Comprehensive characterization of raw and processed quinoa from conventional and organic farming by label-free shotgun proteomics

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Abstract

Quinoa, a dicotyledonous plant native to the Andes, is recognized as a high-quality food due to its outstanding nutritional properties, including complete proteins. However, there is a lack of information on how the proteomic profile of raw quinoa is influenced by processing methods such as boiling and extrusion, as well as by conventional and organic farming conditions. Here, proteins from both raw (seeds and grains) and processed (boiled and extruded) white quinoa cultivated under conventional and organic farming were extracted, trypsinized, and analyzed by nanoliquid chromatography-tandem mass spectrometry (nanoLC-MS/MS). The mass spectra data were then scrutinized against a dedicated quinoa database from The National Center for Biotechnology Information (NCBI) via MaxQuant/Andromeda, leading to the identification and quantification of 1,796 proteins. Finally, qualitative and quantitative data interpretation tools were employed for data inspection and visualization, unveiling for the first time, similarities and differences at the proteomic level among the studied samples.

KEYWORDS: Conventional farming; NanoLC-MS/MS; Organic farming; Processed quinoa; Raw quinoa; Shotgun proteomics

1. Introduction

Quinoa (*Chenopodium quinoa* Willd.) can be regarded as an excellent food choice due to its remarkable nutritional properties, particularly its high-quality protein composition with a well-balanced profile of essential amino acids (Chaudhary et al., 2023; Hussain et al., 2021). Additionally, unlike most cereals, quinoa is gluten-free and non-allergenic (Alvarez-Jubete et al., 2010; Hussain et al., 2021). In recent years, the outstanding nutritional benefits of quinoa, combined with its adaptability to diverse agroecological conditions, have led to a substantial increase in demand and the global expansion of its cultivation (Alandia et al., 2020; Ceyhun Sezgin & Sanlier, 2019; Hussain et al., 2021).

Although quinoa seeds are a valuable source of nutrients and bioactive compounds, including minerals, amino acids, polyphenols, terpenes, and proteins (Aloisi et al., 2016; Chaudhary et al., 2023), they need to be processed before being incorporated into the human diet to enhance their digestibility. For this purpose, after separating the grains from the pericarp and washing to remove saponins (responsible for the bitter taste), several processing methods can be applied, with boiling and extrusion being the most commonly used (Kowalski et al., 2016; Kuktaite et al., 2022; Motta et al., 2019). Boiling, a traditional and simple method used to prepare quinoa, consists of cooking the grains in an excess of water for around 15 min (Van de Vondel et al., 2022). In contrast, extrusion involves exposing quinoa grains to heat, mechanical energy, and pressure, ultimately forcing them through a die to shape the final product (Kowalski et al., 2016). Compared to other food-processing methods, such as roasting and steam pre-conditioning, boiling and extrusion are simpler and exhibit shorter processing times (Van de Vondel et al., 2022; Kowalski et al., 2016). However, due to the application of heating and pressure treatments, the

physicochemical properties of the resulting food products are affected, often leading to protein denaturation, oxidation, and aggregation (Santé-Lhoutellier et al., 2008; Soladoye et al., 2015).

Another factor that can influence the proteomic profile of raw crops is the farming type. Nowadays, with the improvement of living standards, consumer demands have shifted from basic dietary needs to higher nutritional requirements. In this evolving context, organic farming has emerged as a significant influence. Regulated by legislation encouraging practices such as crop rotations and the prohibition of synthetic herbicides, pesticides, and fertilizers, organic farming aims to produce healthier and more sustainable foods (Gomiero, 2018; Gomiero et al., 2011; Xiao et al., 2019). This new trend is evident in the current substantial demand for organic quinoa internationally, particularly from the United States, Australia, Canada, and the European Union (Alandia et al., 2020; Cancino-Espinoza et al., 2018; Hussain et al., 2021). Interestingly, the consumption of organic quinoa has also experienced a considerable surge in traditional country producers, such as Peru (Cancino-Espinoza et al., 2018).

The literature on quinoa proteomics is relatively recent and has mainly focused on raw seeds and grains. In our previous research, we described a shotgun proteomics approach to characterize four commercially available quinoa grains (black, red, and white quinoa from Peru, and white quinoa from Bolivia, also known as royal), allowing to establish a comprehensive quinoa grain map comprising 1,211 proteins (Galindo-Luján, Pont, Minic, et al., 2021). This study served as a groundwork for developing a simple data mining strategy aimed at identifying quinoa grain proteins with potential immunonutritional bioactivities, including those related to cancer (Galindo-Luján et al., 2023). A recent

study also described a shotgun proteomics approach to evaluate changes associated with water limitation (rainfed conditions) when compared to full irrigation (irrigated conditions) in quinoa seed samples, revealing a total of 2,577 proteins (Poza-Viejo et al., 2023). Other studies have demonstrated the usefulness of untargeted proteomics approaches for the characterization of quinoa proteins after subjecting non-edible parts of the plant, such as the leaves or guard cells, to mitovirus infection (Di Silvestre et al., 2022) or salinity treatments (Derbali et al., 2021; Rasouli et al., 2021). Nevertheless, none of the aforementioned studies have explored the impact of different processing and farming procedures on the raw quinoa proteome. In this study we employed, for the first time, a label-free nanoliquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) shotgun proteomics approach to extensively examine the proteome of both raw (seeds and grains) and processed (boiled and extruded) white quinoa (Salcedo variety) cultivated under conventional and organic farming conditions. The proposed methodology provides a comprehensive and detailed set of 1,796 proteins, offering potential utility in enhancing the nutritional value of raw quinoa under diverse processing or farming conditions.

2. Materials and methods

2.1. Chemicals

All the chemicals were of analytical reagent grade or better. Sodium hydroxide (NaOH, ≥99.0%), hydrochloric acid (HCl, 37% (v/v)), boric acid (H_3BO_3, ≥99.5%), β-mercaptoetanol (≥99.0%), sodium dihydrogen phosphate (NaH_2PO_4, ≥99.0%), water (LC-MS grade), acetonitrile (ACN, LC-MS grade), bovine serum albumin (BSA, relative molecular mas (M_r) of 66,000), formic acid (FA, 99.0%), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, ≥99.5%), urea (≥99.0%), Triton™ X-100

(laboratory grade), glycerol (≥99.5%), tris(2-carboxyethyl)phosphine hydrochloride (TCEP, ≥98.0%), sodium sodecyl sulfate (SDS, ≥99.8%), and iodoacetamide (IAA, ≥99.0%) were provided by Merck (Darmstadt, Germany). Trypsin/Lys-C enzyme mix (MS grade) was supplied from Promega (Madison, WI, USA).

2.2. Sample treatment

White quinoa seeds (Salcedo variety, National Institute of Agricultural Innovation of Peru) were cultivated in 2018 under conventional and organic conditions in La Molina, Lima, Peru (latitude 12° 04' 36"S, longitude 76° 56' 43"W, altitude 241 m above sea level (masl)) and Omas, Lima, Peru (latitude 12° 33' 25.6"S, longitude 76° 19' 9"W, altitude 1227 masl), respectively. In conventional soil fertilization, a mixture of urea, diammonium phosphate, and potassium chloride was applied. In contrast, organic soil fertilization employed 'bokashi,' a fermented food-based fertilizer prepared with ingredients such as animal dung, molasses, and other organic materials. In order to separate the grain from the pericarp, quinoa seeds were polished for 5 min using a scarifier machine (Vulcano, Lima, Peru). After that, the obtained quinoa grains were washed three times for 5 min in a quinoa-to-water ratio of 1:10 (m/v) at room temperature (rt). Finally, the washed quinoa grains were dried at 40ºC in an oven (Memmert, Schwabach, Germany) and stored at rt in a dry environment.

2.2.1. Boiling process

White quinoa grains from both conventional and organic farming were ground with an ultra-centrifugal mill (Restch, Schwabach, Germany) at 18,000 rpm for 30 s. The sieving

operation was conducted using a mesh with a 0.5 mm opening during the grinding process. The resulting quinoa grain flour was dispersed in water before boiling to prevent lump formation, ensuring a homogenous mixture. A flour-to-water mixture (1:20, m/v) was heated in a cooking pot for 20 min at 100ºC with continuous stirring. After the process, the boiled grains were cooled for 20 min, dried at 40ºC for 72 h, and subsequently stored in polyethylene (PE) bags at rt until further analysis.

2.2.2. Extrusion process

White quinoa grains from both conventional and organic farming were extruded using a co-rotating twin screw extruder (Inbramaq, São Paulo, Brazil). The extruder comprised a feeding zone, a heating zone, and a die zone. The overall length of the extruder barrel was 960 mm, with a screw diameter of 30 mm, and a cylindrical die diameter of 10 mm. The temperature was configured as follows: the extruder feeding zone was set at 30ºC, progressing to 40ºC and, then, 50ºC. The heating zone exhibited variations at 70ºC, 85ºC, and 100ºC, while the die zone was maintained at temperatures of 100ºC, 110ºC, and 125ºC. The grain feeding rate was set at 14 kg/h, with the screw speed held constant at 800 rpm. The retention time was maintained between 10 and 15 s, and the cut frequency was configured at 17 Hz. After the process, the extruded grains were cooled for 15 min and subsequently stored in PE bags at rt until further analysis.

2.2.3. Protein extraction

Proteins from raw (i.e., seeds and grains), boiled, and extruded quinoa from conventional (C_{seed}, C_{grain}, C_{boiled}, and $C_{extruded}$) and organic farming (O_{seed}, O_{grain}, O_{boiled}, and $O_{extruded}$)

were extracted as in our previous work (Galindo-Luján, Pont, Sanz-Nebot, et al., 2021), with some modifications. Briefly, 250 mg of each sample were mixed with 2 mL of water and 39 µL of 1 M NaOH (final pH of 10.0) using a vortex Genius 3 (Ika®, Staufen, Germany) for 3 h at rt. Separation of soluble proteins from the insoluble residue was performed by centrifugation at 23,000 x g for 60 min at 4ºC in a cooled Rotanta 460 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). For protein purification, the supernatant pH was adjusted with 22 µL of 1 M HCl to obtain a final pH value of 5.0. After centrifugation at 30,000 x g for 30 min at 4ºC, precipitated proteins were resuspended in 1 mL of a solution of 60 mM H_3BO_3 (pH adjusted to 9.0 with NaOH). The resulting solution was filtered through 0.22 µm nylon filters (MSI, Westboro, MA, USA) before analysis. All pH measurements were made using a Crison 2002 potentiometer and a Crison electrode 52-03 (Crison Instruments, Barcelona, Spain).

2.3. Total protein content analysis

The total amount of protein in the quinoa extracts was estimated spectrophotometrically using a capillary electrophoresis (CE) instrument equipped with a diode-array detector (7100 CE, Agilent Technologies, Waldbronn, Germany). Samples (two independent replicates from C_{seed}, C_{grain}, C_{boiled}, $C_{extruded}$, O_{seed}, O_{grain}, O_{boiled}, and $O_{extruded}$ quinoa) were injected at 50 mbar for 10 s using a 58 cm total length (L_T) × 50 µm internal diameter (i.d.) × 365 µm outer diameter (o.d.) fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA). A calibration curve was established by analyzing BSA standard solutions at concentrations ranging between 100 and 1,000 µg/mL. Flow injection experiments were carried out without voltage, mobilizing the sample plug by applying 50

mbar of pressure after the injection. Absorbance was measured at 214 nm from the area of the detected protein peaks.

2.4. Proteolytic digestion

Quinoa protein extracts were digested using a modified filter-aided sample preparation (FASP) protocol designed for proteomic analysis (Wiśniewski et al., 2009). In this process, 50 µg of protein sample was diluted to a volume of 100 µL using a denaturation buffer consisting of 8 M urea and 25 mM HEPES (pH 8.0). After vortexing briefly, samples were transferred to 10,000 M_r cut-off (MWCO) centrifugal filters (Millipore, Molsheim, France). The sample volume was reduced to 20 µL through centrifugation for 20 min at 14,000 x g, followed by protein reduction with the addition of 4 mM TCEP in 100 µL of denaturation buffer. Incubation at 25°C for 30 min was followed by a 15-min centrifugation step at 14,000 x g. Proteins were then alkylated using 20 mM IAA in 100 µL of denaturation buffer, followed by a 40-min incubation at 25°C and a 15-min centrifugation at 14,000 x g. Subsequently, 100 µL of digestion buffer (0.6% (v/v) glycerol and 25 mM HEPES, pH 8.0) were added to the filter and, after a 15-min centrifugation at 14,000 x g, the filter was transferred to a clean collection tube. Proteolytic digestion was achieved by adding MS-grade trypsin/Lys-C mix at an enzyme-to-protein ratio of 1:300 (m/m), followed by incubation in the dark under shaking at 600 rpm at 37°C for 12 h. Peptides were separated in the filtrate by centrifugation at 14,000 x g for 15 min, and digestion was stopped by adding 1% (v/v) FA and centrifuged for 2 min at 15,000 x g. The digested proteins collected from the supernatant were desalted using disposable TopTip C-18 columns (Glygen, Columbia, MD, USA), evaporated to dryness, and reconstituted in 20 µL of water containing 1% (v/v) FA.

2.5. NanoLC-MS/MS

NanoLC-MS/MS analyses were performed on an Ultimate3000 nanoRLSC (Thermo Scientific) coupled to an Orbitrap Fusion™ (Thermo Scientific). Two µL of protein digests were injected and separated on a column (15 cm L_T x 75 µm i.d. x 365 µm o.d. fused silica capillary, Polymicro Technologies) packed in-house with Luna C18 particles (Luna C18(2), 3 µm, 100 Å, Phenomenex, Torrance, California, USA). The mobile phase consisted of a mixture of water/ACN/0.1% (v/v) FA, working at a flow rate of 0.30 µL/min (0-7 min, 2-2% ACN; 7-107 min, 2-38% ACN; 107-112 min, 38-98% ACN; 112-122 min, 98-98% ACN; 122-130 min, 98-2% ACN; 130-140 min, 2-2% ACN). The mass spectrometer was operated in electrospray ionization (ESI) positive mode under the following parameters: ion source temperature 250°C, ion spray voltage 2.1 kV, top speed mode, and full-scan MS spectra acquired with a resolution of 60,000 over 350–2,000 *m/z*. Precursor ions were selectively filtered through monoisotopic precursor selection, considering a charge state range of +2 to +7, and dynamic exclusion parameters (30 s with a ± 10 ppm window). The automatic gain control settings were configured at $5*10^5$ for the full scan and $1*10^4$ for MS/MS scans. Fragmentation was achieved using collision induced dissociation (CID) in the linear ion trap. Isolation of precursors utilized a 2 *m/z* isolation window, followed by fragmentation with a normalized collision energy set at 35%.

2.6. Data analysis

MaxQuant (Thermo Scientific, version v1.6.17.0) (Cox & Mann, 2008) in combination with the search engine Andromeda (Cox et al., 2011) was used for protein and peptide

identification in all the MS/MS raw files. Trypsin was selected as the proteolytic enzyme, permitting a maximum of two missed cleavages, peptide charges spanning from +2 to +7, a 10 ppm precursor mass tolerance, and a 0.5 Da fragment mass tolerance. In addition, search parameters were set to allow for dynamic modifications, including methionine oxidation, acetylation on the N-terminus, and fixed cysteine carbamidomethylation. The search database consisted of a non-redundant quinoa protein sequence FASTA file containing the 63,370 entries from *Chenopodium quinoa* found in the reference sequence (RefSeq) project from The National Center for Biotechnology Information database (NCBI, https://www.ncbi.nlm.nih.gov/). Normalized label-free quantification (LFQ) values were obtained by applying the in-built MaxLFQ algorithm (Cox et al., 2014).

Data interpretation was done through the use of Venn diagrams, distribution bar graphs, heat maps, volcano plots, and Gene Ontology (GO) classification graphs. Specifically, Venn diagrams were generated considering the number of identified proteins using the Venn diagram R package (version 1.7.3). Distribution bar graphs were constructed considering the percentage of identified proteins within different M_r ranges (below 20,000, between 20,000-40,000, between 40,000-60,000, between 60,000-80,000, between 80,000-100,000, and above 100,000). The construction of the heat maps was achieved considering the LFQ values of the identified proteins through the freely available web server Heatmappper (http://www.heatmapper.ca). Volcano plots were generated considering the LFQ values of the identified proteins through the use of different freely available R packages, including tidyverse (version 2.0.0) for data manipulation and visualization, ggpubr (version 0.6.0) for plot generation, and rstatix (version 0.7.2) for t-test statistical analyses. Finally, GO analyses were performed using the PANTHER classification system (http://www.pantherdb.org). However, as

Chenopodium quinoa is not available in PANTHER, which works primarily with UniProt identifiers and modeled organisms, the NCBI accession numbers (IDs) of the identified proteins were blasted against the UniProt database (https://www.uniprot.org/) of *Arabidopsis thaliana*, a model plant organism.

3. Results and discussion

3.1. Protein extraction

In our previous work (Galindo-Luján, Pont, Sanz-Nebot, et al., 2021), we employed a simple protein extraction protocol, which consisted of solubilizing proteins at pH 10.0 (extracting 250 mg of sample in 1 mL of water and 39 µL of 1 M NaOH), followed by a 1-h incubation at 36°C, isoelectric precipitation at pH 5.0 (with the addition of 22 µL of 1 M HCl), and subsequent redissolution of the protein precipitate in 60 mM H_3BO_3 at pH 9.0 (1 mL). Unfortunately, when assessing the total protein content of boiled and extruded quinoa samples (from both conventional and organic farming), minimal protein amounts were quantified in the extracts. This observation can be attributed to the protein denaturation process that takes place when subjecting raw quinoa grains to heat and pressure treatments (Fischer, 2004; Van de Vondel et al., 2022). To solve this issue, we explored an alternative extraction solvent described in the literature for the analysis of processed quinoa grains (Chen et al., 2011; Fischer, 2004; Kuktaite et al., 2022), which consisted of a water solution containing 0.035 M NaH_2PO_4 (pH 7.0), 0.1 M 2-mercaptoethanol, and 1.5% (v/v) SDS. However, protein extractability was not improved under these conditions. Finally, the best results were obtained by making some adjustments to our previously described method, i.e., increasing the water volume in the extraction solvent to 2 mL, extending the incubation time to 3 h, and augmenting speed

rates and time during the centrifugation steps. Under the optimized protocol, the total protein content analysis yielded the following values: 5.5% (m/m) for Cs_{eed}, 4.6% (m/m) for C_{grain}, 0.9% (m/m) for C_{boiled}, 1.1% (m/m) for $C_{extruded}$, 5.3% (m/m) for O_{seed}, 4.5% (m/m) for O_{grain}, 0.5% (m/m) for O_{boiled}, and 0.6% (m/m) for $O_{extruded}$.

3.2. Shotgun proteomics

3.2.1. NanoLC-MS/MS

In this study, as in our previous work with commercially available grains (Galindo-Luján, Pont, Minic, et al., 2021), an Orbitrap Fusion™ mass spectrometer was used for the comprehensive characterization of proteins from raw (seeds and grains) and processed (boiled and extruded) white quinoa samples cultivated under conventional and organic farming conditions. This state-of-the-art mass spectrometer, in contrast to earlier generation Orbitraps, significantly enhanced sensitivity, resolution, and scan speed, leading to a noteworthy increase in the number of identified peptides (Zhu et al., 2018). Furthermore, in this work, we implemented a FASP protocol and extended the chromatographic gradient to optimize both sample preparation and chromatographic separation. Under these refined conditions, two independent protein extracts from C_{seed}, C_{grain}, C_{boiled}, $C_{extruded}$, O_{seed}, O_{grain}, O_{boiled}, and $O_{extruded}$ quinoa were analyzed by nanoLC-MS/MS, and the raw files were subjected to rigorous data analysis.

3.2.2. Data analysis

The MaxQuant/Andromeda environment, in combination with a non-redundant quinoa protein sequence FASTA file containing 63,370 entries from the RefSeq NCBI database,

was used for protein identification and label-free quantification across the entire set of MS/MS raw data files. Considering all the quinoa samples (C_{seed}, C_{grain}, C_{boiled}, $C_{extruded}$, O_{seed}, O_{grain}, O_{boiled}, and $O_{extruded}$), a total of 1,796 quinoa proteins were successfully identified (169 of them uncharacterized), improving the coverage obtained in our previous work with commercial quinoa grains, where 1,211 quinoa proteins were identified (Galindo-Luján, Pont, Minic, et al., 2021). Supplementary Table S-1 provides detailed information about the protein group level, the ID, the protein name, the M_r, the Andromeda score, the number of peptides, the sequence coverage, and the normalized LFQ intensity for the 1,796 quinoa proteins identified in the studied samples. It is worth mentioning that for every quinoa sample, only proteins found in the two replicates were reported. Additionally, the number of peptides, the sequence coverage, and the normalized LFQ intensity obtained for all the quinoa samples is presented as an average value for the different protein extract samples (in all cases, relative standard deviation (%RSD) was lower than 10%). As can be observed in Supplementary Table S-1, the 1,796 quinoa proteins were identified at the group level with different reliabilities, with Andromeda score values ranging between 323 and 2.

Venn diagrams

For a simple representation of the results, we initially employed Venn diagrams. To enhance comprehension without complicating the visualization, two Venn diagrams were created. Figure 1-A illustrates the relationship between the number of identified proteins in raw quinoa (seeds and grains) cultivated under both conventional and organic farming. Notably, a greater number of proteins were identified in organic raw quinoa (1,637 proteins considering both O_{seed} and O_{grain}) compared to conventional raw quinoa (1,320

proteins considering both C_{seed} and C_{grain}). Likewise, the number of identified proteins was only slightly greater for the seeds compared to the grains. Among these proteins, 945 (56% of the total) were identified across all the samples, while 750 (44% of the total) were only present in some of them. Regarding proteins identified in only one sample, 31 proteins were exclusively identified in C_{seed}, 11 in C_{grain}, 186 in O_{seed}, and 60 in O_{grain}. Moving to Figure 1-B, which depicts the relationship between the number of identified proteins in processed quinoa (boiled and extruded) cultivated under both conventional and organic farming, a notable reduction in the number of identified proteins compared to raw quinoa was observed (a total of 957 vs. 1,695 proteins, Figure 1-B and 1-A, respectively). Furthermore, as can be seen in Figure 1-B, a greater number of proteins were identified in extruded quinoa (898 proteins considering both $C_{extruded}$ and $O_{extruded}$) compared to boiled quinoa (388 proteins considering both C_{boiled} and O_{boiled}). In contrast, almost no differences were observed in the number of identified proteins considering organic and conventional farming. Among these proteins, 176 (18% of the total) were identified in all the samples, while 781 (82% of the total) were only present in some of them. Regarding proteins identified in only one sample, 28 proteins were exclusively identified in C_{boiled}, 85 in $C_{extruded}$, 13 in O_{boiled}, and 92 in $O_{extruded}$. All these observations suggested differences at the proteome level between conventional and organic raw quinoa seeds and grains, but specially after boiling and extuding quinoa grains.

Distribution bar graphs

In order to assess differences in the M_r protein profile between the studied quinoa samples, a distribution bar graph was constructed considering the percentage of identified proteins in all the sample classes at different M_r ranges (Figure 2). As can be seen in

Figure 2, raw and boiled quinoa samples from both conventional and organic farming (C_{seed}, C_{grain}, C_{boiled}, O_{seed}, $O_{grain,}$ and O_{boiled}) predominantly exhibited proteins with M_r ranging between 20,000 and 40,000 (34, 34, 33, 35, 36, and 33%, respectively), and between 40,000 and 60,000 (28, 27, 25, 27, 27, and 24%, respectively). It is important to note that these two M_r ranges encompass the major storage quinoa seed proteins, including 11S globulins (M_r around 36,000), 7S globulins (M_r around 46,000), and 13S globulins (M_r around 55,000) (Supplementary Table S-1) (Galindo-Luján, Pont, Minic, et al., 2021; Poza-Viejo et al., 2023). Regarding extruded quinoa samples from both conventional and organic farming ($C_{extruded}$ and $O_{extruded}$), they predominantly exhibited proteins with M_r between 20,000 and 40,000 (37% and 37%, respectively), and below 20,000 (34% and 30%, respectively), highlighting a notable disparity in the M_r protein profile when subjecting quinoa grains to extrusion processes. It is worth mentioning that the M_r protein profile of boiled quinoa was different from that obtained for extruded quinoa. This emphasizes the idea that extrusion processes, which are subjected to higher temperatures and pressures than boiling procedures, are more prone to induce protein unfolding and denaturation of higher M_r proteins (>40,000), hence poorer solubilities, recoveries, or bioavalabilites (Gao et al., 2022; Van de Vondel et al., 2022).

Heat maps

To ensure a more confident discrimination between the different samples, it was necessary to consider protein differences at the concentration level. Therefore, a heat map was generated using the data matrix of average normalized LFQ intensities for the 174 proteins (rows) that were identified in all studied quinoa samples (columns). In a heat map, both rows and columns are rearranged to bring together those with similar profiles,

with each row's z-score entry in the data matrix represented by a distinct color. This visualization facilitates a graphical exploration of relationships and patterns within the dataset. Moreover, many heat maps employ an agglomerative hierarchical clustering algorithm to group data based on the observed characteristic profiles, presenting the information through a dendrogram. When two clusters merge, a connecting line is drawn at a height reflecting the similarity between the clusters (Benno Haarman et al., 2015; Key, 2012; Krentzman et al., 2011). As can be observed in Figure 3, each sample exhibited a distinctive protein concentration profile, with green, red, and black boxes representing up-regulated, down-regulated, and unchanged expression proteins, respectively. The dendrograms depicted in the heat map revealed that, according to their protein concentration profile, raw (seeds and grains) and processed (boiled and extruded) quinoa samples were separated into two differentiated groups, regardless of the farming conditions. Within raw quinoa, C_{grain} and C_{seed} quinoa samples were clustered together, followed by O_{seed} and, finally, O_{grain} quinoa, which, according to the clusters, was the least closely related sample based on the quantified protein groups. Within processed quinoa, C_{boiled}-O_{boiled} and $C_{extruded}$-$O_{extruded}$ were clustered together, suggesting a notable change in the protein concentration profile between boiled and extruded quinoa samples, regardless of the farming conditions. This observation supported our previous findings with the Venn diagrams and the distribution bar graph, where boiled and extruded samples presented a small percentage of common proteins and a different M_r protein profile.

Volcano plots

Despite the usefulness of Venn diagrams, distribution bar graphs, and heat maps, a differential statistical analysis was mandatory to determine whether there were

quantitative differences regarding protein abundance in raw and processed quinoa samples cultivated under conventional and organic farming conditions. Consequently, distinct volcano plots were constructed (Figure 4 A-C), wherein X-axes represented the log_2fold-change (log_2FC) values (FC calculated as the ratio between the average LFQ values for two compared conditions, represented as condition 1-condition 2), and Y-axes depicted the -log *p*-values (computed using statistical R packages, as detailed in section 2.6). It is essential to highlight that only proteins meeting the criteria of FC > 1.5 and *ρ*-values < 0.05 were deemed significant for the differentiation. The application of the previously mentioned data interpretation tools revealed that the most significant differences between conventional and organic farming practices were evident in raw quinoa samples, without any significant distinctions between seeds and grains. This observation prompted the creation of a dedicated volcano plot for the C_{raw}-O_{raw} comparison (Figure 4-A). To further explore variations associated with the processing methods (i.e., boiling and extrusion), additional volcano plots were generated based on the differentiated clusters observed in the heat map (Figure 3): Raw-Boiled (including samples from both conventional and organic farming, Figure 4-B) and Raw-Extruded (including samples from both conventional and organic farming, Figure 4-C). Supplementary Table S-2 shows the protein group level, the ID, the protein name, and the protein expression (up-regulated in condition 2 ("+"), up-regulated in condition 1 ("-"), and non-statistically significant ("n.s.")) for the quinoa proteins represented in the different volcano plots (C_{raw}-O_{raw}, Raw-Boiled, and Raw-Extruded, Figure 4 A-C).

Examining Figure 4-A, which distinguishes between conventional and organic farming in raw quinoa samples, it was determined that, out of the total 1,262 represented proteins, 109 were up-regulated in C_{raw} (green dots, "-" symbol in Supplementary Table S-2), 72

were up-regulated in O_{raw} (red dots, "+" symbol in Supplementary Table S-2), and 1,081 were considered non-statistically significant for the differentiation (grey dots, "n.s." acronym in Supplementary Table S-2). In the Raw-Boiled comparison (Figure 4-B), out of the total 381 represented proteins, 166 displayed overexpression in raw quinoa, while a considerably lower number (22) exhibited overexpression in boiled quinoa (Supplementary Table S-2). A similar pattern was noted in Raw-Extruded (Figure 4-C), where out of the total 822 represented proteins, 284 demonstrated up-regulation in raw quinoa, whereas a lower number (152) were up-regulated in extruded quinoa (Supplementary Table S-2). The results obtained from the volcano plots revealed quantitative variations in protein abundance between raw quinoa samples cultivated under conventional and organic farming, showing a comparable number of different proteins up-regulated in both conditions. In addition, there were quantitative variations in protein abundance between raw and processed quinoa samples, notably indicating downregulation of protein expression in processed quinoa, especially after boling.

GO classification graphs

Taking into accounnt the results derived from the volcano plots, we considered it appropriate to conduct GO analysis at the molecular function, biological process, and protein class levels (Figure 5 A-C, respectively) for the following groups: up-regulated proteins in C_{raw} and O_{raw} (i), up-regulated proteins in raw and boiled quinoa (ii), and up-regulated proteins in raw and extruded quinoa (iii). However, the PANTHER-GO system primarily works with UniProt identifiers, and the UniProt database for *Chenopodium quinoa* contains a dataset that is relatively limited when compared to the extensive NCBI database necessary for this proteomics study. Consequently, the NCBI IDs of the

identified proteins underwent a BLAST search against the UniProt database of *Arabidopsis thaliana*, a model plant organism. Supplementary Table S-3 presents the 1,627 quinoa proteins (derived from the total of 1,796 identified proteins after excluding the 169 uncharacterized proteins detailed in Supplementary Table S-1), along with their correspondence to 1,527 UniProt IDs from *Arabidopsis thaliana* (average percent identity was 72%±16% (±standard deviation, s). It is important to note that only UniProt IDs corresponding to proteins up-regulated in the conditions outlined in the volcano plots were subjected to GO analysis (these proteins are marked in Supplementary Table S-3).

Regarding the molecular function category (Figure 5-A), comparing C_{raw}-O_{raw} (Figure 5 A-i), the highest number of hits in both conditions were associated with catalytic activities (57% in C_{raw} and 35% in O_{raw}), followed by binding activities (32% in C_{raw} and 27% in O_{raw}). Additionally, slight differences were noted in the less represented categories, with a small number of hits associated with translation regulator, structural molecule, and antioxidant activities in C_{raw} (11%), and structural molecule, ATP-dependent, transporter, and antioxidant activities in O_{raw} (38%). Interestingly, the percentage of proteins with catalytic activities was significantly higher in C_{raw}, while in O_{raw} the less represented activities displayed greater diversity, contributing with a significantly higher number of hits. Comparing Raw-Boiled (Figure 5 A-ii), great differences emerged. While raw quinoa exhibited a higher number of hits associated with catalytic and binding activities (46% and 30%, respectively), boiled quinoa only showcased hits linked to binding, transcription regulator, and structural molecule activities. Comparing Raw-Extruded (Figure 5 A-iii), the greater number of hits in raw quinoa were associated with catalytic activities (39%), closely followed by binding activities (32%). In contrast, extruded quinoa exhibited an opposite trend, with higher number of hits predominantly linked to

binding activities (65%) and, to a lesser extent, catalytic activities (15%). Concerning the less represented hits and, in comparison to boiled and extruded quinoa, raw quinoa showcased proteins with a wider variety of molecular functions. These observations suggested that quinoa grain processing and, specially boiling, greatly depleted enzymes involved in catalytic activities and decreased protein variety.

Concerning the biological process category (Figure 5-B), minimal differences were noted in all comparisons. Analyzing C_{raw}-O_{raw} (Figure 5 B-i), the majority of hits in both classes were predominantly associated with metabolic and cellular processes (in total, 84% in C_{raw} and 76% in O_{raw}). This pattern persisted in Raw-Boiled (Figure 5 B-ii) and Raw-Extruded (Figure 5 B-iii), with the higher number of hits highlighting the same processes (around 80% in raw quinoa, 71% in boiled quinoa, and 73% in extruded quinoa). In this case, no clear-cut trends affecting biological processes were identified, likely due to the highly heterogeneous protein classes involved in these biological processes.

Finally, the protein class category (Figure 5-C) showed notable differences. Focusing on the C_{raw}-O_{raw} comparison (Figure 5 C-i), 49% and 18% of the hits in C_{raw} were classified as metabolite interconversion enzymes and protein-modifying enzymes, respectively. In contrast, 41% and 28% of the hits in O_{raw} were classified as metabolite interconversion enzymes and translational proteins. Indeed, translational proteins and protein-modifying enzymes only accounted for 5% and 5% of the hits in C_{raw} and O_{raw}, respectively. This suggested that, while protein classes associated with enzymatic functions were predominant in both quinoa classes, organic farming appeared to favor the presence of proteins related to protein translation rather than protein enzymatic modification. The prevalence of protein classes associated with enzymatic functions in C_{raw} agreed with our

previous observation about the enhancement of catalytic activity molecular functions. The natural enzymatic modification of quinoa grain proteins may have important implications not only at the nutritional level, but also at the bioactivity and techno-functional levels (Shen et al., 2022). In the comparison of Raw-Boiled (Figure 5 C-ii) and Raw-Extruded (Figure 5 C-iii), a similar trend to that observed before for the molecular function category emerged, and protein classes associated with enzymatic functions and protein diversity decreased after processing, specially after boiling. In the case of raw quinoa, the majority of up-regulated hits were categorized as metabolic interconversion enzymes (around 40%), followed by translational proteins (around 20%). Conversely, boiled and extruded quinoa displayed a different pattern, with a higher number of hits classified as tranlational proteins in boiled quinoa (27%), and the same number of hits classified as translational proteins and metabolite interconversion enzymes in extruded quinoa (23% each). Concerning the less represented hits and, in comparison to boiled and extruded quinoa, raw quinoa showcased a wider variety of protein classes, including scaffold/adaptor proteins, cytoskeletal proteins, DNA metabolism proteins, transfer/carrier proteins, and cell adhesion molecules, among others.

Al these observations not only supported, but also complemented our earlier findings, highlighting that the up-regulated proteins in raw and processed quinoa cultivated under conventional and organic farming exhibit characteristic molecular functions and protein classes, whereas less differences are found at the biological process level. These variations can potentially exert a significant influence on the characteristics of the studied quinoa samples, particularly impacting the nutritional, techno-functional, and bioactive properties of the end products intended for human consumption.

4. Conclusions

We outlined a nanoLC-MS/MS shotgun proteomics approach to comprehensively characterize the proteome of raw and processed white quinoa (Salcedo variety) cultivated under conventional and organic farming. In total, 1796 proteins from a non-redundant NCBI quinoa database were identified and quantified. To explore relationships among the studied quinoa samples, we integrated a diverse set of qualitative data interpretation tools, such as Venn diagrams, distribution bar graphs, and GO classifications graphs, as well as advanced quantitative data analysis tools based on the LFQ intensities of the identified proteins, including heat maps and volcano plots. The number of identified proteins greatly decreased after quinoa processing rather than farming, specially after boiling. Additionally, extrusion affected the typical M_r distribution of the identified proteins, resulting in a significant increase of proteins with M_r below 20,000. In the comparison between the up-regulated proteins in conventional and organic raw quinoa, C_{raw} exhibited a significantly higher presence of proteins with catalytic activities, while O_{raw} displayed a greater diversity of molecular functions and protein classes. When comparing raw and processed samples, raw quinoa demonstrated a higher prevalence of proteins with catalytic activities and a broader range of molecular functions and protein classes in the less represented hits, suggesting that quinoa processing, specially boiling, depleted enzymes and diminished protein diversity. Overall, the proposed methodology provides, for the first time to the best of our knowledge, a comprehensive analysis of the quinoa proteome exposed to different processing and farming procedures, providing essential information for improving the nutritional, techno-functional, and bioactive properties of quinoa. This enhancement may be achieved by selecting quinoa varieties,

improving cultivar yield under diverse agroecological conditions, or optimizing the industrial processing procedures.

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Data Availability Statement: The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD050043.

Figure legends

Figure 1. Venn diagram analysis of the identified proteins in (A) raw quinoa (including C_{seed}, C_{grain}, O_{seed}, O_{grain}) and (B) processed quinoa (including C_{boiled}, $C_{extruded}$, O_{boiled}, $O_{extruded}$).

Figure 2. Distribution bar graph constructed considering the percentage of identified proteins in the studied quinoa samples within different M_r ranges (below 20,000, between

20,000-40,000, between 40,000-60,000, between 60,000-80,000, between 80,000-100,000, and above 100,000).

Figure 3. Heat map obtained using the row z-score normalized LFQ intensities of the identified proteins in the studied quinoa samples (C_{seed}, C_{grain}, O_{seed}, O_{grain}, C_{boiled}, $C_{extruded}$, O_{boiled}, $O_{extruded}$).

Figure 4. Volcano plots for discriminating between two conditions, represented as condition 1-condition 2: (A) C_{raw}-O_{raw}, (B) Raw-Boiled (including samples from both conventional and organic farming), and (C) Raw-Extruded (including samples from both conventional and organic farming). X-axes represent the log_2fold-change (log_2FC) values (FC calculated as the ratio between the average LFQ values for the two compared conditions), and Y-axes depict the -log *p*-values (computed using statistical R packages, as detailed in section 2.6). Only proteins with FC > 1.5 and *p*-values < 0.05 are considered statistically significant for the differentiation. Up-regulated proteins in condition 1 are represented as green dots ("-" in Supplementary Table S-2), up-regulated proteins in condition 2 are represented as red dots ("+" in Supplementary Table S-2), and non-statistically significant proteins are represented as grey dots ("n.s." in Supplementary Table S-2).

Figure 5. Gene Ontology (GO) graphs classified by (A) molecular function, (B) biological process, and (C) protein class for the quinoa proteins up-regulated in (i) C_{raw}-O_{raw}, (ii) Raw-Boiled, and (iii) Raw-Extruded, and blasted against the Uniprot database of *Arabidopsis thaliana*.

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