein profile, and protein quality of chia seeds cultivated in two distinct locations: Mexico and the UK. By undertaking this research, we aim to contribute to a more comprehensive understanding of the chia protein landscape, elucidating the influence of extraction and fractionation processes. This research aims to provide knowledge on the intricate interplay between extraction methodologies and resulting protein attributes and presence and/or absence of certain antinutrients, thereby advancing the broader comprehension of chia proteins' applicability, protein quality and digestibility.

2. Materials and methods

Chia seeds (*Salvia hispanica* L.) were procured from two locations: British seeds grown at Great Tey in Essex in 2019, provided by Hodmedod's, Essex, UK; and the Mexican seeds were provided by producers located in Guadalajara, Mexico, these seeds belong to the January 2019 harvest. Reagents were analytical grade and purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA) and Merck (Darmstadt, Germany), unless otherwise specified.

2.1. Sample preparation

2.1.1. Sample pre-treatment

The seeds were ground to produce raw chia flour using a Fritsch P11 Knife Mill (Fritsch GmbH, Germany) before the nutritional assessment was carried out (Raw sample, R). Afterwards, for the degummed and defatted chia flours, the method reported by Salazar Vega et al., 2020 was used with slight modifications. Chia seeds were immersed in distilled water in a ratio of 1:40 (w/v) under constant stirring (IKA, Staufen, Germany) for 2 h at room temperature to allow mucilage formation. Samples were sonicated at an amplitude of 50%/750 W in an ultrasound bath (SONICS, Tacoma, Washington, USA) with intervals of 4 min to eliminate the mucilage adhered to the seeds. Then, the degummed chia seeds were dried overnight in an oven at 55 °C and afterwards the mucilage was manually separated from the seeds with the assistance of a sieve (200 mm/30 mesh) and ground into flour using a Fritsch P11 Knife Mill (Fritsch GmbH, Germany). Subsequently, the degummed chia flour (DF) was defatted by mixing flour with hexane in a ratio of 1:5 (w/v) and stirring for 1 h at room temperature under a fume cupboard. Afterwards, the slurry was centrifuged at 4816 *g* for 20 min at 4 °C, the supernatant was discarded, while the pellet was recovered and mixed with hexane at least 3 times as previously described. Finally, the recovered degummed-defatted chia flour (DDF) was left overnight under the fume cupboard to evaporate remaining hexane. Both, the Mexican (MDDF) and British (BDDF) degummed-defatted chia flours were stored at 4 °C until further use.

2.1.2. Protein concentrates production

Chia proteins were isolated from MDDF and BDDF by alkaline solubilization coupled to isoelectric precipitation using the method reported by Timilsena et al. (2016a) with some modifications. As a result, Mexican (MPC) and British chia protein concentrates (BPC) were obtained. Briefly, the DDF was dispersed with distilled water in a ratio of 1:10 (w/v), adjusted pH of the slurry to 10 with 1 N NaOH under constant stirring for 1 h at room temperature, followed by centrifugation using an Avanti J-30I high-speed centrifuge (Beckman Coulter, Brea, California, USA) at 8288 *g* for 15 min at 4 °C. Supernatant was collected for isoelectric precipitation, the pH of the supernatant was adjusted to 4.5 with 1 N HCl and the slurry was stirred continuously and maintained for 1 h at room temperature. After that, the slurry was centrifuged (8288 *g*, 15 min, 4 °C), supernatants were discarded and pellets were recovered and stored at −80 °C and freeze dried (Labconco, Kansas, MO, USA). Then freeze-dried powders were grinded, MPC and BPC were stored at 4 °C in vacuum-sealed bags for further use.

2.1.3. Protein fractionation

Protein fractions from MDDF and BDDF were extracted based on their solubility according to Osborne classification using the method reported by Grancieri et al., 2019b with slightly modifications. DDF was mixed with the corresponding extraction solutions at 1:40 (w/v) and 1:10 (w/v) ratios for albumin and globulin extraction, respectively. Albumin fraction (Alb) was extracted with distilled water and constant stirring for 1 h at 4 °C. The suspension was centrifuged at 13,000 *g* for 20 min at 4 °C and supernatant (albumin fraction). Then, the pellet was resuspended in 50 mM Tris/0.4 M NaCl pH 8.0 solution at constant stirring for 1 h at 4 °C, the suspension was centrifuged at 13,000 *g* for 20 min at 4 °C. The supernatant obtained was the globulin fraction (Glo). Again, the pellet was resuspended in 70% of isopropanol and constant stirring for 1 h at 4 °C, followed by centrifugation of 13,000 *g* for 20 min at 4 °C. The supernatant obtained was prolamin (Pro). Afterwards the resulting pellet was resuspended in 0.1 mol/L $Na_2B_4O_7{\cdot}H_2O$ (pH 10) at constant stirring for 1 h at 4 °C, the suspension was centrifuged at 13,000 *g* for 20 min at 4 °C. The supernatant obtained was glutelin (Glu). All

protein fractions were stored at −80 °C and freeze-dried (Labconco, Kansas, MO, USA) for further analysis.

2.2. Nutritional characterization

Proximate composition of raw (R), degummed (DF) and degummed-defatted (DDF) chia flours was determined according to the official methods of the AOAC (2016). For total lipids estimation, an acid hydrolysis was carried out using 50 mL of 4 mol/L HCl, followed by Soxhlet extraction. Results were expressed as g/100 g of dry weight (dw). Briefly, 2 g of samples were placed in crucibles and thermally treated in a Phoenix microwave furnace (PS6854, Germany) at 800 °C for 5 h for the determination of ash. For moisture, 5 g of sample were dehydrated in an oven at 105 °C for 240 min. Total nitrogen content R, DF, DDF, PC, Alb, and Glo from Mexican and British chia seeds was measured using an Elementar Vario Max Cube (Elementar-Straße 1, Germany) following the Dumas combustion method (AOAC, 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 the samples with high protein content, ethylenediaminetetraacetic acid (EDTA) was used as standard, while for samples with low protein content, rice flour was used as standard.

2.3. Total amino acids

For estimation of total amino acids, 2 mg of DDF, PC, Alb, and Glo from Mexican and British chia seeds were hydrolyzed in 6 N HCl (4 mL) at 110 °C for 24 h in tubes sealed under nitrogen. Tryptophan was analyzed by HPLC after basic hydrolysis according to Yust et al. (2004). Amino acids were determined after derivatization with diethyl ethoxymethylenemalonate by HPLC according to the method of Alaiz et al. (1992), using D,L-α-aminobutyric acid 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).

2.4. Protein quality estimation

2.4.1. Amino acid score

Amino acid score (AAS) was estimated using the following equation (FAO/WHO/UNU, 1985):

$$AAS = \frac{mg\ of\ amino\ acids\ in\ 1\ g\ of\ total\ protein}{mg\ of\ amino\ acids\ in\ 1\ g\ requirement\ pattern} \times 100$$

2.4.2. Essential amino acid index

Essential amino acid index (EAAI) was calculated using the amino acid composition of a standard (whole egg protein) (Amza et al., 2013):

$$EEAI = \sqrt[9]{\frac{(Lys \times Thr \times Val \times Met \times Ile \times Leu \times Phe \times His \times Trp)a}{(Lys \times Thr \times Val \times Met \times Ile \times Leu \times Phe \times His \times Trp)b}}$$

where "a" the content of amino acids in test sample and "b" the content of the same amino acids in the standard (%).

2.4.3. Predicted biological value

Predicted biological value (BV) was calculated according to Amza et al. (2013) using the following equation:

$$BV = 1.09(EAAI) - 11.7$$

2.4.4. Protein efficiency ratio

Protein efficiency ratio (PER) values were obtained from the amino acid composition of chia samples based on the following five equations (Amza et al., 2013):

$$PER\ 1 = -0.684 + 0.456\ (Leu) - 0.047(Pro)$$

$$PER\ 2 = -0.468 + 0.454(Leu) - 0.105(Tyr)$$

$$PER\ 3 = -1.816 + 0.435(Met) + 0.780(Leu) + 0.211(His) - 0.944(Tyr)$$

$$PER\ 4 = 0.08084(Thr + Val + Met + Ile + Leu + Phe + Lys) - 0.1094$$

$$PER\ 5 = 0.0632(Thr + Val + Met + Ile + Leu + Phe + Lys + His + Arg + Tyr) - 0.1539$$

2.4.5. In vitro protein digestibility (IVPD)

Samples were digested following the method reported by Tinus et al. (2012), with few modifications. Briefly, the equivalent of 62.5 mg of protein was rehydrated in 10 mL of Milli-Q water, heated to 37 °C and adjusted to pH 8.0. The samples were monitored for 10 min to record the stability of the pH, followed by the addition of a multienzyme cocktail containing trypsin (16 mg, 13,000–20,000 *N*α-Benzoyl-L-arginine ethyl ester (BAEE)U/mg protein), chymotrypsin (31 mg, 40 U/mg protein) and protease (50–100 U/g solids). After the addition of the digestive cocktail, the subsequent pH drop was recorded for 10 min. Subsequently, the samples were transferred in a water boiling bath for 15 min and cooled down in an iced bath. Then the samples were centrifuged at 4 °C, 6000 *g* for 30 min, and the supernatants were recovered. The IVDP was calculated as follows:

$$IVPD\ (\%) = 65.66 + 18.10 \times (pH_{0\ min} - pH_{10\ min})$$

Meanwhile, the *in vitro* protein-digestibility corrected amino acid score (IVPDCAAS) was calculated as a product of the AAS and IVPD (Nosworthy et al., 2018).

2.5. FPLC-gel filtration chromatography

An FPLC AKTA Purifier system (GE Healthcare, Uppsala, Sweden) equipped with a Superose 12 column (GE Healthcare) was used to analyze the molecular weight distribution of DDF, PC, Alb, and Glo from both chia seeds in non-denaturating conditions. 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 during 30 min at room temperature. 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 at 0.5 mL/min. Elution of protein was monitored at 214 nm. Molecular masses were determined using blue dextran (2000 kDa), cytochrome C (12.5 kDa), aprotinin (6512 Da), bacitracin (1450 Da), cytidine (246 Da) and glycine (75 Da) as molecular weight standards (Amersham Pharmacia LKB Biotechnology, Uppsala, Sweden).

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

SDS-PAGE was performed according to Laemmli (1970) using a Minin-Protean 3 Gel Electrophoresis Unit (Bio-Rad) and Criterion TGX™ Prec ast Any kD gel (5,671,124 Bio-Rad Laboratories, Inc., CA, USA). 2.5 mg of sample was dissolved in 1 mL Laemmli buffer (0.1 M Tris-Tricine, pH 6.8, 2% SDS, 5% β-mercaptoethanol and 0.025% bromophenol blue), stirred for 1 h, boiled for 5 min, and then centrifuged at 10,000 *g* for 1 min and loaded onto the gel (20 μg protein premixed with Stained Protein Standard/well) and run at 150 kV. Gel was stained using 0.125% Coomassie Brilliant Blue R-250 in 7% acetic acid and 40% MeOH (v/v) solution and destained in 7% acetic acid and 30% EtOH (v/v) solution. As a molecular marker, Precision Plus Protein™ standard (10–250 kDa, Bio-Rad Laboratories Inc., CA, USA) was used.

2.7. Antinutrients

2.7.1. Total soluble phenolic compounds

100 mg of sample were dispersed in 1 mL of 80% MeOH + 0.1%

formic acid. The mixture was incubated at 30 °C for 15 min in a Thermomixer C (Eppendorf, Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, samples were centrifuged (Eppendorf Centrifuge model 5424 R, Thermo Fisher Scientific, Waltham, MA, USA) at 2000 rpm at 30 °C for 15 min. The supernatant was collected, and the extraction process was repeated. An aliquot of 1 mL of supernatant was mixed with 100 μL of freshly prepared 0.1% Fast Blue BB and 100 μL of 5% NaOH. Reaction mixtures were incubated 2 h in the dark without shaking, and absorbance was read at 420 nm in a microplate reader (Biotek Instruments, Winooski, VT, USA). The results were expressed as mg of gallic acid equivalents (GAE)/100 g dw.

2.7.2. Phytic acid

Phytic acid (PA) content of samples was determined using the Phytic Acid (Phytate)/Total Phosphorus Assay kit (Megazyme, Wicklow, Ireland). The results were expressed as g/100 g dw.

2.7.3. Trypsin inhibitory activity (TIA)

Trypsin inhibitory activity (TIA) was determined according to Sueiro et al. (2015). Briefly, 100 mg of samples were dispersed in 5 mL 0.01 M NaOH (pH 8.4–10) and incubated at 20 °C for 3 h with constant stirring in a Thermomixer (Eppendorf, Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, the resulting volume was adjusted to 10 mL with Milli-Q water, mixed and left to stand for 15 min. Followed by aliquoting 1.0 mL mixed slurry and then diluted to a factor resulting in 40–60% inhibition of trypsin. An Eppendorf tube was used to mix 200 μL of 0.015 mg/mL trypsin working solution (1.5 mg of trypsin in 100 mL of 1 mM HCl) with 100 μL of diluted sample slurry. Then, 500 μL of a pre-heated (to 37 °C) Nα-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (DL-BAPNA) solution (40 mg in 1 mL dimethylsulfoxide, diluted to 100 mL using preheated tris-buffer at pH 8.2) was added to each tube. Following a 10-min incubation at 37 °C in a Thermomixer (1000 rpm), the reaction was terminated by adding 100 μL of 30% acetic acid. The samples were subsequently centrifuged in an Eppendorf microcentrifuge at 2500 *g* for 10 min at 37 °C, and the absorbance of the supernatant was measured at 410 nm using a microplate reader (Biotek Instruments, Winooski, VT, USA). Acetic acid was added first to the sample blank, while the control was conducted without adding the sample. The results were expressed as TIU/mg dw and calculated using the following equation:

$$TIA = \left(SAMPLE - CTR * \frac{10}{0.01}\right) * \frac{5\ (dilution\ factor)}{sample\ weight\ (mg)}$$

2.8. Statistical analysis

All experiments were performed in three replicates. Data were expressed as the mean ± standard deviation. The data were examined using analysis of variance (ANOVA). The significant differences were determined at 0.05 probability level, and $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Sample pre-treatment, recovery yield, and nutritional composition

Mucilage of chia seeds is present on the outer surface of the seeds coat (Muñoz et al., 2012). The objective of the degumming process was to remove the mucilage layer that could impede the extraction of proteins. When seeds are immersed in water, the mucilage forms a transparent coating around the seed, providing structural support and protection. However, this coating can create a physical barrier, hindering nutrient absorption and bioavailability. Following ultrasonic treatment, the cotton-like mucilage layer was efficiently removed from the seed (Fig. 1). The content of mucilage in Mexican (MR) and British (BR) raw chia seeds was 11.6 g/100 g dw and 12.5 g/100 g dw, respectively (data not presented). The mucilage removal yield achieved in this study was greater than that reported by Muñoz et al. (2012) and Urbizo-Reyes et al. (2019), who obtained yields of 6.97% using controlled temperature and pH methods, 4.21% by freeze-drying, 3.65% by oven drying, and 1.03–1.86% by heat extraction. A higher mucilage removal may have positive effects in the accessibility to nutrients and bioactive compounds contained within the inner structures of chia seeds.

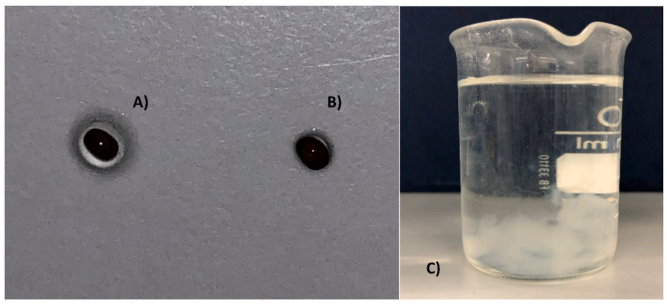

Fig. 1. Chia mucilage. A) chia mucilage forms after hydration; B) degummed chia seed; C) chia mucilage after ultrasound assisted extraction.

Proximate composition of raw (R), degummed (DF) and degummed-defatted (DDF) chia seed samples is summarized in Table 1. Proteins represented 20.40 g/100 g dw for MR and 22.16 g/100 g dw for BR, with non-statistical difference ($p > 0.05$) among them. Ashes represent 4.37 g/100 g dw and 3.98 g/100 g dw, for MR and RB, respectively. After degumming, the proportion of lipids increased by 17.85% in MDF and by 10.39% in BDF. Proteins exhibited a slight statistical increase of 5.29% in MDF and 8.24% in BDF ($p < 0.05$). However, once degumming-defatting steps were carried out, lipids decreased by 90.59% in Mexican chia and 89.39% in British chia seeds compared to MR and BR chia, respectively. Protein increased by 43.13% in MDDF compared to MR chia, and by 44.99% in BDDF compared to BR chia.

3.2. Protein extraction and fractionation

As mentioned above, protein content in Mexican and British chia seeds increased significantly to 43.13% and 44.95%, respectively, with the pretreatments used, thus, DDF flours were used as starting material to produce chia protein concentrates (PC). Mexican (MPC) and British (BPC) chia protein concentrates showed protein contents of 88.32 and 89.20 g/100 g dw, respectively. Under similar conditions, Timilsena et al. (2016b) obtained chia protein isolates with a protein content of 90.5–91.2 g/100 g dw. While Malik & Riar (2022) produced black (Hyderabad, India) and white chia (Mysore, India) protein isolates, with a protein content of 90.65 and 90 g/100 dw, respectively, by means of isoelectric precipitation, being close to the results obtained in this study.

In order to have a more comprehensive understanding of various

Table 1
Proximate composition of raw, degummed and degummed-defatted flours from Mexican and British chia samples (g/100 g dw).

Compound	Locations	Raw	DF	DDF
Ash	Mexican	$4.37 \pm 0.09^{ab,A}$	$4.06 \pm 0.12^{b,A}$	$6.00 \pm 0.07^{a,A}$
	British	$3.98 \pm 0.07^{b,A}$	$3.43 \pm 0.06^{b,A}$	$6.38 \pm 0.29^{a,A}$
Lipid	Mexican	$32.95 \pm 0.81^{b,B}$	$40.11 \pm 0.37^{a,A}$	$2.94 \pm 0.01^{c,A}$
	British	$35.97 \pm 0.78^{b,A}$	$40.14 \pm 0.49^{a,A}$	$3.58 \pm 0.14^{c,A}$
Protein	Mexican	$20.40 \pm 0.17^{c,B}$	$21.54 \pm 0.68^{b,B}$	$37.87 \pm 0.41^{a,B}$
	British	$22.16 \pm 0.26^{c,B}$	$24.15 \pm 0.34^{b,A}$	$40.28 \pm 2.31^{a,A}$

DF, degummed chia flour; DDF, degummed-defatted chia flour. Data are the mean and SD of three replicates. Different lowercase letter within the row indicates statistical differences among different chia samples ($p < 0.05$, Tukey test). Different uppercase letter within the column indicates statistical differences among chia seeds from different place of origin ($p < 0.05$, Tukey test).

aspects of chia protein functionality and its ability to be used for different food formulations, main protein fractions present in chia seeds should be fully characterized as techno-functional properties can vary widely between chia seeds. Furthermore, harvest time, geographical location, environmental conditions, and processing conditions/methods used, will induce differences in structure, protein fractions ratios (globulins, albumins, prolamins and glutelins) and nutritional value of chia ingredients (flour, concentrate, or isolate). Thus, a more in-depth characterization is imperative to understand the different factors that influence protein functionality and nutritional quality of chia protein ingredients. The protein content of albumins was determined in MDDF and BDDF, which presented 37.96% and 56.75%, respectively, while the protein content of globulins was 44.24% and 39.34%, showing statistical differences ($p < 0.05$) between seeds from two locations. However, the protein content and recovery of prolamins and glutelins in both Mexican and British chia of this study were very low, thus, these fractions were not considered for further analysis (data not shown). It can be observed that the main protein fractions presented in chia seeds may vary based on different extrinsic factors as mentioned above, besides the growing conditions, the protein fractions obtained will be highly affected by the method/conditions of extraction used. According to previous studies, the most abundant protein fraction in chia samples were globulins (~52%) and albumins (~18%) (Hernández-Pérez et al., 2020; Orona-Tamayo et al., 2015; Sandoval-Oliveros & Paredes-López, 2013). However, Olivos-Lugo et al. (2010) found that a Mexican chia seeds was composed mostly of 53.8% prolamins and 23% glutelins. While, Julio et al. (2019) observed that the most abundant fraction in chia seeds (from state of Yucatan, Mexico) was globulins (64.86%), followed by glutelins (20.21%).

3.3. Amino acid profile and protein quality parameters

The amino acid profile of DDF, PC, Alb, and Glo from both chia seeds are displayed in Table 2. In all samples, Asp + Asn, Arg and Glu + Gln were the major amino acids (>8.4% of total amino acid content). Because of the potential ability to improve immunity and enhance athletic performance, food sources rich in glutamine have been of great interest (Olivos-Lugo et al., 2010). Food sources rich in Arg have been reported to have the potential ability to prevent cardiovascular diseases (Nitz et al., 2019). Essential amino acids (EAA) represented 44.58–48.89% of total amino acids, with the highest values in BPC (48.04%), BDDF (48.40%), MGlo (48.55%) and MPC (48.89%), while the lowest values were observed in BGlo (44.58%). Except for Trp and Lys, chia samples covered the daily amino acid requirements for children (FAO/WHO/UNU, 1985). In this sense, the consumption of 100 g of each of these chia samples may reach the amino acid requirements of EAA for children (0.48–2.01-fold) and adults (0.7–5.4-fold) (Table 2).

The composition of chia samples reveals that the highest content of sulphur amino acids (Met and Cys), Gly, Thr, Ala, Pro, and Lys is presented in Alb fraction, meanwhile, the lowest content in Ser, His, Arg, Ile, Leu, and Phe appeared in the same protein fraction. The abundance of sulphur amino acids in Alb indicates that they may take part in maintaining the tertiary and quaternary structure of this fraction (Chen et al., 2023; Sandoval-Oliveros & Paredes-López, 2013), and is of interest to the food industry due to the role they play in hormonal regulation (Julio et al., 2019). The contribution of EAA by chia proteins provides 100% of the requirements of sulphur amino acids suggested by the FAO/WHO. The amino acid composition of both PC is characterized by a high content of aromatic amino acids (Phe, Trp, and Tyr), His, Ile, and Leu when compared to DDF, while the content in Lys was lower. Within these, Trp and Phe are EAA that are present in fair proportions in meat and other animal products and play an important role in the organism, such as regulation of appetite and mood (Górska-Warsewicz et al., 2018; Rodríguez Lara et al., 2021). Additionally, it can be noticed that Trp contents of BAlb and BGlo were significantly higher ($p < 0.05$) than Mexican fractions, while the content in Pro of BGlo was significantly lower ($p < 0.05$) than Mexican fractions. Overall, BDDF and BAlb samples showed better amino acid balance than the Mexican chia seeds, whereas BPC and BGlo samples of chia presented opposite results.

The diversity of amino acids also may influence parameters of protein quality of foods, but above all to reach the daily requirements of essential amino acids (Adhikari et al., 2022). In this way, EAAI ranged in 189.40–496.73% in contrast to standard (white egg protein) (Table 3), which means that all protein chia samples have almost or more than 2-fold EAAI than the reference protein source. Highest EAAI were observed in BAlb (475.42%) and BGlo (496.73%) samples. The AAS also

Table 2
Amino acid profile of degummed-defatted flour, protein concentrates, and albumin and globulin fractions from Mexican and British chia samples (g/100 g protein).

Amino Acids	DDF		PC		Alb		Glo		FAO/WHO (1985)	
	Mexican	British	Mexican	British	Mexican	British	Mexican	British	Children	Adults
Asp + Asn	9.39 ± 0.23^{b}	8.72 ± 0.06^{cd}	8.57 ± 0.00^{d}	9.47 ± 0.02^{b}	8.41 ± 0.01^{d}	8.48 ± 0.04^{d}	8.80 ± 0.02^{c}	10.00 ± 0.04^{a}		
Glu + Gln	16.71 ± 0.05^{d}	17.09 ± 0.04^{c}	17.10 ± 0.03^{c}	17.22 ± 0.15^{c}	17.24 ± 0.08^{bc}	16.92 ± 0.02^{c}	17.43 ± 0.05^{b}	18.23 ± 0.08^{a}		
Ser	5.90 ± 0.09^{b}	5.93 ± 0.02^{b}	5.91 ± 0.01^{b}	5.88 ± 0.08^{b}	5.55 ± 0.01^{c}	5.54 ± 0.04^{c}	6.14 ± 0.005^{a}	6.23 ± 0.03^{a}		
His	2.61 ± 0.03^{c}	2.84 ± 0.00^{b}	3.05 ± 0.021^{a}	2.94 ± 0.05^{ab}	2.07 ± 0.05^{d}	2.43 ± 0.08^{c}	2.78 ± 0.07^{bc}	3.09 ± 0.02^{a}		
Gly	4.66 ± 0.03^{b}	4.98 ± 0.01^{a}	4.38 ± 0.01^{c}	4.56 ± 0.06^{bc}	4.99 ± 0.03^{a}	4.82 ± 0.00^{ab}	4.28 ± 0.06^{c}	4.32 ± 0.01^{c}		
Thr	3.89 ± 0.07^{b}	3.93 ± 0.03^{b}	3.78 ± 0.01^{bc}	3.88 ± 0.01^{b}	4.20 ± 0.13^{a}	4.23 ± 0.08^{a}	3.59 ± 0.04^{c}	3.65 ± 0.00^{c}	3.4	0.9
Arg	10.63 ± 0.02^{d}	11.05 ± 0.01^{c}	11.43 ± 0.02^{b}	11.38 ± 0.07^{b}	9.57 ± 0.02^{e}	9.29 ± 0.01^{f}	12.25 ± 0.04^{a}	12.22 ± 0.01^{a}		
Ala	5.42 ± 0.09^{b}	5.52 ± 0.03^{b}	5.27 ± 0.00^{c}	5.33 ± 0.02^{bc}	5.64 ± 0.09^{ab}	5.73 ± 0.01^{a}	5.02 ± 0.01^{d}	5.27 ± 0.01^{c}		
Pro	5.05 ± 0.25^{d}	5.06 ± 0.11^{d}	4.75 ± 0.04^{e}	5.34 ± 0.22^{c}	5.55 ± 0.11^{b}	6.62 ± 0.33^{a}	5.02 ± 0.24^{d}	0.33 ± 0.07^{f}		
Tyr	2.70 ± 0.02^{b}	2.68 ± 0.00^{b}	3.15 ± 0.00^{a}	3.06 ± 0.01^{a}	3.21 ± 0.01^{a}	3.09 ± 0.01^{a}	3.12 ± 0.01^{a}	3.17 ± 0.01^{a}		
Val	7.02 ± 0.15^{a}	5.04 ± 0.02^{f}	5.10 ± 0.04^{ef}	5.25 ± 0.15^{e}	5.48 ± 0.03^{d}	5.75 ± 0.00^{c}	4.99 ± 0.15^{f}	6.71 ± 0.05^{b}	3.5	1.3
Met	2.21 ± 0.01^{d}	2.84 ± 0.01^{cd}	2.95 ± 0.04^{c}	1.27 ± 0.01^{e}	4.67 ± 0.01^{a}	3.76 ± 0.02^{b}	2.96 ± 0.02^{c}	2.32 ± 0.03^{d}		
Cys	1.66 ± 0.01^{c}	1.65 ± 0.03^{c}	1.63 ± 0.00^{c}	1.20 ± 0.01^{d}	2.38 ± 0.01^{a}	1.88 ± 0.02^{b}	1.67 ± 0.01^{c}	1.48 ± 0.00^{c}	2.5	1.7
Ile	3.73 ± 0.10^{c}	3.87 ± 0.04^{bc}	4.18 ± 0.00^{a}	4.22 ± 0.04^{a}	3.34 ± 0.03^{d}	3.58 ± 0.03^{c}	3.95 ± 0.02^{b}	4.12 ± 0.00^{ab}	2.8	1.3
Trp	0.73 ± 0.03^{b}	0.68 ± 0.00^{b}	0.77 ± 0.02^{ab}	0.79 ± 0.03^{ab}	0.75 ± 0.03^{ab}	0.91 ± 0.04^{ab}	0.79 ± 0.00^{ab}	0.94 ± 0.09^{a}	0.8	0.5
Leu	7.28 ± 0.05^{c}	7.51 ± 0.04^{b}	7.61 ± 0.01^{ab}	7.74 ± 0.02^{a}	6.59 ± 0.01^{d}	6.62 ± 0.00^{d}	7.09 ± 0.01^{c}	7.33 ± 0.01^{bc}	6.6	1.9
Phe	5.59 ± 0.01^{d}	5.84 ± 0.01^{c}	6.18 ± 0.03^{b}	6.41 ± 0.02^{a}	4.65 ± 0.01^{e}	4.80 ± 0.01^{e}	6.04 ± 0.01^{bc}	6.30 ± 0.01^{ab}	6.3	1.9
Lys	4.83 ± 0.02^{b}	4.78 ± 0.00^{b}	4.20 ± 0.00^{c}	4.08 ± 0.02^{c}	5.72 ± 0.02^{a}	5.56 ± 0.02^{a}	4.10 ± 0.02^{c}	4.27 ± 0.00^{c}	5.8	1.6

DDF, degummed-defatted chia flour; PC, protein concentrate; Alb, albumin fraction; Glo, globulin fraction. Asp + Asn, aspartic acid + asparagine; Glu + Gln, glutamic acid + glutamine; Ser, serine; His, histidine; Gly, glycine; Thr, threonine; Arg, arginine; Ala, alanine; Pro, proline; Tyr, tyrosine; Val, valine; Met, methionine; Cys, cysteine; Ile, isoleucine; Trp, tryptophan; Leu, leucine; Phe, phenylalanine; Lys, lysine. Different lowercase letter within the column indicates statistical differences among chia seeds from different place of origin ($p < 0.05$, Tukey test).

Table 3
Protein quality parameters of degummed-defatted flour, protein concentrates, and albumin and globulin fractions from Mexican and British chia samples.

Sample	Locations	AAS (%)	EAAI (%)	BV	PER_1	PER_2	PER_3	PER_4	PER_5	IVPD (%)	IVPDCASS (%)
DDF	**Mexican**	133.72	310.52	326.77	2.40	2.55	2.83	2.68	3.04	78.03 ± 0.28^{c}	58.35
	British	131.03	325.36	342.94	2.50	2.66	3.35	2.62	3.03	81.65 ± 5.72^{b}	62.31
PC	**Mexican**	134.62	406.98	431.90	2.57	2.66	3.08	2.64	3.11	91.66 ± 0.84^{a}	68.09
	British	130.31	189.40	194.75	2.59	2.72	2.51	2.55	3.02	91.91 ± 0.65^{a}	70.53
Alb	**Mexican**	131.74	362.62	383.56	2.06	2.19	2.76	2.69	2.97	67.65 ± 0.51^{f}	51.35
	British	135.27	475.42	506.50	2.02	2.21	2.58	2.66	2.95	69.73 ± 0.13^{d}	51.55
Glo	**Mexican**	129.18	296.95	311.98	2.31	2.42	2.64	2.54	3.06	68.86 ± 0.38^{e}	53.31
	British	140.09	496.73	529.74	2.64	2.53	2.57	2.70	3.21	67.83 ± 0.95^{ef}	48.42

AAS, amino acids score; EAAI, essential amino acid index; BV, biological value; PER, protein efficient ratio; IVPD, *in vitro* protein digestibility; IVPDCAAS, *in vitro* protein digestibility-corrected amino acid score. DDF, degummed-defatted chia flour; PC, protein concentrate; Alb, albumin fraction; Glo, globulin fraction. Different letters in same column indicate statistical differences by Tuckey's test ($p < 0.05$).

showed values above those of the standard (130.31–140.09%), which refers to the desirable content of EAA in a protein (RAD, 1989).

Moreover, the BV of chia samples also showed high values ranging between 194.75 and 529.74. A BV above 100 is considered as a good reference to the possible biological importance of the aminoacidic profile of a protein (Oser, 1959). The higher BV were found again in BAlb (506.50) and BGlo (529.54) samples, which indicates a relationship between EAAI and BV.

Protein efficiency ratio (PER) values were estimated in 2.02–3.35 in chia samples. PER is based on theorical efficiency of a protein according to selected EAA (Amza et al., 2013). Low content of Leu in MAlb and BAlb impacts in the relatively low PER_1 and PER_2 (2.02–2.19), which it is compensated by a high content of Thr (4.20–4.23 g/100 g protein), Ala (5.64–5.73 g/100 g protein) and Lys (5.56–5.72 g/100 g protein), while BGlo and BPC had the highest PER_1 (2.64) and PER_2 (2.72) values, respectively. Elevated content in Leu (>7.00 g/100 g protein) and Arg (>10 g/100 g protein) mainly favored the high PER_5 values in chia samples.

In vitro protein digestibility (IVPD) of Mexican and British DDF were 78.03% and 81.65%, respectively. After protein extraction, MPC showed a IVPD of 91.66% (14.85% higher than MDDF) and BPC of 91.91% (>11.16% higher than BDDF). IVPD of albumins and globulins from both chia seeds were below 70%. Mohammed et al. (2019) conducted an assessment of IVPD of defatted chia flour grown in USA and Egypt, showing an IVPD value of approximately 66% for both samples. Monroy-Torres et al. (2008) and Sandoval-Oliveros & Paredes-López (2013) estimated the IVPD of DDF samples (~80%), obtaining a close IVPD value to that of this study, thus degumming-defatting treatment seems to not affect the chia protein digestibility. IVPD of PC increased notably in both chia seeds, but Olivos-Lugo et al. (2010) reported values of 49.4% of IVPD in a chia protein isolate, which is markedly lower than the values observed in PC, Alb and Glo samples from both seeds in this study. This could be due to the vast variety of *in vitro* protein digestibility methodologies, which makes it difficult to compare results, as enzymatic conditions change widely between methods. However, globulin fraction presented an IVPD of 82.5% in a defatted chia sample (Sandoval-Oliveros & Paredes-López, 2013). Protein digestibility is a crucial factor for protein quality, as it provides valuable information on the ability of digestive enzymes to break down proteins to release amino acids and small peptides from native proteins (López et al., 2018b). However, protein digestibility may be influenced by the interaction with different compounds present naturally in chia seeds known as antinutritional factors (Salgado et al., 2022).

Compared to previous studies, the IVPD observed in MDDF is similar to the reported by Sandoval-Oliveros & Paredes-López (2013) (77.5–78.9%) possibly because these are also Mexican genotypes, however, both Mexican and British DDF showed higher IVPD values than American and Egyptian defatted chia seed samples (Mohammed et al., 2019). Fat removal is of outmost importance, as the reduction of the protein digestibility in samples containing fair quantities of fat could be linked to the formation of protein-lipid complexes (Alvarez-Barajas et al., 2023). Information about IVPD of protein fractions of chia is scarce, but data for other seeds has indicated that IVPD from wheat flour and protein fractions is highly influenced by non-protein components and the albumin proportion present; additionally it was observed that these must be considered for the development of wheat cultivars with higher protein digestibility and thus impacting food product development (Ma & Balk, 2021; Orlien et al., 2023).

PDCAAS is a protein quality assessment method that takes into account both human amino acid requirements and protein digestibility. Protein concentrates from both chia seeds also showed the highest IVPDCAAS, with 68.09% and 70.53% for the Mexican and British samples, respectively, followed by the DDF (58.35–62.31%) and protein fractions (48.42–53.31%) (Table 3), only the globulin fraction from British chia had IVPDCAAS lower than 50%. The prediction suggests a good digestibility of chia proteins, which could represent an enhanced release and bioavailability of small peptides and free amino acids (Shaghaghian, 2022). Monroy-Torres et al. (2008) evaluated the digestibility of chia seeds subjected to common cooking conditions and concluded that chia's proteins have intermediate and low digestibility (values not reported), which could be due to the absence of degumming-defatting steps in the preparation of the samples analyzed.

IVPD of chia proteins can vary depending on the specific location of chia seeds and the processing methods used. It is important to highlight that IVPD may not necessarily reflect the real protein digestibility in the human body, as it does not consider several factors such as the gut microbiota and individual differences in digestive physiology. However, *in vitro* protein digestibility values can still provide useful information about the potential nutritional value of a protein source.

3.4. FPLC profile of chia

Fast protein liquid chromatography (FPLC) was carried out in order to analyze the molecular weight distribution of the DDF, PC, Alb and Glo from Mexican and British chia seeds in non-denaturing conditions (Fig. 2). MDDF and BDDF presented a similar profile of proteins, but in MDDF the largest peak reached 101 kDa, while in BDDF, it barely exceeds 90 kDa. Other polypeptides of 8.4 and 7.7 kDa may be identified in MDDF and BDDF, respectively. Interestingly, in these samples large proteins can be found along small peptides (0.44 kDa in MDDF and 0.36 kDa on BDDF).

Proteins of high MW, 99.4 and 107.4 kDa, were identified in MPC and BPC, respectively, these peaks may be 6S globulins (~104 kDa). Globulins polypeptides aggregate−disaggregate due to different phenomena during the protein extraction procedure (i.e., temperature, alkaline pH, and freeze drying), and especially the pH is involved in the structural changes of globulins, producing association and dissociation of the hexamer subunits of this protein fraction (Sandoval-Oliveros & Paredes-López, 2013). However, these peaks did not present the highest signals for MPC and BPC. Instead, polypeptides with a MW of 15.4 kDa and 16.4 kDa displayed the largest peaks, for MPC and BPC, respectively. These peaks may belong to the glutelin fraction (4 bands from 20

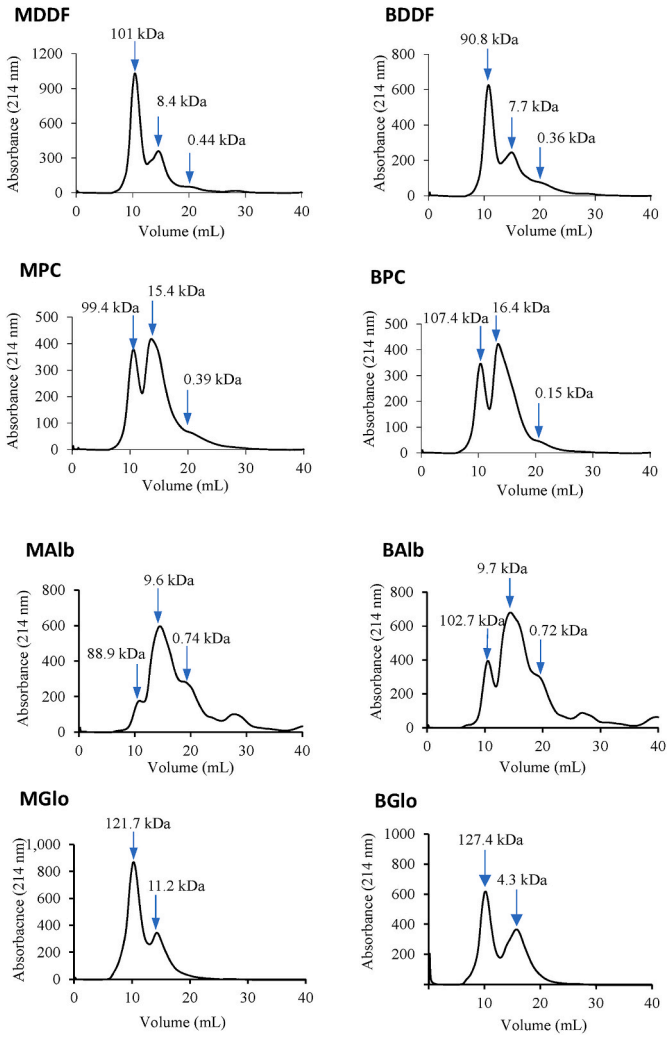

Fig. 2. FPLC gel filtration analysis of chia samples. MDDF, Mexican degummed-defatted chia flour; BDDF, British degummed-defatted chia flour; MPC, Mexican protein chia concentrate; BPC, British protein chia concentrate; MAlb, albumin fraction from Mexican chia; BAlb, albumin fraction from British chia; MGlo, globulin fraction from Mexican chia; BGlo, globulin fraction from British chia.

to 30 kDa) that can be classified either as globulins or glutelins, this is attributed mainly to the difficulty to solubilize this protein fraction and to the possible denaturation caused by the processing/extraction method used (Sandoval-Oliveros & Paredes-López, 2013; Verfaillie et al., 2023; Wang et al., 2023). This indicates that the protein extraction procedure used led to an enrichment of the main chia protein fractions for both PC. High MW proteins are still present in PC, but in BPC proteins above 100 kDa were more abundant than in BDDF, while in MPC proteins of about 90 kDa were dominant after the protein extraction step. Additionally, in both chia seeds low MW peaks are observed, about 0.39 kDa in MPC and 0.15 kDa in BPC, which means di- and tripeptides may be found in these samples.

In albumin samples, dominant signals of about 9.6–9.7 kDa were observed, in consistency with molecular weights reported for albumins in other studies (Sandoval-Oliveros & Paredes-López, 2013). The second largest signal corresponded to 88.9 kDa in MAlb and 102.7 kDa in BAlb. The other outstanding peaks were estimated between 0.72 and 0.74 kDa, which can be related to small peptides of 4–7 amino acids.

Finally, MGlo and BGlo showed dominant peaks above 120 kDa, followed by signals of 11.4 and 4.3 kDa, respectively. It can be observed that DDF and MGlo showed larger molecular weight distribution than the British chia seeds, while the British chia seeds showed a wider molecular weight range than Mexican for PC and Alb. A previous study has confirmed that the major component of the globulin fraction is a 11S globulin, whose molecular weight was between 300 and 400 kDa (Sandoval-Oliveros & Paredes-López, 2013), however, this cannot be accurately determined by FPLC in this study. Additionally, is important to consider protein solubility, as proteins are extracted from flours by adjusting the pH and/or the ionic strength of the solubilization media to promote protein solubility, thus, proteins will have different behaviors depending on their structural features and extraction conditions.

All these results provide an indication that protein extraction and fractionation processes did not have negative modifications on chia protein samples; on the contrary, protein fractionation may allow to improve the concentration/isolation of target protein fractions and/or peptides from heterogeneous protein solutions.

3.5. SDS-PAGE

The electrophoresis of MDDF and BDDF is displayed in Fig. 3A. Under non-reducing conditions, in Mexican samples the main bands observed corresponded to 42 (24.3%), 12 (14%) and 10 (12.3%) kDa, while in British samples the major protein bands had a MW of 45 (7.7. %), 12 (13.1%), 10 (12.8%) and 9 (16.18%) kDa. However, under reducing conditions, both chia seeds showed similar band distributions, these were grouped in two MW distribution groups: 26.3–28.7 kDa (20.3–24.9%) and 3.7–8.1 kDa (29.2–41.3%), respectively.

On the other hand, Fig. 3B shows the protein profile of MPC and BPC. Both protein concentrates under non-reducing conditions showed similar protein bands of 42.2 kDa (26.7%) and another of 10.5–15.0 kDa (35.8–37.2%), but in the British sample, two other bands of no less importance appeared with 43.3 (30.3%), 26.6 (14.7%) and 7.7 (20.3%) kDa. As was observed in reducing conditions in DDF samples, both protein concentrates showed similar pattern of proteins of 25.2–26.5 (27.7–32.7%), 16.9–17.5 (11.3–11.4%) and 2.5–5.8 (19.6–21.4%) kDa.

For non-reducing conditions, MAlb showed a band of 7.1 kDa (77.9%), while for BAlb the main band was found at 6.3 kDa (55.1%) (Fig. 3B). In reducing conditions, albumin from Mexican chia seeds showed three main bands of 10.1 kDa (29.9%), 3.6 kDa (14.5%) and <2 kDa (36.2%), while the British sample presented bands of 9.9 kDa (21%), 5.6 kDa (15.6%), 3.6 kDa (12.8%) and <2 kDa (19.9%). A similar pattern of bands was detected by Sandoval-Oliveros & Paredes-López (2013) in albumins from Mexican chia seeds.

Under non-reducing conditions, the main band of MGlo was found with a MW of 50 kDa (31.4%), while in the British the main bands were reported with 30 (17.7%), 28 (10.7%), 26 (35.2%) and 16 (22.7%) kDa (Fig. 3B). For reducing conditions, MGlo showed the main bands of 25.9–28.2 (34.2%), 18 (12.5%) and 6 (17.1%) kDa, while BGlo main bands had a MW of 28 (13.7%), 21 (12.1%), 18 (14.6%), 6 (29.6%) kDa. These MWs correspond to 11S and 7S subunits from chia globulins, both described by Sandoval-Oliveros & Paredes-López (2013) for Mexican chia under reduced and non-reduced conditions.

SDS-PAGE of DDF and PC samples under reducing conditions showed a similar protein profile, while under non-reducing conditions it is not appreciated. However, this behavior was not detected when protein fractions were compared among reducing and non-reducing conditions. Bands of 42–50 kDa were observed in MDDF and MPC, but also were present as the main bands in MGlo (even at reducing conditions), which indicates that globulins may represent the most abundant proteins in chia. Globulins were also detected in British samples, but mainly at a MW of 25–35 kDa. As is appreciated in Fig. 3B, albumins are present in both chia seeds, but their presence did not stand out as globulins were. Most intense bands were also related to globulins by Grancieri et al. (2019a) in Brazilian DDF in non-reducing conditions and similar to the reported by Sandoval-Oliveros & Paredes-López (2013) in MDDF at reducing conditions. In these two studies, authors reported that albumin's bands were less intense in comparison to globulins and raw

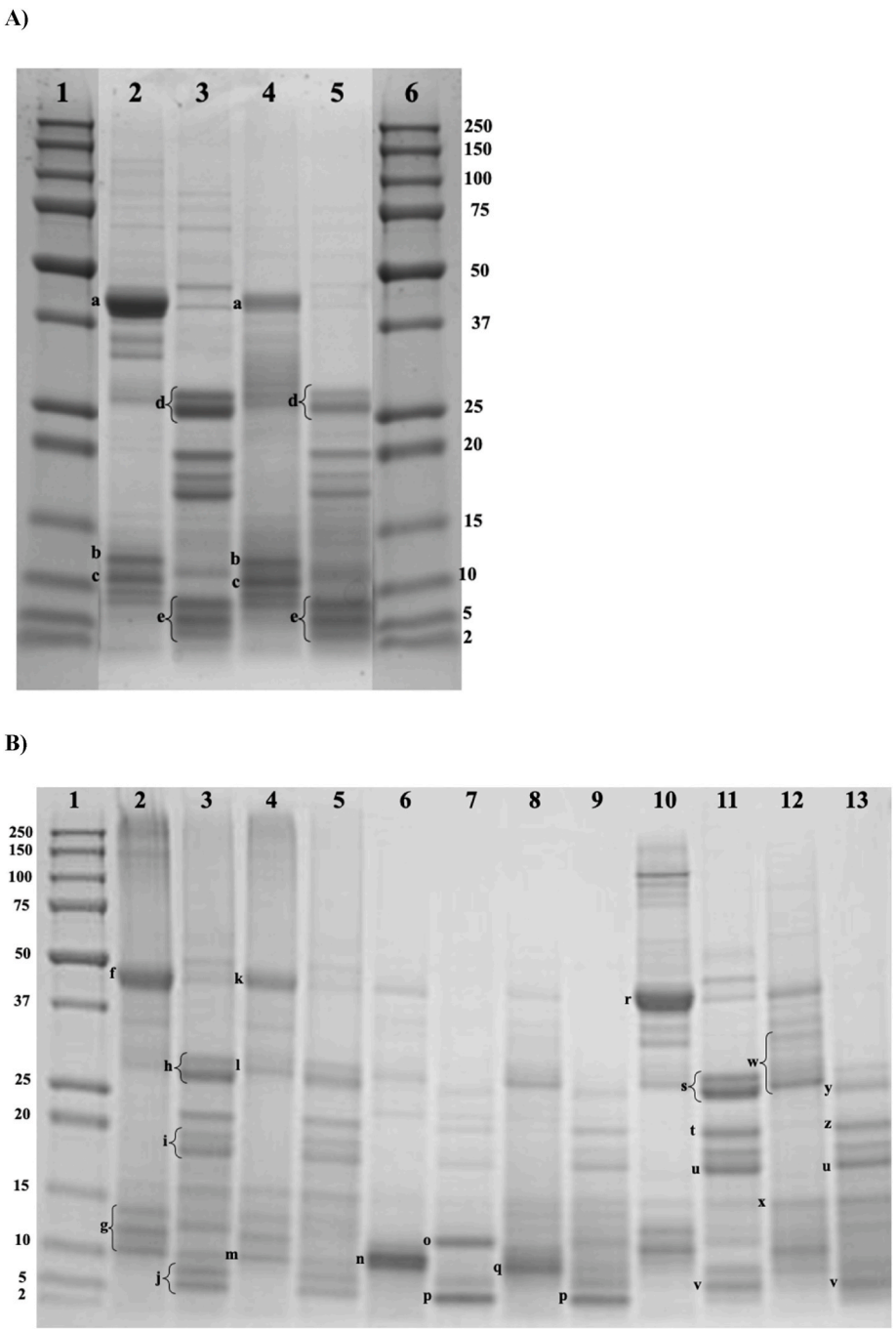

Fig. 3. SDS-PAGE of protein samples from Mexican and British chia samples. **A)** SDS-PAGE of degummed-defatted chia samples. Lines: **1** and **6**) protein reference (kDa); **2**) Mexican chia-degummed-defatted (non-reducing); **3**) Mexican chia-degummed-defatted (reducing); **4**) British chia-degummed-defatted (non-reducing); **5**) British chia-degummed-defatted (reducing). **B)** SDS-PAGE of protein concentrates and fractions. Lines: **1**) protein reference (kDa); **2**) Mexican chia protein concentrate (non-reducing); **3**) Mexican chia protein concentrate (reducing); **4**) British chia protein concentrate (non-reducing); **5**) British chia protein concentrate (reducing); **6**) Mexican albumin fraction (non-reducing); **7**) Mexican albumin fraction (reducing); **8**) British albumin fraction (non-reducing); **9**) British albumin fraction (reducing); **10**) Mexican globulin fraction (non-reducing); **11**) Mexican globulin fraction (reducing); **12**) British globulin fraction (non-reducing); **13**) British globulin fraction (reducing). **a**, 45 kDa; **b**, 12 kDa; **c**, 10 kDa; **d**, 26.3–28.7 kDa; **e**, 3.7–8.1 kDa; **f**, 42.2 kDa; **g**, 10.5–15.0 kDa; **h**, 25.2–26.5 kDa; **i**, 16.9–17.5 kDa; **j**, 2.5–5.8 kDa; **k**, 43.3 kDa, **l**, 26.6 kDa; **m**, 7.7 kDa; **n**, 7.1 kDa; **o**, 10.1 kDa; **p**, 3.7 kDa; **q**, 6.3 kDa; **r**, 50 kDa; **s**, 25.9–28.2 kDa; **t**, 21 kDa; **u**, 18 kDa; **v**, 6 kDa; **w**, 26–30 kDa; **x**, 16 kDa; **y**, 28 kDa, **z**, 21 kDa.

protein extract, which agree with the results obtained this study.

Both Mexican and British chia samples showed a large number of bands with a wide range of molecular sizes, which indicates that during the protein extraction and fractionation processes these remained intact and are not washed away within the different steps. However, in the albumin fractions, bands with high intensity are faintly observed, these belong to the globulins fraction, as is not possible to guarantee the absence of globulins in this fraction (Segura-Nieto et al., 1992).

3.6. Antinutrient content

Fig. 4 shows the composition of antinutrients in Mexican and British DDF, PC, Alb, and Glo. Compared to other chia samples, TPC showed the highest values for MAlb and BAlb, (4884 and 3338 mg GAE/100 g,

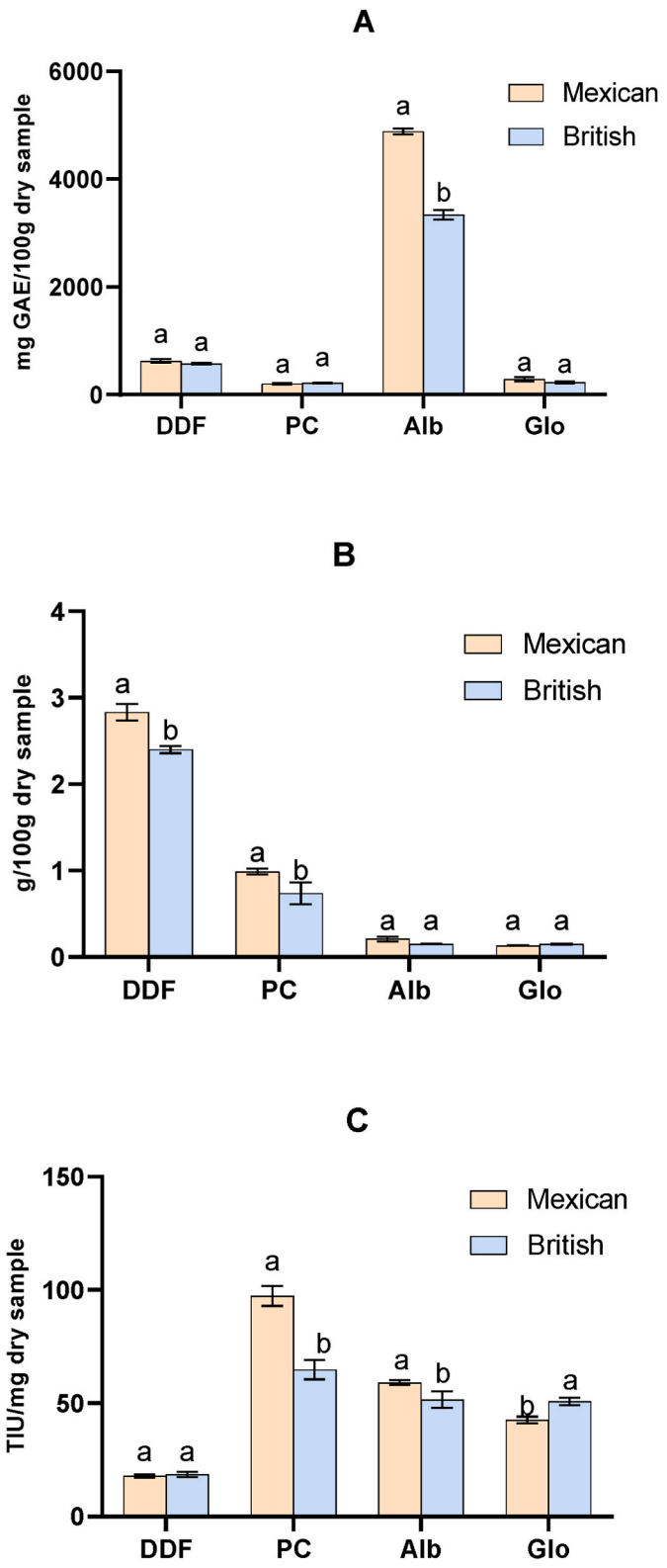

Fig. 4. Antinutritional factors in chia degummed-defatted chia flour (DDF), protein concentrate (PC), albumin (Alb), and globulin (Glo) fractions. A: total soluble phenolic compounds (TPC, mg GAE/100 g dry sample); B: phytic acid (g/100 g dry sample); C: trypsin inhibitory activity (TIA, TIU/mg dry sample).

respectively) followed by DDF (628.64 and 579.84 mg GAE/100 g for Mexican and British chia seeds, respectively), PC (306.99 mg GAE/100 g in and 248.34 mg GAE/100 g in MPC and BPC, respectively), MGlo and BGlo (209.94 mg GAE/100 g and 213.55 mg GAE/100 g, respectively) (Fig. 4A). As can be observed, the protein extraction step decreased 51.16% TPC in MPC and 57.17% in the BPC, however, these values increased dramatically with albumin fractionation (7.77-fold for MAlb and 5.76-fold in BAlb) compared to DDF. This could be due to the possible removal of water-soluble phenolic compounds during protein fractionation, such as flavonoids and tannins.

Rahman et al. (2017) obtained a TPC in Canada defatted chia meal of 1422 mg GAE/100 g, while Tunçil & Çelik (2019) reported that the TPC in defatted Argentinian black and white chia seeds were 352 and 342 mg GAE/100 g, respectively. Ikumi et al. (2022) observed that the TPC from chia seeds grown in Kenya ranged between 73 and 87 mg GAE/100 g. The differences could be due to the presence of mucilage in Argentinian and Canadian seeds which may hinder the extraction of phenolic compounds, as this was not carried out. Moreover, previous studies have proven that growing/environmental conditions and geographical location significantly impact the composition, amount and diversity of phenolic compounds of chia seeds (Ayerza, 2010). Additionally, differences in the extraction methods employed across different studies have led to notable variations in reported TPC values (Silva et al., 2015). Furthermore, processing conditions (i.e. heating, particle size, and pressure) and the solvent (i.e. water, methanol, acidified ethanol, ethyl acetate, etc.) used will also affect the concentration, chemical nature, polarity, and solubility of these compounds in the different chia products (Silva et al., 2015). Phenolics removal from protein matrixes with low or null impact on proteins is still a gap in plant-based protein products.

Phenolic compounds offer various health benefits, including their capacity to act as potential antioxidants and free radical scavengers, along with other significant physiological and biological properties (Alcântara et al., 2019, Oliveira-Alves et al., 2017; Reyes-Caudillo et al., 2008; Goli et al., 2005). However, protein-phenolic interactions can have negative effects on the physicochemical properties of chia proteins, as well as their techno-functionality, digestibility, protein quality, bioaccessibility (and their consecutive bioavailability and bioactivity), among other parameters (Czubinski & Dwiecki, 2017; Yan et al., 2023). These types of interactions may decrease the number of reactive groups in proteins and peptides, especially on free amino, free thiol groups and tryptophan residues (Sęczyk et al., 2019). Same effects could also be observed on globulins (Sęczyk et al., 2019), however, in the studied samples this phenomenon was totally different. Albumins from chia may present a high amount of free tryptophan residues, which they have affinity to bind to phenolics of low MW, such as hydroxycinnamic and hydroxybenzoic acids, as well as they may interact with glycosidic residues from aglycones of flavanols and flavonoids (Czubinski & Dwiecki, 2017). The high content of TPC in albumins could be due to the fractionation process, as albumins are water soluble proteins. Certain water-soluble phenolic compounds are extracted along with albumins, such as gallic acid, catechin, epicatechin, procyanidin and quercetin, which have been identified in chia seeds (Abdel-Aty et al., 2021). Globulins do not have these characteristics, hence protein chelation by phenolics is less frequent in these proteins (Sęczyk, et al., 2019).

PA contents of chia samples were gradually decreased with protein concentration-fractionation processes (Fig. 4B). The PA content in DDF was significantly higher compared to other samples, with values of 2.83 and 2.40 g/100 g for Mexican and British samples, respectively. Then, the PA content in MPC and BPC decreased by 65.12% and 69.21%, respectively. MAlb exhibited a decrease of 92.68% compared to MDDF, while BAlb showed a decrease of 93.68% compared to MDDF. MGlo showed a decrease of 95.34% compared to MDDF, while BGlo showed similar content to MAlb. The protein concentration/fractionation processes successfully allowed the reduction (a decrease of >90%) of PA in chia samples. This achievement can be attributed to the presence of PA

primarily within the outer layer of the chia seeds. A comprehensive study by Sahu et al. (2023) confirmed this point by illustrating the exclusive localization of PA in distinct seed layers across various plant species. For instance, in barley, rice, wheat, and millet, PA is stored within the aleurone layer and germ. In the context of wheat, the outer pericarp and aleurone layers enclose PA, while in maize, its presence is notable in the germ region.

Egli et al. (2002) used a consecutive soaking-germination process for three pseudocereals (amaranth, buckwheat, and quinoa), obtaining a PA content of 0.97–1.42 g/100 g in untreated seeds, 1.03–1.43 g/100 g in soaked seeds and 0.85–1.43 in germinated seeds. Thus, soaking-germination process did not effectively reduce the PA content. Furthermore, authors also observed this behavior in 10 cereals, 11 pulses and 2 oilseeds subjected to this treatment. However, Levent (2017) found a PA content in noodles made with 0–30% chia seed flour + DATEM (diacetyl tartaric esters of mono/di glycerides) as emulsifier of 0.17–1.06 g/100 g, these results are notably lower than ours, possibly because they used a lower amount of chia seeds for the noodle formulation.

PA exhibits a dual behavior concerning complex formation with proteins. At low pH and low cation concentration, direct electrostatic interactions lead to the formation of phytate-protein complexes. Conversely, at pH levels above 6 to 7, a ternary phytic acid-protein complex is formed, which subsequently dissociates in the presence of high Na^+ concentrations. These complexes are associated with reduced protein bioavailability and display heightened resistance to proteolytic digestion under low pH conditions (Castro-Alba et al., 2019; Zhang et al., 2020). Although PA exhibits beneficial properties such as antioxidant activity, anticancer, cholesterol and blood sugar reduction, and DNA damage protection (Campos-Vega et al., 2010; Nissar et al., 2017), it is important to consider its impact from a nutritional perspective, particularly in food products. PA possesses chelating capabilities, which can hinder the absorption of essential minerals, making it less desirable for individuals or populations with mineral deficiencies (Castro-Alba et al., 2019; Egli et al., 2002; Kumar et al., 2010; Sánchez-Velázquez et al., 2021). This issue becomes particularly relevant for vegans and vegetarians who might consume PA-rich foods and, consequently, are more prone to experiencing mineral deficiencies.

Contrary to TPC and PA contents, TIA increased with extraction/fractionation process on chia seed samples (Fig. 3C). Thus, TIA of DDF presented the lowest values (17.91 and 18.65 TIU/mg in MDDF and BDDF, respectively), while MPC and BPC showed a TIA increase of 5.43 and 4.72-fold, respectively. Followed by Alb and Glo for both Mexican (3.3–2.37-fold increase) and British (2.76–2.72-fold) seeds, respectively. This phenomenon may be attributed to the predominant presence of trypsin inhibitors (TIs) within the chia protein concentrates and fractions, with the extraction and fractionation process enhancing the concentration of TIs content within these protein fractions.

Avilés-Gaxiola et al. (2018) found that proteinase inhibitors (TIA) in soy and *faba* beans, are mostly located in the cotyledon (>90%). However, in chickpeas, TIA are distributed across multiple anatomical parts, including cotyledon (77.2%–75.8%), embryonic axis (11.9%–15.5%), and seed coat (10.9%–8.7%). Thus, the cellular localization of proteinase inhibitors has been found within protein bodies, cell walls, intercellular spaces, and cytosol (Avilés-Gaxiola et al., 2018). For mung beans, TIA are only localized in the cytoplasm and not within protein bodies (Krishnan et al., 2022), while for soybean, inhibitors (BBTI and KTI) were also found in the nucleus (Hernández-Nistal et al., 2009). This study showed that chia seeds contain high levels of TI in the protein concentrates, albumins, and globulins, indicating that protein bodies and cotyledon are potentially the primary locations of TI in the seeds.

During digestion, trypsin is produced as an inactive form called trypsinogen in the pancreas, it is a globular protein consisting of 220 amino acid residues with a molecular weight of 24 kDa. Upon entering the small intestine, trypsinogen is inactive and converted into its active form, trypsin (Divyapicigil et al., 2020). Thus, consuming foods rich in TIs can result in the irreversible formation of a complex between trypsin and TI, leading to a decreased trypsin activity in the intestine and reduced protein digestibility (Sánchez-Velázquez et al., 2021).

The protein concentration/fractionation process successfully reduced the content of PA for both PC and protein fractions, while TPC showed a similar behavior, but this was not observed for the albumin fractions as discussed above. While the content of TIs was increased. Thus, depending on the methods used for the extraction and/or fractionation of plant proteins, these will impact on the presence or absence of certain antinutrients, therefore, reducing and/or enhancing the protein digestibility and nutritional quality of plant protein ingredients.

Results of chia flour obtained in this study are in some extent different to those published, which could be associated with the degumming and defatting processing steps used, the extraction and fractionation methods applied and to the environmental/growing conditions and geographical locations of the seeds analyzed.

4. Conclusions

To the best of our knowledge, this is the first study that evaluates the effect of degumming-defatting, protein extraction and fractionation of Mexican and British chia seeds on the proximate and amino acid composition, protein quality and antinutrient content. The main protein fractions of both chia seeds were albumins and globulins. The protein contents of chia fractions were 37.9% (MAlb), 56.8% (BAlb), 44.2% (MGlo) and 39.3% (BGlo), respectively. BDDF and BAlb showed an improved amino acid profile than the Mexican chia seeds, while BPC and BGlo samples showed opposite results. The EAAI indicated that all chia protein samples provided more than the double of white egg protein (protein reference), ranging from 189.4% to 496.7%. BV values showed the same trend as EAAI, indicating the potential biological value of chia, due to its complete amino acid profile. The PER values were influenced by EAA and ranged from 2.02 to 3.35 in chia samples. IVPD of Mexican and British chia samples increased after protein extraction but decreased during fractionation, likely due to concentration of TI and phenolic compounds. Albumins from both seeds showed the highest TPC, possibly due to the extraction of certain water-soluble phenolic compounds during albumin fractionation. The protein extraction/fractionation process successfully reduced PA content but increased TIA. The presence of antinutrients in chia samples indicates that protein digestibility could be reduced to some extent during gastrointestinal digestion due to the presence of certain antinutrients. In general, the findings suggest that both Mexican and British chia protein fractions are potential ingredients for the development of food formulations with enhanced nutritional traits. Furthermore, subsequent research should focus on optimizing extraction and fractionation methods, in order to explore the increase and/or reduction of certain antinutrients. Depending on the protein fraction of interest, tailor made optimized foods can be developed by incorporating chia protein fractions into palatable, digestible and nutritionally enhanced foods. Chia proteins promise to play a pivotal role in crafting functional foods that could fulfill diverse dietary preferences and promote health benefits.

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.

Data availability

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

References

ABDEL-ATY, A. M., Elsayed, A. M., Salah, H. A., Bassuiny, R. I., & Mohamed, S. A. (2021). Egyptian chia seeds (Salvia hispanica L.) during germination: Upgrading of phenolic profile, antioxidant, antibacterial properties and relevant enzymes activities. *Food Science and Biotechnology, 30*, 723–734.

ADHIKARI, S., Schop, M., DE Boer, I. J., & Huppertz, T. (2022). Protein quality in perspective: A review of protein quality metrics and their applications. *Nutrients, 14*, 947.

ALAIZ, M., Navarro, J. L., Girón, J., & Vioque, E. (1992). Amino acid analysis by high-performance liquid chromatography after derivatization with diethyl ethoxymethylenemalonate. *Journal of Chromatography A, 591*, 181–186.

Alcântara, M. A., Polari, I. D. L. B., de Albuquerque Meireles, B. R. L., de Lima, A. E. A., da Silva Junior, J. C., de Andrade Vieira, É., … de Magalhães Cordeiro, A. M. T. (2019). Effect of the solvent composition on the profile of phenolic compounds extracted from chia seeds. *Food Chemistry, 275*, 489–496.

ALI, N.M., Yeap, S.K., Ho, W.Y., Beh, B.K., Tan, S.W., & Tan, S.G. (2012). The promising future of chia, Salvia hispanica L. Journal of Biomedicine and Biotechnology. 2012; 2012:171956.

ALVAREZ-BARAJAS, A., Perez-Carrillo, E., & DE LA Rosa-Millan, J. (2023). Physicochemical, thermal and in vitro digestion characteristics of cricket-(Acheta domesticus) substituted corn tortillas. *International Journal of Food Science and Technology, 58*, 1821–1831.

AMZA, T., Balla, A., Tounkara, F., Man, L., & Zhou, H. (2013). Effect of hydrolysis time on nutritional, functional and antioxidant properties of protein hydrolysates prepared from gingerbread plum (Neocarya macrophylla) seeds. *International Food Research Journal, 20*, 2081.

AOAC. (2016). *Official methods of analysis of AOAC International*. Rockville, MD: AOAC International.

AOAC Official Methods of. (1995). *Analysis; Association of Official Analytical Chemists*. Arlington, Washington, DC.

AVILÉS-GAXIOLA, S., Chuck-Hernández, C., & Serna Saldivar, S. O. (2018). Inactivation methods of trypsin inhibitor in legumes: A review. *Journal of Food Science, 83*, 17–29.

AYERZA, R. (2010). Effects of seed color and growing locations on fatty acid content and composition of two chia (Salvia hispanica L.) genotypes. *Journal of the American Oil Chemists' Society, 87*, 1161–1165.

BOS, C., Gaudichon, C., & Tomé, D. (2000). Nutritional and physiological criteria in the assessment of milk protein quality for humans. *Journal of the American College of Nutrition, 19*, 191S–205S.

CAMPOS-VEGA, R., Loarca-Piña, G., & Oomah, B. D. (2010). Minor components of pulses and their potential impact on human health. *Food Research International, 43*, 461–482.

CAMPOS, B. E., Ruivo, T. D., Da Silva Scapim, M. R., Madrona, G. S., & Bergamasco, R. D. C. (2016). Optimization of the mucilage extraction process from chia seeds and application in ice cream as a stabilizer and emulsifier. *LWT–Food Science and Technology, 65*, 874–883.

CAPITANI, M. I., Ixtaina, V. Y., Nolasco, S. M., & Tomás, M. C. (2013). Microstructure, chemical composition and mucilage exudation of chia (Salvia hispanica L.) nutlets from Argentina. *Journal of the Science of Food and Agriculture, 93*, 3856–3862.

CASTRO-ALBA, V., Lazarte, C. E., Perez-Rea, D., Carlsson, N. G., Almgren, A., Bergenståhl, B., & Granfeldt, Y. (2019). Fermentation of pseudocereals quinoa, canihua, and amaranth to improve mineral accessibility through degradation of phytate. *Journal of the Science of Food and Agriculture, 99*, 5239–5248.

CHEN, J., Zhu, L., Wu, Q., Chen, Y., Wu, G., & Zhang, H. (2023). Structure characterization and bioactivities of protein hydrolysates of chia seed expeller processed with different proteases in silico and in vitro. *Food Bioscience*, Article 102781.

CISTERNAS, C., Farías, C., Muñoz, L., Morales, G., & Valenzuela, R. (2022). Composición química, características nutricionales y beneficios asociados al consumo de chía (Salvia hispanica L.). *Revista Chilena de Nutricion, 49*, 625–636.

CZUBINSKI, J., & DWIECKI, K. (2017). A review of methods used for investigation of protein–phenolic compound interactions. *International Journal of Food Science and Technology, 52*, 573–585.

DA SILVA, B. P., Dias, D. M., DE Castro Moreira, M. E., Toledo, R. C. L., Da Matta, S. L. P., Lucia, C. M. D., Martino, H. S. D., & Pinheiro-Sant'Ana, H. M. (2016). Chia seed shows good protein quality, hypoglycemic effect and improves the lipid profile and liver and intestinal morphology of Wistar rats. *Plant Foods for Human Nutrition, 71*, 225–230.

DE FALCO, B., Amato, M., & Lanzotti, V. (2017). Chia seeds products: An overview. *Phytochemistry Reviews, 16*, 745–760.

DIVYAPICIGIL, M., Gokturk, S. S. P., Ergenoglu, B., Yucel, F., & Akcael, E. A. (2020). Development of mutant human immunoreactive trypsinogen 1 (IRT1) and mutant human immunoreactive trypsinogen 2 (IRT2) for use in immunoassays. *Protein Expression and Purification, 169*, Article 105572.

EGLI, I., Davidsson, L., Juillerat, M., Barclay, D., & Hurrell, R. (2002). The influence of soaking and germination on the phytase activity and phytic acid content of grains and seeds potentially useful for complementary feedin. *Journal of Food Science, 67*, 3484–3488.

FAO/WHO/UNU. (1985). *Energy and protein requirements. Report of a joint FAO/WHO/UNU Expert Consultation*.

FELISBERTO, M. H. F., Wahanik, A. L., Gomes-Ruffi, C. R., Clerici, M. T. P. S., Chang, Y. K., & Steel, C. J. (2015). Use of chia (Salvia hispanica L.) mucilage gel to reduce fat in pound cakes. *LWT–Food Science and Technology, 63*, 1049–1055.

GOLI, A. H., Barzegar, M., & Sahari, M. A. (2005). Antioxidant activity and total phenolic compounds of pistachio (Pistachia vera) hull extracts. *Food Chemistry, 92*, 521–525.

GÓRSKA-WARSEWICZ, H., Laskowski, W., Kulykovets, O., Kudlińska-Chylak, A., Czeczotko, M., & Rejman, K. (2018). Food products as sources of protein and amino acids—the case of Poland. *Nutrients, 10*, 1977.

GRANCIERI, M., Martino, H., & DE Mejia, E. (2019a). Chia seed (salvia hispanica L.) as a source of proteins and bioactive peptides with health benefits: A review. *Comprehensive Reviews in Food Science and Food Safety, 18*, 480–499.

GRANCIERI, M., Martino, H. S. D., & Gonzalez DE Mejia, E. (2019b). Chia (salvia hispanica L.) seed total protein and protein fractions digests reduce biomarkers of inflammation and atherosclerosis in macrophages in vitro. *Molecular Nutrition & Food Research, 63*. n/a-n/a.

HERNÁNDEZ-NISTAL, J., Martín, I., Jiménez, T., Dopico, B., & Labrador, E. (2009). Two cell wall Kunitz trypsin inhibitors in chickpea during seed germination and seedling growth. *Plant Physiology and Biochemistry, 47*, 181–187.

HERNÁNDEZ-PÉREZ, T., Valverde, M. E., Orona-Tamayo, D., & Paredes-Lopez, O. (2020). Chia (salvia hispanica): Nutraceutical properties and therapeutic applications. MDPI *Proceedings, 17*.

IKUMI, P. W., Mburu, M., Njoroge, D., Gikonyo, N., & Musingi, B. M. (2022). *Physical, total phenolic and total flavonoid properties of chia (salvia hispanica L.) seeds grown in Kenya*.

JOOD, S., & SINGH, M. (2001). Amino acid composition and biological evaluation of the protein quality of high lysine barley genotypes. *Plant Foods for Human Nutrition, 56*, 145–155.

JULIO, L., Ruiz-Ruiz, J., Tomás, M., & Segura-Campos, M. (2019). Chia (salvia hispanica) protein fractions: Characterization and emulsifying properties. *Journal of Food Measurement and Characterization, 13*, 3318–3328.

KAUR, S., & BAINS, K. (2020). Chia (salvia hispanica l.)–a rediscovered ancient grain, from aztecs to food laboratories: A review. *Nutrition & Food Science, 50*, 463–479.

KOTECKA-MAJCHRZAK, K., Sumara, A., Fornal, E., & Montowska, M. (2020). Oilseed proteins–properties and application as a food ingredient. *Trends in Food Science & Technology, 106*, 160–170.

KRISHNAN, H. B., Kim, S., Pereira, A. E., Jurkevich, A., & Hibbard, B. E. (2022). Adenanthera pavonina, a potential plant-based protein resource: Seed protein composition and immunohistochemical localization of trypsin inhibitors. *Food Chemistry X, 13*, Article 100253.

KUMAR, V., Sinha, A. K., Makkar, H. P., & Becker, K. (2010). Dietary roles of phytate and phytase in human nutrition: A review. *Food Chemistry, 120*, 945–959.

LAEMMLI, U. (1970). Cleavage of structural proteins during the assembly of the head ofbacteriophage T_4 Nature (Lond). *Laemmli680227Nature (Lond), 227*, 680–685.

LEVENT, H. (2017). Effect of partial substitution of gluten-free flour mixtures with chia (Salvia hispanica L.) flour on quality of gluten-free noodles. *Journal of Food Science and Technology, 54*, 1971–1978.

LÓPEZ, D. N., Galante, M., Raimundo, G., Spelzini, D., & Boeris, V. (2019). Functional properties of amaranth, quinoa and chia proteins and the biological activities of their hydrolyzates. *Food Research International, 116*, 419–429.

LÓPEZ, D. N., Galante, M., Robson, M., Boeris, V., & Spelzini, D. (2018a). Amaranth, quinoa and chia protein isolates: Physicochemical and structural properties. *International Journal of Biological Macromolecules, 109*, 152–159.

LÓPEZ, D. N., Ingrassia, R., Busti, P., Bonino, J., Delgado, J. F., Wagner, J., Boeris, V., & Spelzini, D. (2018b). Structural characterization of protein isolates obtained from chia (Salvia hispanica L.) seeds. *Lebensmittel-Wissenschaft & Technologie, 90*, 396–402.

LQARI, H., Vioque, J., Pedroche, J., & Millán, F. (2002). Lupinus angustifolius protein isolates: Chemical composition, functional properties and protein characterization. *Food Chemistry, 76*, 349–356.

MA, F., & BAIK, B. K. (2021). Influences of grain and protein characteristics on in vitro protein digestibility of modern and ancient wheat species. *Journal of the Science of Food and Agriculture, 101*, 4578–4584.

MALIK, A. M., & RIAR, C. S. (2022). Difference in the nutritional, in vitro, and functional characteristics of protein and fat isolates of two Indian chia (Salvia hispanica L) seed genotypes with variation in seed coat color. *Journal of Food Science, 87*, 3872–3887.

MARINELI, R. D. S., Moraes, É. A., Lenquiste, S. A., Godoy, A. T., EBERLIN, M. N., & Maróstica, M. R., JR. (2014). Chemical characterization and antioxidant potential of Chilean chia seeds and oil (Salvia hispanica L). *LWT–Food Science and Technology, 59*, 1304–1310.

MARTÍNEZ-CRUZ, O., & PAREDES-LÓPEZ, O. (2014). Phytochemical profile and nutraceutical potential of chia seeds (Salvia hispanica L.) by ultra high performance liquid chromatography. *Journal of Chromatography A, 1346*, 43–48.

MOHAMMED, O., Bekhet, M., EL-Razek, A., Amal, M., & Moharram, Y. (2019). Evaluation of chia (Salvia hispanica L.) seeds meal as a source of bioactive ingredients. *Alexandria Science Exchange Journal, 40*, 177–189.

MONDOR, M., & HERNÁNDEZ-ÁLVAREZ, A. J. (2022). Processing technologies to produce plant protein concentrates and isolates. *Plant Protein Foods. Springer.61-108*.

MONROY-TORRES, R., Mancilla-Escobar, M. L., Gallaga-Solórzano, J. C., & Santiago-García, E. J. (2008). Protein digestibility of chia seed Salvia hispanica L. *Revista Salud Pública Y Nutrición, 9*, 1–9.

MUÑOZ, L. A., Cobos, A., Diaz, O., & Aguilera, J. M. (2012). Chia seeds: Microstructure, mucilage extraction and hydration. *Journal of Food Engineering, 108*, 216–224.

NISSAR, J., Ahad, T., Naik, H., & Hussain, S. (2017). A review phytic acid: As antinutrient or nutraceutical. *Journal of Pharmacognosy and Phytochemistry, 6*, 1554–1560.

NITZ, K., Lacy, M., & Atzler, D. (2019). Amino acids and their metabolism in atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology, 39*, 319–330.

NOSWORTHY, M. G., Medina, G., Franczyk, A. J., Neufeld, J., Appah, P., Utioh, A., Frohlich, P., & House, J. D. (2018). Effect of processing on the in vitro and in vivo protein quality of beans (Phaseolus vulgaris and Vicia Faba). *Nutrients, 10*, 671.

OLIVEIRA-ALVES, S. C., Vendramini-Costa, D. B., Cazarin, C. B. B., Júnior, M. R. M., Ferreira, J. P. B., Silva, A. B., Prado, M. A., & Bronze, M. R. (2017). Characterization of phenolic compounds in chia (Salvia hispanica L.) seeds, fiber flour and oil. *Food Chemistry, 232*, 295–305.

OLIVOS-LUGO, B. L., Valdivia-López, M.Á., & Tecante, A. (2010). Thermal and physicochemical properties and nutritional value of the protein fraction of Mexican chia seed (salvia hispanica L). *Food Science and Technology International, 16*, 89–96.

ORLIEN, V., Aalaei, K., Poojary, M. M., Nielsen, D. S., Ahrne, L., & Carrascal, J. R. (2023). Effect of processing on in vitro digestibility (IVPD) of food proteins. *Critical Reviews in Food Science and Nutrition, 63*, 2790–2839.

ORONA-TAMAYO, D., Valverde, M. E., Nieto-Rendón, B., & PAREDES-López, O. (2015). Inhibitory activity of chia (Salvia hispanica L.) protein fractions against angiotensin I-converting enzyme and antioxidant capacity. *LWT–Food Science and Technology, 64*, 236–242.

OSER, B. L. (1959). An integrated essential amino acid index for predicting the biological value of proteins. *Protein and amino acid nutrition*, 281.

RAD. (1989). *Recommended dietary allowances*. Washington,DC,USA: National Research Council-National Academy Press.

RAHMAN, M. J., DE Camargo, A. C., & Shahidi, F. (2017). Phenolic and polyphenolic profiles of chia seeds and their in vitro biological activities. *Journal of Functional Foods, 35*, 622–634.

REYES-CAUDILLO, E., Tecante, A., & Valdivia-Lopez, M. A. (2008). Dietary fibre content and antioxidant activity of phenolic compounds present in Mexican chia (Salvia hispanica L.) seeds. *Food Chemistry, 107*, 656–663.

RODRÍGUEZ LARA, A., Mesa-García, M. D., Medina, K. A. D., Quirantes Piné, R., Casuso, R. A., Segura Carretero, A., & Huertas, J. R. (2021). Assessment of the phytochemical and nutrimental composition of dark chia seed (Salvia hispánica L). *Foods, 10*, 3001.

SAHU, A., Verma, R., Gupta, U., Kashyap, S., & Sanyal, I. (2023). An overview of targeted genome editing strategies for reducing the biosynthesis of phytic acid: An anti-nutrient in crop plants. *Molecular Biotechnology*, 1–15.

SALAZAR VEGA, I. M., Quintana Owen, P., & Segura Campos, M. R. (2020). Physicochemical, thermal, mechanical, optical, and barrier characterization of chia (Salvia hispanica L.) mucilage-protein concentrate biodegradable films. *Journal of Food Science, 85*, 892–902.

SALGADO, V. D. S. C. N., Zago, L., Antunes, A. E. C., & Miyahira, R. F. (2022). Chia (salvia hispanica L.) seed germination: A brief review. *Plant Foods for Human Nutrition, 77*, 485–494.

SÁNCHEZ-VELÁZQUEZ, O. A., Ribéreau, S., Mondor, M., Cuevas-Rodríguez, E. O., Arcand, Y., & Hernández-Álvarez, A. J. (2021). Impact of processing on the in vitro protein quality, bioactive compounds, and antioxidant potential of 10 selected pulses. *Legume Science, 3*, e88.

SANDOVAL-OLIVEROS, M. R., & PAREDES-LÓPEZ, O. (2013). Isolation and characterization of proteins from chia seeds (Salvia hispanica L). *Journal of Agricultural and Food Chemistry, 61*, 193–201.

Sęczyk, Ł., Świeca, M., Kapusta, I., & Gawlik-Dziki, U. (2019). Protein–phenolic interactions as a factor affecting the physicochemical properties of white bean proteins. *Molecules, 24*(3), 408.

SEGURA-CAMPOS, M. R. (2019). Isolation and functional caharacterization of chia (Salvia hispanica) proteins. *Food Science and Technology, 40*, 334–339.

SEGURA-NIETO, M., Vazquez-Sanchez, N., Rubio-Velazquez, H., Olguin-Martinez, L. E., Rodriguez-Nester, C. E., & Herrera-Estrella, L. (1992). Characterization of amaranth (Amaranthus hypochondriacus L.) seed proteins. *Journal of Agricultural and Food Chemistry, 40*, 1553–1558.

SHAGHAGHIAN, S., Mcclements, D. J., Khalesi, M., Garcia-Vaquero, M., & Mirzapour-Kouhdasht, A. (2022). Digestibility and bioavailability of plant-based proteins intended for use in meat analogues: A review. *Trends in Food Science &, 129*, 646–656.

SILVA, A., Schimdt, M., Scapin, G., Prestes, R., Ferreira, S., & Da Rosa, C. (2015). Effect of extract of chia seed (Salvia hispanica) as an antioxidant in fresh. *pork, 22*(3), 1195–1202.

SUEIRO, S., Hermida, M., González, M., Lois, A., & Rodríguez-Otero, J. (2015). A comparison of the ISO and AACC methods for determining the activity of trypsin inhibitors in soybean meal. *Journal of the American Oil Chemists' Society, 92*, 1391–1397.

TIMILSENA, Y. P., Adhikari, R., Barrow, C. J., & Adhikari, B. (2016a). Microencapsulation of chia seed oil using chia seed protein isolate chia seed gum complex coacervates. *International Journal of Biological Macromolecules, 91*, 347–357.

TIMILSENA, Y. P., Adhikari, R., Barrow, C. J., & Adhikari, B. (2016b). Physicochemical and functional properties of protein isolate produced from Australian chia seeds. *Food Chemistry, 212*, 648–656.

TINUS, T., Damour, M., VAN Riel, V., & Sopade, P. (2012). Particle size-starch–protein digestibility relationships in cowpea (Vigna unguiculata). *Journal of Food Engineering, 113*, 254–264.

TUNÇIL, Y. E., & ÇELIK, Ö. F. (2019). Total phenolic contents, antioxidant and antibacterial activities of chia seeds (Salvia hispanica L.) having different coat color. *Akademik Ziraat Dergisi, 8*, 113–120.

URBIZO-REYES, U., San Martin-González, M. F., Garcia-Bravo, J., Vigil, A. L. M., & Liceaga, A. M. (2019). Physicochemical characteristics of chia seed (Salvia hispanica) protein hydrolysates produced using ultrasonication followed by microwave-assisted hydrolysis. *Food Hydrocolloids, 97*, Article 105187.

VALDIVIA-LÓPEZ, M.Á., & TECANTE, A. (2015). Chia (salvia hispanica): A review of native Mexican seed and its nutritional and functional properties. *Advances in Food & Nutrition Research, 75*, 53–75.

VÁZQUEZ-OVANDO, A., Betancur-Ancona, D., & Chel-Guerrero, L. (2013). Physicochemical and functional properties of a protein-rich fraction produced by dry fractionation of chia seeds (Salvia hispanica L). *CyTA - Journal of Food, 11*, 75–80.

VERFAILLIE, D., Janssen, F., VAN Royen, G., & Wouters, A. G. (2023). A systematic study of the impact of the isoelectric precipitation process on the physical properties and protein composition of soy protein isolates. *Food Research International, 163*, Article 112177.

WANG, L., Zhang, Y., Li, R., & Xiang, D. (2023). L-lysine moderates thermal aggregation of coconut proteins induced by thermal treatment. *Scientific Reports, 13*, Article 13310.

YAN, X., Zeng, Z., Mcclements, D. J., Gong, X., Yu, P., Xia, J., & Gong, D. (2023). A review of the structure, function, and application of plant-based protein–phenolic conjugates and complexes. *Comprehensive Reviews in Food Science and Food Safety, 22*, 1312–1336.

YUST, M. A. M., Pedroche, J., Girón-Calle, J., Vioque, J., Millán, F., & Alaiz, M. (2004). Determination of tryptophan by high-performance liquid chromatography of alkaline hydrolysates with spectrophotometric detection. *Food Chemistry, 85*, 317–320.

ZHANG, Z., He, S., Liu, H., Sun, X., Ye, Y., Cao, X., Wu, Z., & Sun, H. (2020). Effect of pH regulation on the components and functional properties of proteins isolated from cold-pressed rapeseed meal through alkaline extraction and acid precipitation. *Food Chemistry, 327*, Article 126998.