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Characterization of Bioactive Phenolic Compounds in Seeds of Chilean Quinoa (*Chenopodium quinoa* Willd.) Germplasm

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Abstract: In recent years, quinoa (*Chenopodium quinoa* Willd.), an ancient Andean region crop, has received increased research attention because it is an excellent source of nutrients and also of bioactive phenolic compounds, which are potentially beneficial for human health. However, variation in the content and type of these metabolites in quinoa genetic resources remains, to a large extent, unexplored. We evaluated the composition of free and bound phenolic forms in the seeds of 111 Chilean quinoa accessions by using LC-DAD-MS/MS. The relative phenolic content ranged from 35.51 mg/100 g to 93.23 mg/100 g of seed dry weight. The free phenolic fraction accounted for 72% of the total phenolic content, while the bound fraction represented the remaining 28% of the total phenolic content. Our study also revealed a significant degree of variation in terms of individual phenolic compounds such as rutin, vanillic acid, quercetin, and their derivatives, which can have important implications for quinoa's nutritional and functional properties. We conclude that our data reveal a significant phenotypic variation of bioactive phenolic content in the examined germplasm, which could be exploited in current and future genetic improvement programs in quinoa.

Keywords: quinoa; free and bound phenolic compounds; HPLC-DAD-MS/MS

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1. Introduction

In recent years, there has been a growing interest in the nutritional and health benefits of traditional and underutilized crops. Among these, *Chenopodium quinoa* Willd. (Quinoa), a pseudocereal native to the Andean region of South America, has gained significant attention due to its exceptional nutritional profile and potential health-promoting properties. Quinoa has been cultivated for centuries by various indigenous communities and has served as a staple food source owing to its high protein content, essential amino acid composition [1,2], and adaptability to diverse agroecological conditions [3,4]. Beyond their macronutrient composition, *C. quinoa* seeds also contain a range of bioactive compounds, particularly phenolic compounds, which have been linked to various health benefits [5,6].

Phenolic compounds, a class of secondary metabolites found abundantly in plant tissues, have been widely recognized for their diverse physiological and pharmacological properties. These compounds exhibit antioxidant, anti-inflammatory, antimicrobial, and anticancer activities, among others, making them highly valuable components in the context of functional foods and nutraceuticals [6]. Plant phenolic compounds, or polyphenols, play a significant role in the field of agronomy due to their various important functions in plant

growth, development, soil nutrient cycling, and defense mechanisms [7–10]. Moreover, these compounds have been reported to have anti-herbivory characteristics in Amaranthus species of the Chenopodiaceae family [11]. For example, rutin and kaempferol act as phagostimulants and feeding deterrents, respectively, and can alter the growth of aphids. Vanillic acid sugar ester glucoside is more effective on the offspring of aphids, while tannins reduce the savoriness of tissues due to their astringent characteristics [12–16].

In recent years, several studies on phenolic compounds in *C. quinoa* seeds have shown that phenolic compounds exist in both free and bound forms. Usually, free phenolics are flavonoids or proanthocyanidins and their glycoside derivatives, and, to a lesser extent, glucosides of ferulic and vanillic acids. Whereas the fraction of bound phenolics includes phenolic acids such as, e.g., benzoic acid, ferulic acid, and vanillic acid, which are building blocks of lignin as part of the cell walls [12,17]. Both free and bound phenolic forms can be extracted for analytical assessment by alkaline and acid hydrolysis. To date, phenolic compounds from *C. quinoa* have been evaluated mainly by spectrophotometric methods like the Folin-Ciocalteu assay [18–21]. However, the accuracy of this assay can be influenced by a number of interfering substances, and, in addition, there is a lack of standardization. These aspects lead to an overestimation of phenolic compounds and insufficient comparability of results, respectively [22]. To improve analyses, over the past few years, methodologies based on nuclear magnetic resonance (NMR), high-pressure liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), gas chromatography (GC), and gas chromatography-mass spectrometry (GC-MS) have been used for identifying and quantifying phenolic compounds in *C. quinoa* seeds [23–28]. High-performance liquid chromatography with diode array detection (DAD) and electrospray ionization (ESI) tandem mass spectrometry (MS/MS) are currently the methods of choice for the analysis of phenolic compounds. The use of MS/MS not only allows the identification of individual phenolic compounds by their fragmentation patterns but also their quantification with high reliability [17,29–31].

The Chilean *C. quinoa* germplasm, encompassing a diverse range of quinoa accessions adapted to the specific climatic and soil conditions of the Chilean Andean region [12], presents a unique opportunity to investigate the variability in bioactive phenolic compounds. However, these secondary metabolites are formed in the plant as an adaptive response to environmental factors, and their concentrations and levels can significantly vary depending on factors such as genetic background, specific environmental factors, and their interactions [32–34]. Recent investigations revealed the effects of agroecological factors on bioactive phenolic compounds in other diverse crops such as *Brassica* species, tomatoes, baby leaf lettuces, and strawberry fruits [35–38]. While the nutritional value of *C. quinoa* has been extensively studied, a comprehensive understanding of the phenolic profile of Chilean *C. quinoa* germplasm is still in its nascent stages. Elucidating the types and concentrations of phenolic compounds present in these seeds could provide valuable insights into their potential health benefits and contribute to the overall appreciation of *C. quinoa* as a functional food. Moreover, shedding light on genetic factors that influence the phenolic composition of Chilean *C. quinoa* could facilitate the selection and breeding of *C. quinoa* varieties with optimized phenolic profiles.

In the present work, we hypothesized that the types and contents of the bioactive phenolic compounds in the quinoa seeds are strongly influenced by inherent genetic factors, and as a result, the Chilean *C. quinoa* germplasm exhibits significant variation in the composition and concentration of phenolics present in their seeds. To this purpose, we analyzed the phenolic profiles of *C. quinoa* germplasm collected from two different biomes within Chile to verify if such bioactive phytochemicals in seed extracts vary according to geographical origin. We evaluated the composition of both free and conjugated phenolics among 111 *C. quinoa* genotypes. The seeds were collected during field trials under agroecological conditions in the southern Atacama desert region in northern and central Chile and further selected from the INIA breeding program. Our results provide a comprehensive overview of the existing variation of bioactive phenolic compounds present in the seeds of Chilean

C. quinoa germplasm. Ultimately, a deeper understanding of the phenolic compounds in Chilean *C. quinoa* germplasm could open new avenues for utilizing this ancient grain as a functional food with enhanced health-promoting properties. Moreover, the present knowledge endeavors to bridge the knowledge gap regarding marker-assisted selection and determine genomic regions that might be linked to bioactive phenolic compounds to support future *C. quinoa* breeding programs.

2. Materials and Methods

2.1. Chenopodium quinoa Germplasm

In the present study, the phenolic compounds were assessed in 111 different *C. quinoa* genotypes, including advanced breeding lines, in the framework of the ongoing Instituto de Investigaciones Agropecuarias's Quinoa Breeding Program (INIA's IQBP) in Chile. This *C. quinoa* collection was initially established by INIA, Chile, through mass selection and self-pollination of individual lines for at least two growing seasons. This diversity panel included 7 genotypes (salares ecotype) originally collected in the Chilean Altiplano (highlands region), 2 south Altiplano genotypes from the Cancosa area, and 102 genotypes originating from the Chilean coastal-lowland regions (Figure S1). The detailed information is reported in a previous publication [39]. Most of the genotypes were cultivated and harvested at the Huasco experimental station (28°3′ S, 70°4′ W), located in the southern Atacama desert region. Typically, environmental conditions at this research station during the quinoa growing season are characterized by high solar irradiance, cool nights, and virtually no precipitation, requiring irrigation throughout crop growth [40]. The soil at the experiment station was consistent with La Compañia string, a sandy loamy class of soil with low organic matter, and showed an alkaline pH and the following macronutrient amounts: N (45 mg/kg), P (21 mg/kg), and K (311 mg/kg).

2.2. Chemicals

Caffeic acid, cinnamic acid, coumaric acid, ferulic acid, gallic acid, 4-hydroxybenzoic acid, kaempferol, quercetin, quercetin-3-O-glucuronide, quercetin 3-O-sambubioside, rutin, syringic acid, and vanillic acid were purchased from VWR (Darmstadt, Germany). Acetonitrile, ethyl acetate, hexane, methanol, water (all LC-MS grade), hydrochloric acid, and sodium hydroxide were supplied by VWR (Darmstadt, Germany).

2.3. Extraction of Free Phenolic Compounds from Quinoa Seeds

Free phenolic compounds were extracted from quinoa seeds according to Gómez-Caravaca et al. [17], with some modifications. Hundred milligrams of ground quinoa seeds were extracted with 1.5 mL of water:methanol (3:1) containing 0.1% formic acid by using a vortex for 1 min, followed by an ultrasonic bath for 20 min. After centrifugation at 12,000 rpm for 2 min, 1 mL of supernatant was withdrawn and filtered through a 0.2-micrometer PTFE filter. The pellet was dried in a stream of nitrogen gas. Samples were stored at −20 °C until analysis.

2.4. Extraction of Bound Phenolic Compounds from Quinoa Seeds

The remaining pellet from the extraction of the free phenolic compounds was used for the extraction of the bound phenolic compounds following the method of Gómez-Caravaca et al. [17]. Briefly, the dried pellet was resuspended in 1 mL of water and transferred into a bigger sample tube. Powder adherent to the wall of the storage tube was removed three times with 1.5 mL of 2 M sodium hydroxide, which was combined with the suspension. After gently blowing nitrogen gas into the tubes, these were vigorously mixed on a vortex for 1 min and thereafter placed in an ultrasonic bath for 30 min. Furthermore, the suspensions were shaken at room temperature for 20 h. After cooling on ice, the sample solutions were brought to pH 1–2 by the dropwise addition of 32% hydrochloric acid. For the removal of lipids, the samples were extracted with 20 mL of hexane. The phenolic compounds were finally extracted three times with 4 mL of ethyl acetate by vortexing for

1 min. The combined organic fractions were evaporated to dryness and reconstituted in 2 mL of a water:methanol (3:1) mixture. Prior to analysis, the samples were filtered through 0.2-micrometer PTFE filters.

2.5. Identification and Quantification of Phenolic Compounds by Liquid Chromatography-Diode Array Detection-Tandem Mass Spectrometry (LC-DAD-MS/MS) Analysis

LC-DAD-MS/MS was performed on a Waters ACQUITY® UHPLC system (binary pump, autosampler, and diode array detector) coupled to a Waters Xevo TQ-S® triple-quadrupole mass spectrometer (Waters Technologies Corp., Milford, MA, USA). Separation of phenolic compounds was achieved on a Nucleoshell RP18 column (100 × 4.6 mm, 2.7 µm; Macherey-Nagel, Düren, Germany). The column was equipped with a pre-column (Macherey—Nagel, Düren, Germany) and maintained at 40 °C. The mobile phases were water (A) and acetonitrile (B), each containing 0.1% formic acid, at a flow rate of 1.0 mL/min. The gradient program was as follows: 85% A, to 60% A within 7 min, to 2% A within 0.5 min and holding for 2.5 min, back to 85% A within 0.1 min, and holding for 2.9 min. The injection volume was 1 µL. UV spectra were recorded in the wavelength range of 200–400 nm. For the identification of phenolic compounds, the electrospray ionization (ESI) interface of the mass spectrometer was driven in both positive and negative modes. The capillary voltage was set to 2.5 (ESI(+)) and 2.0 kV (ESI(−)), respectively. The desolvation temperature and source temperature were 600 °C and 150 °C, respectively. The desolvation gas flow was set to 1000 L/h and the cone gas flow at 150 L/h, using nitrogen in both cases. MS detection was carried out in full scan mode (*m/z* 50–1000). Identified molecular ion adducts were subjected to collision-induced dissociation in the daughter ion scan mode at collision energies of 8, 10, 15, 20, and 30 eV.

Quantification was performed in the ESI(−) mode by applying the same ESI setting as mentioned above. The triple-quadrupole mass spectrometer was driven in the multiple reaction monitoring (MRM) mode for the detection of selected phenolic acids and flavonoid glycosides (Table 1). Nitrogen was used as the collision gas at a flow rate of 0.15 mL/min.

Table 1. MRM parameters of analyzed phenolic compounds.

No.	Compound	t_R [min]	Precursor Ion $[M-H]^-$ *m/z*	Product Ion (Quantifier/Qualifier) *m/z*	Cone [V]	Collision Energy (Quant./Qual.) [V]
1	4-Hydroxybenzoic acid	2.33	137.0	93.6/65.0	46	20/26
2	Vanillic acid	2.61	167.0	152.0/108.0	22	14/18
3	Syringic acid	2.63	197.0	182.0/123.0	54	14/22
4A,B	Coumaric acid	3.51/3.76	163.0	119.4/93.0	42	17/28
5A,B	Ferulic acid	3.87/4.10	193.0	134.0/178.0	52	16/14
6A,B	Quer-Hex-(DHex-Pent) [a]	[c]	741.0	300.1 [d]/271.0	94	36/62
7A–E	Quer-Hex-DHex [a]	[c]	609.0	300.1 [d]/271.0	86	34/60
8A,B	Quer-Hex-Pent [a]	[c]	595.0	300.1 [d]/271.0	74	30/52
9A,B	Quer-Hex [a]	[c]	463.0	300.1 [d]/271.0	54	36/58
10	Quer-HexA [a]	[c]	477.0	301.1/151.0	80	20/36
11	Quercetin	6.21	301.0	151.0/179.0	76	20/18
I [b]	Cinnamic acid	6.76	147.0	103.0/77.0	32	10/20
II [b]	Gallic acid	1.18	169.0	97.0/69.0	24	18/22
III [b]	Caffeic acid	2.49	179.0	79.0/107.0	20	24/22
IV [b]	Kaempferol	7.57	285.0	151.0/93.0	82	18/30

[a] Que: Quercetin. Pent: pentose; Hex: hexose; DHex: deoxyhexose; HexA: hexuronic acid. [b] Compounds I–IV were <LoD in all samples. [c] see Table 2. [d] Radical aglycon product ion from homolytic cleavage of the glycosidic bond ($[Y_0 - H]^-$, *m/z* 300) [41]

Data acquisition and processing were performed using the software MassLynx 4.2 (Waters Technologies Corp., Milford, MA, USA). The analysis of each quinoa genotype or cultivar was performed in technical duplicates. Quantification was performed by the method of external calibration with standard solutions in the concentration range of 0.1–25 µM. Compounds 1–5, 7, 8, 10, and 11 were quantified using their corresponding standard reference compounds (Table 2). The quercetin glycosides 6 and 9 were quantified by applying the straight-line equation from the rutin calibration. Compounds 4–9 were split into several isomeric peaks (Figure 1). Their total peak areas were formed by adding up the peak areas of the individual isomers.

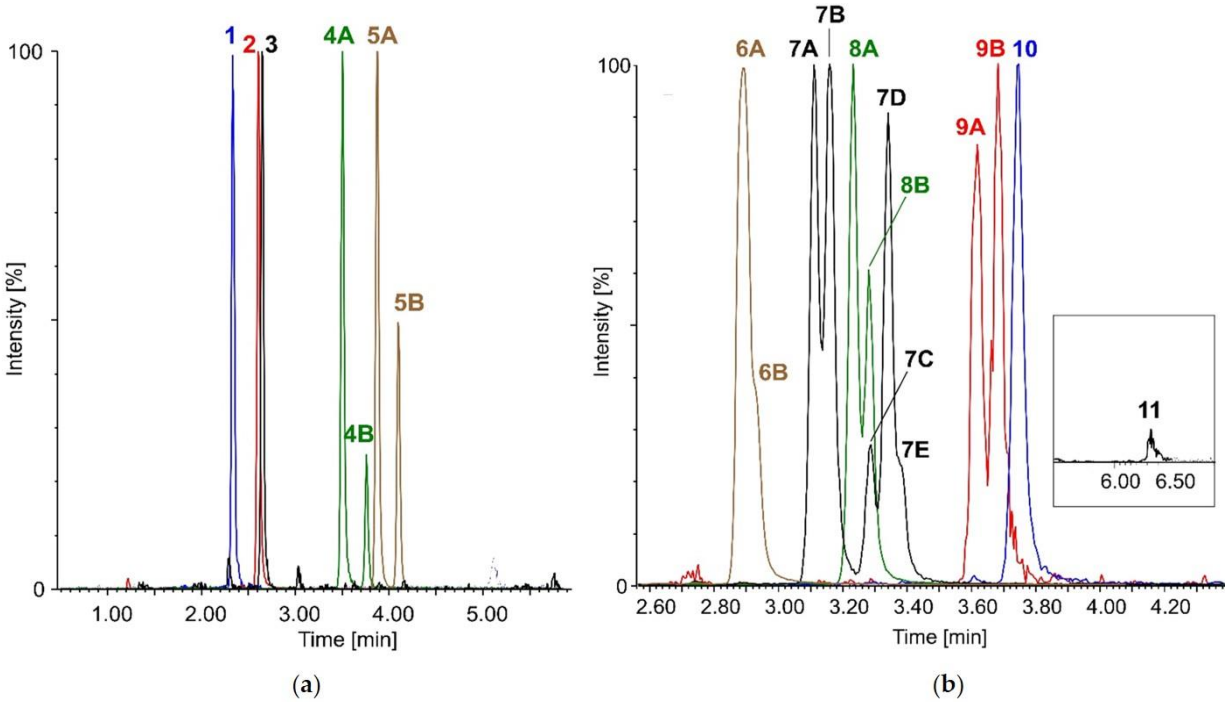

Figure 1. Overlaid MRM chromatograms of phenolic acids from the bound phenolic fraction (**a**) and flavonoid glycosides from the free phenolic fraction (**b**) of quinoa seeds obtained by LC-ESI(−)-MS/MS. The HPLC chromatogram profile shows the separated free and bound phenolic derivatives from *C. quinoa* seed extract. Total phenolic content was quantified by means of their free and bound fractions of phenolics. Fragments of phenolic derivatives were assigned by mass spectra and comparing retention times to the corresponding standards. For the peak assignment of (**a**), see Table 1, and for (**b**), see Table 2.

Table 2. Product ions of $[M + H]^+$ for quercetin glycosides from the free phenolic fraction of quinoa seed extracts.

No.	t_R [min]	Flavonoid Glycoside [a]		*m/z* (% Base Peak Intensity)							CE [eV]	Lit.
				$[M + H]^+$	$[Y_0]^+$	$[Y*]^+$	$[Y_1]^+$	$[Y_2]^+$	$[B_1]^+$	$[B_2]^+$		
6A	2.89	Quer-Hex-DHex-Pent	Quer-7-*O*-Glc-Rha-Xyl	742.76 (100)	303.17 (82.5)	-	465.06 (19.3)	610.83 (5.1)	-	-	15	
6B	2.93		Quer-3-*O*-Glc-Rha-Xyl	742.82 (100)	303.50 (10.6)	-	465.12 (7.5)	-	-	-	15	[42]
7A	3.11	Quer-Hex-DHex or Quer-HexA-Pent	Quer-7-*O*-Glc (6→1)Rha	611.23 (18.8)	303.06 (100)	449.10 (12.1)	465.12 (30.7)	-	147.38 (5.0)	309.03 (11.1)	10	[43]
7B	3.16		Quer-7-*O*-Glc (2→1)Rha	611.10 (40.6)	303.25 (100)	449.10 (32.6)	465.12 (65.0)	-	146.92 (4.3)	-	10	[43]
7C	3.29		Quer-3-*O*-Glc (2→1)Rha	611.17 (40.5)	303.0 (100)	449.17 (6.1)	465.18 (30.3)	-	147.38 (2.3)	309.29 (0.7)	10	[43]
7D	3.34		Quer-3-*O*-GlcA(2→1)Xyl	611.23 (17.2)	303.06 (45.6)	435.32 (2.7)	479.29 (100)	-	-	-	10	[44]
7E	3.38		Quer-3-*O*-Glc (6→1)Rha [b]	611.10 (41.8)	303.25 (100)	449.0 (3.1)	465.18 (44.9)	-	-	-	10	[43]
8A	3.23	Quer-Hex-Pent	Quer-3-*O*-Gal(6→1)Xyl	597.19 (27.1)	303.19 (100)	435.18 (4.3)	465.18 (35.8)	-	133.34 (3.3)	295.11 (3.9)	8	[45]
8B	3.28		Quer-3-*O*-Glc(6→1)Xyl	597.25 (28.9)	303.19 (100)	435.12 (0.7)	465.25 (20.3)	-	133.34 (0.5)	295.25 (0.8)	8	[46,47]
9A	3.62	Quer-Hex	Quer-3-*O*-Gal	464.99 (88.2)	303.22 (100)	-	-	-	-	-	8	[48]
9B	3.68		Quer-3-*O*-Glc	465.19 (27.3)	303.22 (100)	-	-	-	-	-	8	[17,48,49]
10	3.74	Quer-HexA	Quer-3-*O*-GlcA [b]	479.15 (100)	303.17 (99.5)	-	-	-	-	-	8	[17,48]

[a] Quer: Quercetin; Pent: pentose; Hex: hexose; DHex: deoxyhexose; HexA: hexuronic acid. Xyl: xylose; Rha: rhamnose; Glc: glucose; Gal: galactose; GlcA: glucuronic acid. [b] Verified by a reference standard. * is the designation of the ion and no footnote.

2.6. Statistical Analysis

All analyzed data are recorded as the mean ± standard deviation (SD) of single extractions (n = 4). The complete dataset was subjected to the Shapiro-Wilk normality test. To address statistical significance in phenolic content among the studied *C. quinoa* genotypes, the dataset was analyzed with a one-way analysis of variance ANOVA (Tukey's Honestly Significant Difference multiple comparisons, α = 0.05) using the R-package Agricolae [50]. Principal component analysis (PCA) was performed to summarize the whole dataset by means of a smaller set of concise indexes of specific variables based on a correlation distance matrix using R packages (factoextra and FactoMineR) [51,52]. Cluster analysis was performed based on Euclidean distance and the complete grouping method using PCA scores. In addition, the percentage of variance explained by the genotypic effect (Vg) in the total phenotypic variance for phenolic compounds was evaluated with the following linear additive model:

$$y_{ij} = \mu + G_i + \varepsilon_{ij} \quad (1)$$

where y_{ij} is the phenotypic value assessed for the trait y on the plant j of the genotype i; μ refers to the whole mean; G_i is the random influence of genotype i, denoting the effect of each genotype or genotypic effect on trait y; and ε_{ij} is the random residual error per plant j of the genotype i. Vg was measured based on the restricted maximum likelihood (REML) variance components using the lme4 library of R [53]. The relevance of the effects was gauged using the Akaike and Bayesian criteria and probed by the likelihood ratio.

3. Results and Discussion

3.1. Detection of Phenolic Compounds by HPLC-DAD-MS/MS

In the current work, a total of 111 *C. quinoa* genotypes were analyzed for both free and bound fractions of phenolics. To achieve this goal, a C18 core-shell column was selected to determine the existing phenolic compounds, as described previously [17]. All accessible free and bound phenolic fractions were detected (Figure 1). However, an isocratic step with 100% acetonitrile was added to the gradient program to flush out potential lipophilic compounds from the column. An important factor was the choice of solvent composition for the samples. Water:methanol (3:1) turned out to have the best influence on both the sharpness and symmetry of the peaks. However, with an increasing amount of methanol, peak broadening and/or splitting were observed.

3.2. Assessment of the Free Phenolic Fraction in C. quinoa Seeds

By extracting quinoa seeds with water and methanol, three main families of compounds could be obtained: phenolic acids, flavonoid glycosides, and saponins. The results for saponins are described in a previous publication [39].

Nineteen different phenolic compounds have been found and quantified by LC-MS among the studied genotypes (Table 1). Figure 1a illustrates the overlaid multiple-reaction monitoring (MRM) chromatograms of seven phenolic compounds that were found in the free and in much larger quantities in the bound phenolic fraction of *C. quinoa* seeds. Therefore, this phenolic fraction will be discussed in detail in Section 3.3.

The other twelve phenolic compounds could be assigned to the class of flavonoid glycosides (Figure 1b). For their unambiguous characterization, LC-DAD-MS/MS analysis was performed in the positive ESI mode since this mode is more informative than ESI(−) mode for structural evaluation [43]. The UV spectra of compounds 6–10 showed two absorption bands at 250–255 nm and 350–355 nm, respectively, which is typical for quercetin glycosides [54]. After identification of the $[M + H]^+$ ions in the full scan mode, they were submitted to MS^2 fragmentation in order to obtain their product ion spectra (Table 2). Subsequently, the nomenclature described by Domon and Costello [55] was employed for the assignment of the product ions of flavonoid glycosides (Figure 2).

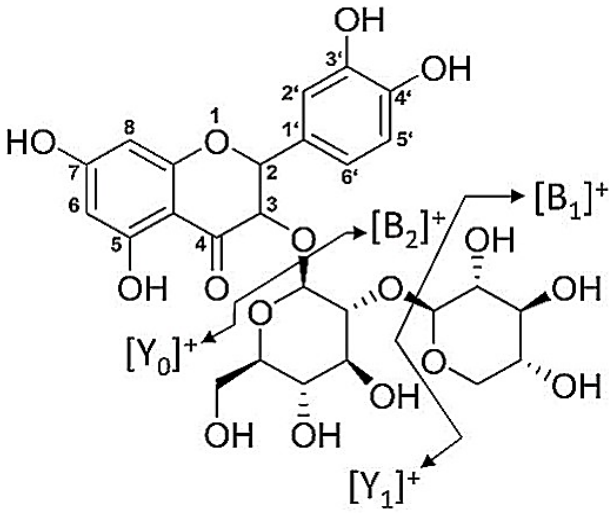

Figure 2. Nomenclature of formed product ions from the fragmentation of flavonoid glycosides. Y_j represents the product ions still containing the aglycon, where j is the number of interglycosidic bonds broken, counting from the aglycone. The glycosidic bond between the sugar unit and the aglycon is numbered 0. B_i are the cleaved sugar moieties, where i represents the number of glycosidic bonds cleaved, counting from the last sugar unit in the molecule.

All MS^2 mass spectra have a fragment profile at *m/z* 303 ($[Y_0]^+$), indicating quercetin as the only aglycon in all identified flavonoid glycosides. In summary, five groups of quercetin glycosides were noted, which were differentiated in the number and type of sugar units (Table 2). Quercetin glycosides with a high degree of glycosylation elute first, followed by those with decreasing glycoside units (Figure 1b). This elution pattern corresponds to the one reported previously [17]. The peaks 6A and 6B (2.89/2.93 min) were tentatively identified as two isomers of tri-glycosylated quercetin. Sequential losses in their fragmentation patterns suggested xylose and rhamnose as the terminal sugars linked to glucose, as described by Price et al. [42]. The difference between both isomers 6A and 6B was their glycosylation of quercetin in positions 7 and 3, respectively. Five diglycosylated quercetins (compounds 7A–E) were eluted between 3.11 and 3.38 min, all showing identical $[M + H]^+$ ions at *m/z* 611. The MS^2 spectra of 7A,B,C, and E looked very similar, showing the subsequent losses of rhamnose ($[M + H - 146]^+$) and glucose ($[M + H - 146 - 162]^+$). The 7E could be identified as rutin by LC-MS analysis of rutin as a reference substance. The other glycosides 7A, 7B, and 7C were elucidated by applying the guideline for characterization of O-di-glycosyl flavonoid isomers with respect to their inter-glycosidic linkage isomeriex and glycosylation positions [43] (Table 2). Compound 7D totally differed from the others in that it was composed of xylose as a terminal sugar linked to glucuronic acid, which had also been found in blueberries and blackberries by Cho et al. [44]. Next, compounds 8A and 8B appeared at 3.23 and 3.28 min, respectively. Their MS^2 spectra both contained the product ions $[M + H - 132]^+$ and $[M + H - 132 - 162]^+$, indicating sequential losses of pentose and hexose. After exclusion of quercetin 3-*O*-[xylosyl-(1→2)-glucoside] (quercetin 3-*O*-sambubioside), which was analyzed as reference standards 8A and 8B, they were tentatively identified by comparison with literature data as quercetin 3-*O*-[xylosyl-(1→6)-galactoside] [45] and quercetin 3-*O*-[xylosyl-(1→6)-glucoside] [46,47], respectively. Compounds 9A and 9B eluted at retention times of 3.62 and 3.68 min, respectively. They showed the loss of hexose ($[M + H - 162]^+$). Compound 9A was tentatively identified as quercetin 3-*O*-galactoside and 9B as quercetin 3-*O*-glucoside, as it had already been reported in pistachio hulls by Ersan et al. [48]. The presence of 9B in quinoa has already been described by Gómez-Caravaca et al. [17,49]. The last quercetin glycoside 10, which occurred at a retention time of 3.74 min, was unambiguously identified as quercetin 3-*O*-glucuronide by comparison to the reference compound. Its presence in quinoa was also reported by Gómez-Caravaca et al. [17,49].

3.3. Assessment of Bound Phenolic Fraction in C. quinoa Seeds

The fraction of bound phenolic compounds was obtained after alkaline hydrolysis of the residue from the water/methanol extraction of ground *C. quinoa* seeds [49]. Five different types of phenolic acids have been identified in this fraction by comparison with the retention times and mass spectra of the corresponding reference substances (Figure 1a, Table 1—No. 1–5). Compounds 1–5 were assigned according to published literature as hydroxybenzoic acid, vanillic acid, syringic acid, coumaric acid, and ferulic acid, respectively [17,27,56]. Remarkably, two fragments at retention times of 3.51 min and 3.76 min have been noted for coumaric acid (Figure 1a, peaks 4A/B) as well as for ferulic acid (Figure 1a, peaks 5A/B) at 3.87 and 4.10 min. Such a phenomenon is likely due to the E/Z-isomerism of the double bond in the vinyl carboxylic acid side chain [57,58]. It is important to note that for the quantification of both phenolics, the fragment areas of both isomers were summed up. Among the five phenolic acids, ferulic acid showed the highest concentration, which is in agreement with previous literature [17]. This result could be explained by its high concentration in the cell walls of *C. quinoa* seeds [57].

3.4. Phenolic Profile of C. quinoa Seeds

In our experimental work, the content of total phenolic compounds in *C. quinoa* seeds was evaluated as the sum of both bound and free fractions. The class of phenolic acids, including hydroxybenzoic acid, vanillic acid, syringic acid, coumaric acid, and ferulic acid, was determined and evaluated in the fractions of free and bound phenolics. The class of quercetin glycosides was only found and quantified in the fraction of free phenolics. Overall, the existence of available phenolic derivatives was in agreement with the previously published literature [17,48,56]. To the best of our knowledge, individual as well as total phenolic contents have not been studied systematically for this collection of Chilean *C. quinoa* genotypes. Therefore, direct comparisons with formerly published articles are not possible at present for the whole dataset. Taking this fact into account, we were nonetheless able to compare results with previously published results for a few previously studied genotypes. The total phenolic content among the studied genotypes ranged from 35.51 mg/100 g to 93.23 mg/100 g of seed dry weight. In the present work, 40.5% of the *C. quinoa* genotypes were found to have an above-average content of phenolic derivatives and were therefore comparatively rich in phenolic content, while 57.6% were found to be comparatively poor in phenolic content with below-average content. Genotypes with high phenolic content could significantly contribute to improved agronomic performance. Numerous research outcomes suggest a positive relationship between the abundance of phenolic compounds in plants and their growth [10,59]. Likewise, several studies demonstrate positive associations between incorporating polyphenol-rich foods into the human diet and the prevention of significant diseases [60]. Detailed statistics are presented as supporting materials (Tables S1 and S2), and Figure 3 shows the overall variation in free and bound phenolic compositions among the studied genotypes. We noted that the differences in total phenolics in the studied *C. quinoa* genotypes were significant ($p < 0.001$) (Figure 3, Tables S1 and S2). In our study, we detected a higher content of total phenolic compounds with 93.23 mg/100 g of seed dry weight in AZ-110 among all genotypes, while the lowest total phenolic content was observed in AZ-18 with 35.51 mg/100 g of seed dry weight. For the few earlier reported *C. quinoa* genotypes, the total mean phenolic content among both central Chilean landraces Cáhuil (AZ-4, AZ-18, AZ-103, and AZ-104) and FARO (AZ-31 and AZ-32) was 47.25 mg/100 g and 46.79 mg/100 g of seed dry weight, respectively, a lower amount than those reported by Vega Gálvez et al. [61] (194 mg/100 g for Cáhuil and 187.79 mg/100 g for FARO). One more experiment carried out by Sobota et al. [62] has also shown a higher value for total phenolics for FARO in comparison with the one reported in our present analysis. These systematic differences in the total phenolic content can be explained by the fact that the determination of phenolic compounds was carried out using a classical approach involving the Folin-Ciocalteu method. Such an assay may overestimate the content of phenolics because of interference

from non-phenolic components [23]. It is important to underline that in both studies reported by Vega Gálvez et al. [61] and Sobota et al. [62], the total phenolics were expressed as gallic acid equivalent (GAE). Similarly, other south Chilean genotypes from the Villarrica region (AZ-19 and AZ-20) have shown a mean of 51.39 mg/100 g and 48.40 mg/100 g of total phenolic content, respectively, which is lower compared with the one reported in a previously published article [61]. As stated above, total phenolic compounds in the reported Villarrica ecotype were analyzed by the Folin-Ciocalteus assay [61]. Finally, south Altiplano ecotypes AZ-3 and AZ-5 that originate from the Cancosa region had a mean of 57.80 mg/100 g and 65.74 mg/100 g of total phenolic content, respectively, values that were less than the ones reported for Cancosa with 112 mg GAE/100 g in the Vega Gálvez et al. [61] experiment. Such contradictory results for these previously reported *C. quinoa* genotypes can arise from several factors, including agronomical conditions as well as the protocols that have been used for the assessment of total phenolic compounds.

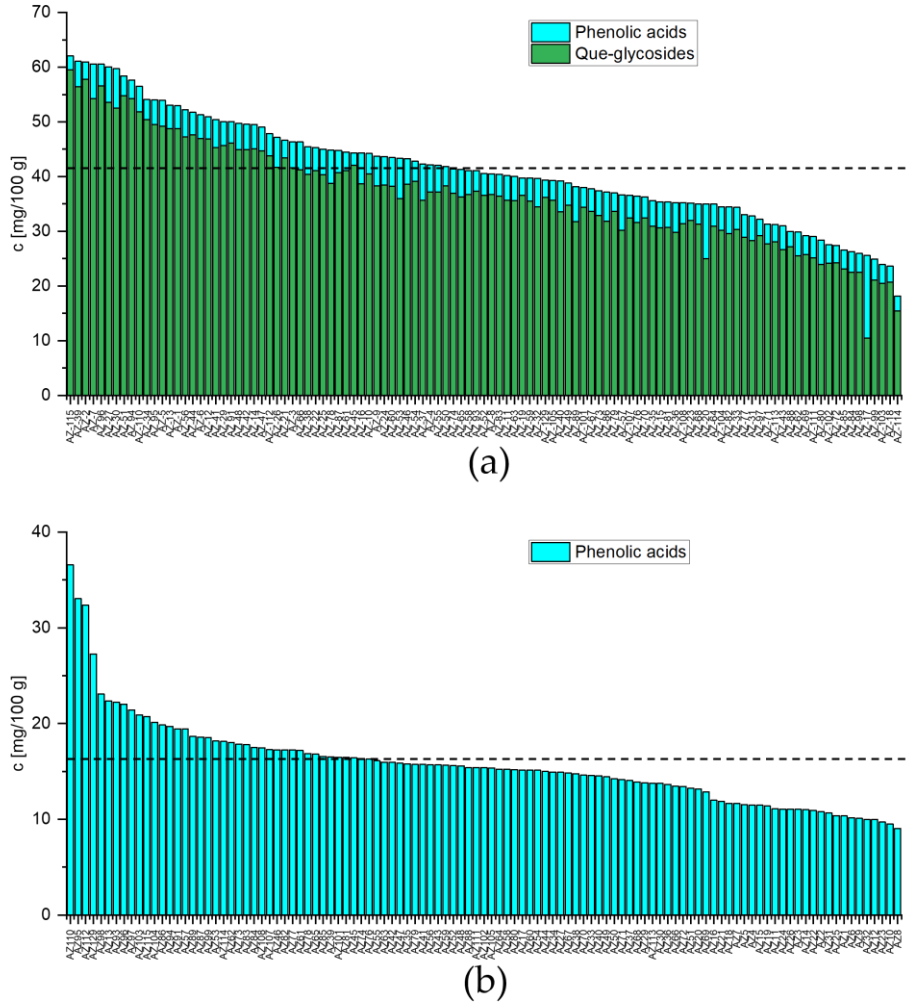

Figure 3. Content of free (**a**) and bound phenolic compounds (**b**) in *C. quinoa* genotypes. The stacked columns show the concentrations of phenolic acids in the fractions of free and bound phenolic compounds as well as quercetin glycosides in the fraction of free phenolic compounds in quinoa samples. Dashed lines in the figures indicate the average values of the summed concentrations of phenolic acids and quercetin glycosides.

Concerning individual compounds, free phenolics have been reported in a range of 18.28 mg/100 g to 62.27 mg/100 g of seed dry weight ($p < 0.001$) (Figure 3a). As reported by Gómez-Caravaca et al. [17], the flavonoid derivatives were the most abundant free phenolics in proportion to 81.3% of the total free phenolics for the studied genotypes. Among previ-

ously reported *C. quinoa* genotypes (Cancosa, Cáhuil, FARO, and Villarrica), south Altiplano ecotypes from the Cancosa region had the highest flavonoid glycoside content (mean of 45.68 mg/100 g), while central-southern ecotypes had the lowest flavonoid glycoside content (mean of 29.98 mg/100 g) (Figure 3a). Relating to these genotypes, the mean flavonoid content in the south Altiplano landraces was 1.5-fold higher compared with the central-southern landraces. In general, this trend is comparable with the results from Graf et al. [63]. In Graf's experiment, flavonoid glycosides were analyzed in the Chilean *C. quinoa* genotypes that originate from different biomes of Chile. In their study, the relative flavonoid content was 2.6 times higher in northern genotypes compared with those originating from the central-southern region of Chile. Similarly, Vega Gálvez et al. [61] also reported the highest flavonoid glycoside content in Cancosa ecotypes (211 mg CAE/100 g) compared to central-southern ecotypes. Such genotypes boasting a remarkable flavonoid content not only excel in agronomic performance [8,9] but also promise substantial health benefits due to their anti-microbial and anti-oxidant properties [6]. Further, the bound phenolics have been reported in a range of 9.03 mg/100 g to 36.57 mg/100 g of seed dry weight ($p < 0.001$) (Figure 3b). Amongst the five phenolic acids mentioned above, ferulic acid was identified as the main compound with 62.3% of the total bound phenolics since the derivatives of hydroxycinnamic acid are monomeric components of lignin, which forms plant cell walls together with cellulose. This result is identical to the previously published articles where ferulic acid was found to be a major compound of bound phenolics in *C. quinoa* [17,19,56]. Previous studies in other cereals have reported that a heightened content of ferulic acid, a potent phenolic compound, has been linked to enhanced defenses against pathogens and pests [64,65]. In addition, ferulic acid emerges as a pivotal catalyst for promoting human well-being and health [66]. Elevated levels of ferulic acid and coumaric acid have been demonstrated to significantly enhance resistance against lodging in other cereals [67]. In addition, the pool of the free phenolic fractions was higher as such, at 72.0%, compared to the bound fractions, at 27.9% of the total phenolic content. Our result is identical to previous results where the free fractions of phenolics were reported as a significant contributor to the total phenolic content compared with the bound fractions [17,56,61,68]. These results could be explained by the fact that free phenols are available on the outer surface of the seed pericarp, whereas bound phenols are attached to cell wall components [12,69,70]. However, on the contrary, a higher content of bound fractions in comparison to free fractions was reported in the Peruvian Altiplano genotypes [70]. Such variation in different phenolic fractions of *C. quinoa* seeds that has been reported in previous studies could occur due to several factors, particularly different germplasm and environmental as well as agronomical conditions [61,70].

The comparison and correlation of total phenolic content with other free and bound compounds can provide insights into the interplay of various bioactive compounds present in the plant (Figure S2). Free phenolic compounds in quinoa, such as phenolic acids and flavonoids, are often correlated with total phenolic content. However, the extraction and bioavailability of bound phenolic compounds are more limited due to their chemical link to cell wall components. As a result, the relationship between total phenols and bound phenolic compounds has been reported as more complex. In the present study, we found that as the total phenolic content increases, it is likely that the concentrations of these individual free and bound phenolic compounds will also increase.

Phenolic compounds and saponins share some common biosynthetic pathways in plants [71]. This shared biosynthetic pathway could lead to co-regulation and might result in some correlation. We explored this hypothesis by correlating the phenolic compound content of this study with the saponin content of the same germplasm panel, which we have previously investigated [39]. We found that there are very weak correlations between total phenolics and total saponin contents, as well as various fractions and specific compounds (Figure S3). The specific correlation between phenolic compounds and saponins may depend on the environmental conditions and genetic factors involved. Therefore, further research is needed to establish specific relationships between phenolic compounds and saponins in *C. quinoa*.

3.5. Principal Component Analysis and Hierarchical Clustering

A principal component analysis (PCA) was performed using a dataset for all variables for each line to represent existing phenotypic variation patterns in the studied *C. quinoa* panel. Principal component analysis was executed with a correlation distance matrix and outlined into a two-dimensional scatter plot. The specific loading factors that outline the principal component are listed in Table S3. As shown in Figure 4a, the first two components, PC1 and PC2, of the principal component analysis explained 25.9% and 22.1% of the total variation, respectively. Based upon the loading factors, phenotypic variables such as total phenolics, several flavonoids, hydroxybenzoic acid (free and bound), vanillic acid (free and bound), and ferulic acid (bound) showed a strong influence on PC1. The remaining variables, syringic acid (free and bound), coumaric acid (free and bound), and ferulic acid (free), showed a strong influence on PC2. The output data for each characteristic and for each accession was grouped into two major clusters consistent with the content of phenolic compounds. Based on the above data, accessions that were abundant in phenolic content are present on the left side of the quadrant, while accessions that had average or below-average phenolic compounds are shown on the right side of the quadrant. Further, hierarchical clustering was carried out based on the Euclidean distance and the complete grouping method using the PCA score (Figure 4b). According to the PCA score, cluster 4 showed the smallest value for PC1. As the first principal component, PC1, correlates negatively with the number of free and bound phenolics and total phenolic content, cluster 4 pooled together those genotypes having a high phenolic content. Moreover, clusters 5 and 1 pooled genotypes that possessed a high content of free fractions of phenolics and, consequently, total phenolics related to the remaining genotypes from other clusters. Contrarily, cluster 3 contains those genotypes that are characterized by low phenolics in both fractions. The remaining cluster, cluster 2, showed scattering close to the central line of PC2. This cluster pooled those genotypes that showed an average amount of total phenolic compounds. However, principal component analysis shows a partially interlinked dispersion for sub-groups of the main clusters. The low phenolics cluster revealed variation within and among sub-groups, which contain genotypes from the south Altiplano and also from coastal-lowland regions. Interestingly, the south Altiplano genotypes are dispersed among different sub-groups within the same cluster, which suggests that the low-land group may have a comparatively higher genetic diversity. Such a result, in the context of existing genetic diversity and comparison of both quality and quantitative traits in *C. quinoa* germplasm, is in agreement with previously published work where data confirmed a comparatively higher genetic variation in lowland genotypes compared with highland ones [72,73]. Overall, our data showed interlinking of the south Altiplano and coastal-lowland genotypes in both PCA and dendrogram. However, interlinking among the genotypes from two different biomes could be due to existing shared alleles [74] and consequently genetic similarity [72] between highland and coastal-lowland quinoa genotypes. Several studies have been carried out that confirmed the extant genetic diversity across the different *C. quinoa* genotypes for various traits, and such genetic variance could be a key reason for possible existing variation in phenolic content [72,74,75]. Therefore, a thorough genetic study of these accessions could help to elucidate the possible genomic variance that leads to comparatively different phenolic compounds in *C. quinoa*.

In general, our data show that the thirteen *C. quinoa* genotypes (AZ-2, AZ-7, AZ-13, AZ-27, AZ-30, AZ-39, AZ-51, AZ-94, AZ-95, AZ-96, AZ-110, AZ-112, and AZ-115) hold 49.7% of total phenolic content. As these genotypes showed a high content of phenolic derivatives, they can be categorized as high-phenolic-content genotypes. Such genotypes could lead to seed enrichment in health-promoting functional bioactive compounds. Further, eighteen genotypes (AZ-1, AZ-5, AZ-6, AZ-29, AZ-34, AZ-41, AZ-42, AZ-44, AZ-47, AZ-48, AZ-53, AZ-56, AZ-61, AZ-78, AZ-87, AZ-91, AZ-93, and AZ-129) showed a total phenolic content above the mean value, and any such genotypes can also be accounted for a comparatively high phenolic source than the remaining ones. Among all genotypes, AZ-15, AZ-17, AZ-18, AZ-20, AZ-23, AZ-31, AZ-36, AZ-43, AZ-62, AZ-68, AZ-69, AZ-71, AZ-72, AZ-80,

AZ-84, AZ-85, AZ-88, AZ-98, AZ-99, AZ-102, AZ-103, AZ-111, AZ-113, and AZ-114 can be categorized as low-phenolic-content genotypes, as they showed a low amount of all assessed phenolic derivatives (i.e., ca 13.5% of total phenolics). The rest of the genotypes had a total phenolic level near the average. It is important to note that the entire categorization was based on the available clustering statistics.

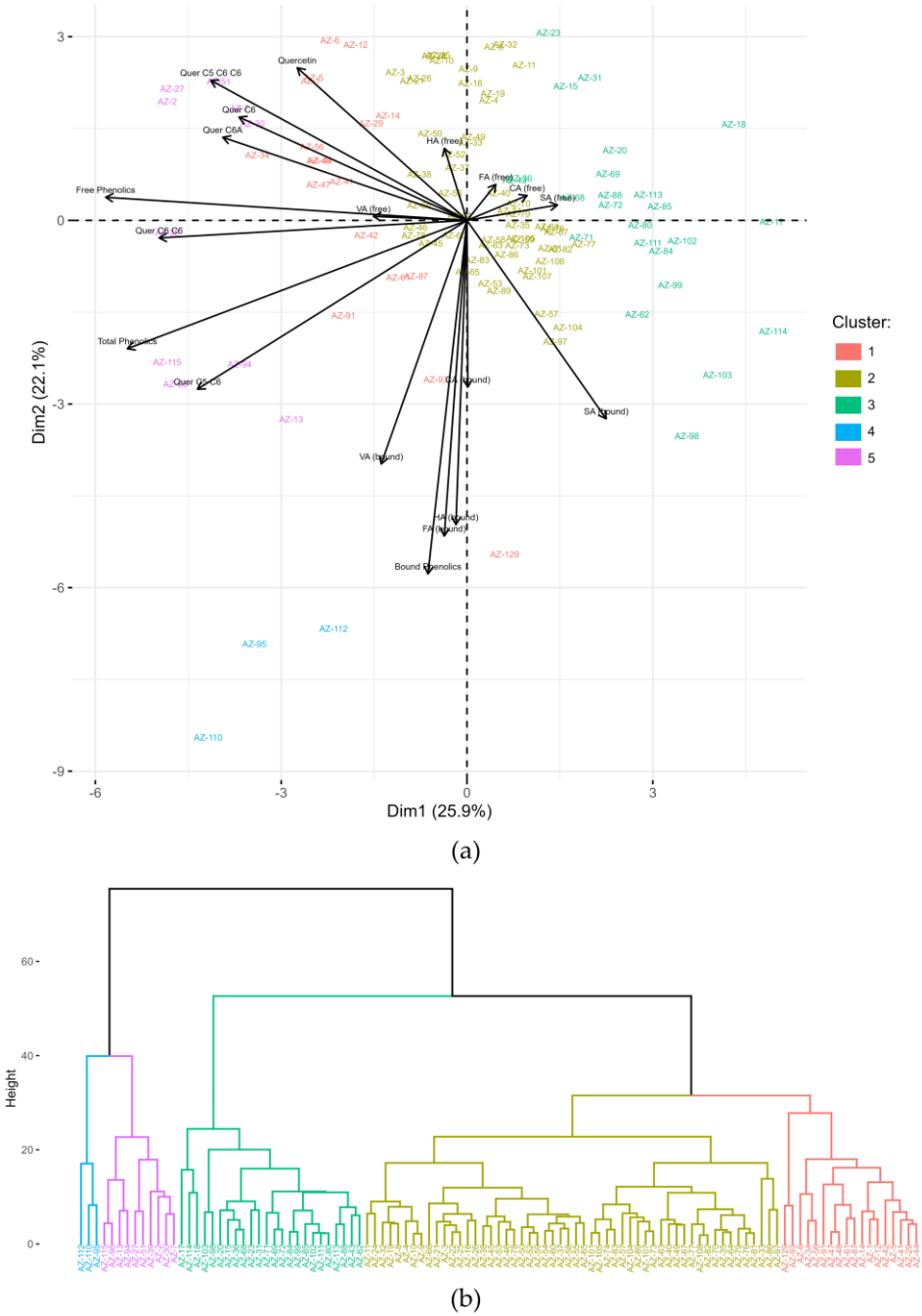

Figure 4. Principal component analysis (**a**) and hierarchical clustering (**b**) of phenolic compounds of *C. quinoa*. The bi-plot shows the main components PC1 and PC2 of PCA, which account for 48.0% of the total phenolic content in *C. quinoa*. Arrows show the phenolic derivatives, and the length of the arrows approximates the variance of the derivatives. The distance between each point explains how similar the observation is, and colors correspond to the clusters.

3.6. Variance Explained by a Genetic Effect

To assess the genotypic effect on trait variation pattern, the percentages of variance described by the genetic effect (Vg) in the total phenotypic variance for agromorphological traits were estimated using a mixed linear model. The variance explained by genetic effect (Vg) was measured according to the restricted maximum likelihood (REML) variance components using the lme4 library of R [53]. In the current study, we noted a significant effect of genetic variance ($p \leq 0.05$), which shows that each accession shows the phenotype of assessed traits distinctly, i.e., there is a genetic variation that would interpret the existing differences in phenolic content. Such a notable genotypic effect by Vg highlights the genetic diversity of 97.4% within the studied Chilean *C. quinoa* genotypes for total phenolic compounds (Table S4). Our data may further contribute to *C. quinoa* breeding programs as they advance toward the development of new cultivars.

4. Conclusions

In summary, this study endeavors to bridge the knowledge gap regarding bioactive phenolic compounds in Chilean quinoa seeds. The results of this study underlie the existence of a wide variation among the Chilean *C. quinoa* germplasm for total phenolic content. The total phenolic content among the studied genotypes ranged from 35.51 mg/100 g to 93.23 mg/100 g of seed dry weight. The identification and characterization of these bioactive compounds not only shed light on the nutritional value and potential health benefits of quinoa but also provide a basis for improving its agronomic performance and nutritional content. From an agronomical perspective, understanding the distribution and variation of bioactive phenolic compounds within different quinoa germplasm can guide the selection of cultivars that exhibit superior phenolic profiles. Among the studied samples, thirteen distinct *C. quinoa* genotypes exhibited a notable abundance of phenolic derivatives, signifying their status as rich sources of these compounds. Additionally, eighteen other genotypes of *C. quinoa* demonstrated comparatively elevated levels of phenolic compounds when contrasted with a subgroup of genotypes possessing lower phenolic content. Notably, the present study revealed that free phenolic fractions exhibited higher levels in comparison to bound fractions within the studied genotypes. Integrating phenolic-rich quinoa into diets as a functional food source could contribute to combating various diet-related ailments and promoting overall well-being. From a future breeding perspective, the comprehensive knowledge of bioactive phenolic compounds offers a promising avenue for targeted breeding strategies. By selecting quinoa varieties with higher phenolic content and optimized profiles, breeders can develop cultivars that are better adapted to diverse environmental conditions and possess enhanced resilience. Concerning future research steps, a genetic study such as genome-wide association studies (GWAS) will be performed in further analyses to determine inherent genomic regions that are associated with phenolic content to be used in quinoa breeding programs through marker-assisted selection.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13082170/s1. Figure S1: Collection localities of. *C. quinoa* germplasm. Figure S2: Pearson's correlation heat map of total phenolics and individual phenolic derivatives. Figure S3: Pearson's correlation between secondary metabolites in *C. quinoa*. Table S1: Phenolicscontent (mg/100 g) in *C. quinoa* seed. Table S2: Tukey's HSD multiple comparisons for phenolic content. Table S3: Loading factors of variables in PCs in PCA. Table S4: Variance by genotypic effect.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author or other co-authors.

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