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Phytoecdysteroids and flavonoid glycosides among Chilean and commercial sources of *Chenopodium quinoa*: variation and correlation to physicochemical characteristics

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Abstract

BACKGROUND—Little is known about varietal differences in the content of bioactive phytoecdysteroids (PE) and flavonoid glycosides (FG) from quinoa (*Chenopodium quinoa* Willd.). The aim of this study was to determine the variation in PE and FG content among seventeen distinct quinoa sources and identify correlations to genotypic (highland vs. lowland) and physicochemical characteristics (seed color, 100-seed weight, protein content, oil content).

RESULTS—PE and FG concentrations exhibited over 4-fold differences across quinoa sources, ranging from 138 ± 11 $\mu\text{g/g}$ to 570 ± 124 $\mu\text{g/g}$ total PE content and 192 ± 24 $\mu\text{g/g}$ to 804 ± 91 $\mu\text{g/g}$ total FG content. Mean FG content was significantly higher in highland Chilean varieties (583.6 ± 148.9 $\mu\text{g/g}$) versus lowland varieties (228.2 ± 63.1 $\mu\text{g/g}$) grown under the same environmental conditions ($P = 0.0046$; t-test). Meanwhile, PE content was positively and significantly correlated with oil content across all quinoa sources ($r = 0.707$, $P = 0.002$; Pearson correlation).

CONCLUSION—FG content may be genotypically regulated in quinoa. PE content may be increased via enhancement of oil content. These findings may open new avenues for the improvement and development of quinoa as a functional food.

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Conflict of Interest

IR has equity in Nutrasorb LLC, which is involved in quinoa R&D.

Supporting Information Available: Supp. Table 1. Harvest data of Chilean quinoa varieties grown in common field experiments in Chiu Chiu, Calama, Northern Chile (Region II); Supp. Table 2. Phytoecdysteroid (PE) and flavonoid glycoside (FG) quantification in leachates vs. traditional extracts of quinoa seeds; Supp. Table 3. 20-Hydroxyecdysone (20HE), total phytoecdysteroid (PE), and flavonoid glycoside (FG) content of quinoa seeds and their saponin powder by-product at various stages of the desaponification process; Supp. Table 4. Eigenvectors from Principle Component Analysis (PCA) of the variables latitude, seed color, 100-seed weight, and contents of protein, oil, 20HE, total PE, and FG among all quinoa sources; Supp. Table 5. 20-Hydroxyecdysone (20HE) content determined in the three major components of the quinoa seed; Supp. Fig. 1. Schematic diagram of the desaponification process performed using the “escarificadora” or “desaponificadora” in San Fernando, Region VI, Chile, to mechanically remove saponin-rich pericarp from quinoa seeds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

breeding; 20-hydroxyecdysone; morphological; nutritional; phytochemical; polyphenol

INTRODUCTION

Quinoa (*Chenopodium quinoa* Willd., Amaranthaceae), a traditional Andean seed crop consumed similarly to staple cereal grains, has recently risen to the forefront of worldwide crop research and development for its nutritive and pharmacological value,^{1, 2} paralleled by a surge in consumer demand.³ The growing importance of quinoa seeds and sprouts as functional foods has triggered an interest in the selection and marketing of varieties with enhanced nutritional quality and increased levels of biologically active phytochemicals (phytoactives), including phytoecdysteroids (PE) and flavonoid glycoside polyphenols (FG)^{4–7} (Fig. 1).

Compared with traditional Poaceae cereal grains, quinoa is the only staple crop reported to contain PE,^{8–10} plant-derived steroids including 20-hydroxyecdysone (20HE) (Fig. 1A) and its structural analogs (minor PE),¹¹ that have demonstrated insulin sensitizing, fat reducing,^{12–14} and fitness enhancing¹⁵ activities in mammals without inducing androgenic or estrogenic effects.^{11, 15–17} Quinoa seeds have also been shown to contain higher levels of polyphenols than rice and wheat.^{2, 18–20} Quinoa-derived polyphenols, among which FG (Fig. 1B) are most prominent,²¹ are powerful antioxidants²² that regulate glucose and lipid metabolism,²³ reduce inflammation, inhibit tumor growth, and promote cardiovascular function.²² Epidemiological studies and randomized clinical trials have suggested a possible role for flavonoids and flavonoid-rich staple crops in the prevention of chronic human diseases such as cardiovascular disease, diabetes, and cancer.^{24, 25} Therefore, increased levels of PE and FG in quinoa seeds may have important implications for human health among quinoa-consuming populations.

Physicochemical characterization of a diverse set of traditionally used and commercialized crop varieties is the first step in the classification of genetic resources with potential utility for the development of new cultivars with improved quality.²⁶ Germplasm banks around the world hold approximately 5000 different quinoa landraces,⁷ each adapted to distinct agroenvironments as a result of natural and artificial selection via trade and migration of quinoa from its origin of domestication (Lake Titicaca) 3000 – 7000 years ago.^{7, 27} Quinoa germplasm studies have revealed that quinoa accessions cluster into highland and lowland ecotypes, representing genetically distinct groups.^{7, 27, 28} This pattern of genetic bifurcation has been particularly well-characterized in Chile, where Northern Chilean varieties (cultivated in the extreme arid Altiplano highlands, 3500 – 4000 m above sea level, 19 °S, 150 – 300 mm annual rainfall, saline soils, frequent frost) are genetically and morphologically distinct from Central-Southern varieties (cultivated in coastal zones, at sea level or low elevation, 34 – 39 °S, 500 – 2000 mm annual rainfall, clayish soils with higher organic content, infrequent frost).^{4, 27, 28}

Since PE and FG are reported to play a role in plant response to environmental stress,^{11, 22, 29} variation in PE and FG content may have evolved among highland versus

lowland genotypes in Chile. Though research is lacking, previous studies have shown variation in PE and FG content among quinoa sources. In one study that employed LC-UV-MS analysis among 46 commercial quinoa sources, 20HE content displayed a range of 184 – 491 µg/g.⁸ FG content of 1 – 2 quinoa cultivars was measured via LC-UV-MS in 3 separate studies, showing total FG contents ranging from 543 – 2561 µg/g.^{10, 21, 30} Furthermore, using spectrophotometric analysis to estimate total phenolic and antioxidant capacities of quinoa seeds, significant differences were observed between different quinoa cultivars produced in their respective regions of origin.^{20, 28, 31} However, spectrophotometric assays are non-specific and subject to high rates of interference by vitamins, amino acids, and sugars.³² To date, total PE and FG contents have not been evaluated using specific quantitative techniques among more than two different quinoa genotypes grown under the same environmental conditions, or among multiple quinoa sources with carefully documented origins. The genetic and environmental factors that influence the concentration of these phytochemicals in quinoa seeds remain largely unknown.^{2, 8, 33}

Additionally, little work has been performed to determine the possible correlations of PE and FG content of quinoa to physicochemical seed characteristics, such as seed color, weight, or oil and protein content. As an Amaranthaceae crop, quinoa seeds have a distinctive seed structure compared to Poaceae cereal crops. The quinoa seed is surrounded by a saponin-rich pericarp, which is usually removed via mechanical abrasion prior to commercial distribution and consumption.^{2, 34} The seed itself is comprised of three major parts: (1) a 2-cell layered seed coat, (2) a circular embryo enveloped at one axis by the endosperm, together containing the protein and lipid reserves of the seed, and (3) a carbohydrate-rich perisperm (Fig. 1C).^{34, 35} Previous reports suggest that PE concentrate in the oil-rich embryo,⁸ whereas FG accumulate in the outer and internal layers of the seed.³⁰ Total PE and FG content in quinoa may, therefore, be correlated with the content of various nutritional components. Knowledge of these relationships may open opportunities for the paralleled improvement of nutritional and phytochemical parameters through crop breeding and agricultural practices.

The objective of this study was to characterize the PE and FG content among quinoa varieties derived from different genotypic pools in Chile (highland vs. lowland) and compare to commercially available sources. Furthermore, correlation analysis and Principle Component Analysis were performed to determine the relationships of PE and FG content to various physicochemical parameters (seed color, 100-seed weight, protein content, oil content), and the relationships of each quinoa variety to one another.

MATERIALS AND METHODS

Chemicals and Reagents

All solvents (95% ethanol, ACS grade acetic acid, and HPLC grade acetone, acetonitrile, hexane, sodium hydroxide, and trichloroacetic acid) were obtained from Sigma (St. Louis, MO). All water used in the experiments was purified using a Millipore reverse osmosis water purification system with a minimum resistivity of 18.2 M cm (Bedford, MA, USA). 20HE (99% pure) was purchased from Bosche Scientific, New Brunswick, NJ). Quercetin 3-

O-(2,6-di- α -L-rhamno-pyranosyl)- β -D-galactopyranoside (98% pure), was isolated from AlterEco Red quinoa seeds as described previously.¹⁰

Collection of Quinoa Sources

Eight Chilean quinoa varieties and nine global commercial sources were selected for study, as described more in detail below. Details regarding the name of each quinoa source, the location from which it originated, and photographs of the seeds are listed in Table 1 and depicted in Figure 2. Seed color intensity was rated on a scale of 1 – 3 via visual inspection, where 1 indicates white - lightly colored seeds, 2 indicates red seeds, and 3 indicates black seeds. Seeds were weighed in 100-seed amounts in triplicate. Seed color and 100-seed weights are reported in Table 2.

Chilean varieties—Eight Chilean varieties were selected to represent typical varieties produced and consumed in Chile, among which four varieties originated from Northern Chile, and four originated from Central-Southern Chile. Previous work has shown that Northern (highland) varieties are genetically distinct from Central-Southern (lowland) varieties, which has been confirmed by morphometric and agronomic differences.²⁸ In order to assess the impact of genotype on PE and FG content among these Chilean varieties, seeds from each variety were grown and harvested in the same location in Chile (Chiu Chiu, Calama, Region II, Northern Chile), where they were all subjected to similar environmental conditions. Details regarding the field conditions in Chiu Chiu have been previously reported.³⁶ (Further information is available upon request from the Chilean Department of Agriculture.) Measurements associated with plant growth, maturity, and seed yield from the harvesting of these varieties is summarized in Supplementary Table 1. All seeds were stored at –5 °C at the germplasm bank of Universidad Arturo Prat, Iquique, Chile, until further analysis. Seeds were not processed to remove the saponin-rich pericarp.

Commercial Sources—Quinoa seeds (0.5 – 4.0 kg quantities) were purchased from local food stores in the USA (NJ and NY) and Ecuador to represent a variety of colors and biogeographic sources. Saponin-rich pericarps were removed from commercial seeds by their respective producers before distribution.

Determination of Total Oil Content

Seeds were washed to remove saponins, dried, and ground to a fine powder with mortar and pestle. Oil extraction was performed as described elsewhere³⁷ with minor modifications. Briefly, 2 g of clean, desaponified quinoa seed powder was extracted with 20 mL hexane for 72 h at 25 °C under gentle agitation, protected from light. Hexane was removed via vacuum filtration, yielding yellow quinoa oil that was weighed. Total oil content was determined in triplicate for each sample and expressed as mg/g of the initial weight of seed powder.

Determination of Total Protein Content

Following oil extraction, quinoa seed solids were filtered from the hexane solvent through a Whatman filter paper and dried at 30 °C for 48 h. Fifty mg of defatted seed powder was suspended in 500 μ L of water. A volume of 500 μ L NaOH (2 M, pH 1.1) was added to the suspended seed powder, vortexed for 30 min at 25 °C, and centrifuged at 20,160 g for 40

min. The supernatant was recovered for protein analysis. A volume of 500 μL TCA (20% in water) was added to the supernatant, vortexed for 5 min at 25 $^{\circ}\text{C}$, incubated at 4 $^{\circ}\text{C}$ for 20 min, and centrifuged at 20,160 g for 40 min at 4 $^{\circ}\text{C}$. The pellet was washed with 300 μL of cold acetone, dried at 40 $^{\circ}\text{C}$ for 48 h, and weighed. Total protein content was determined in triplicate for each sample and expressed as mg/g of the initial weight of seed powder.

Desaponification Samples

In order to determine whether or not desaponification has a significant effect on PE or FG content of quinoa seeds (and should therefore be taken into account when comparing the relative phytochemical content of unprocessed Chilean varieties with that of commercial quinoa sources), we performed LC-UV-MS analysis of the PE and FG content of 8 samples obtained throughout the desaponification process of one Chilean quinoa variety (Faro, C6). Seeds were desaponified in San Fernando, Chile via mechanical abrasion without heat, as depicted in Supplementary Figure 1.

Isolation of Individual Seed Parts

To determine the localization of 20HE within quinoa seeds, AlterEco Red seeds were lightly ground in a mortar and pestle, yielding clearly distinguishable circular embryos. Embryos, enveloped at one axis by the endosperm, were manually separated from the remaining seed material (seed coats and perisperm) with forceps. The seed coat/perisperm mixture was further ground in a mortar and pestle and sifted through wire mesh to separate the red seed coats from the white perisperm powder. Each of the three major components obtained through the seed dissection process (embryo/endosperm, seed coat, perisperm) were prepared as separate samples for LC-UV-MS analysis.

Sample Preparation for LC-UV-MS Analysis

PE and FG were leached and concentrated from all quinoa samples (Chilean varieties, commercial sources, desaponification samples, and isolated seed parts) as previously described.¹⁰ Briefly, 1.6 g of seed or saponin powder were incubated in 8 mL of 70% ethanol for 4 h at 80 $^{\circ}\text{C}$ to leach phytochemicals in triplicate. Saponin powder was sedimented via centrifugation. Then, the liquid leachate was filtered from the intact seeds or sedimented saponin powder into pre-weighed vials and dried by rotary evaporation and lyophilization. Dry leachate weights were recorded for each sample, redissolved to 5 mg/mL in 70% ethanol, and 5 μL was injected for analysis by high performance liquid chromatography – ultraviolet spectroscopy – mass spectrometry (LC-UV-MS). 20HE, total PE, and FG contents in initial seed materials were determined using the following equation: $(\mu\text{g } 20\text{HE, PE, or FG}) / (5 \mu\text{L redissolved leachate}) \times (1000 \mu\text{L redissolve leachate}) / (5 \text{ mg initial leachate}) \times (\text{weight initial leachate}) / (\text{g initial seed material}) = (\mu\text{g } 20\text{HE, PE, or FG}) / (\text{g initial seed material})$.

Our method of leaching intact seeds in 70% ethanol with heat has previously been shown to yield most 20HE available in the seeds, achieving levels equal to or above those obtained from traditional extraction of macerated seed material.¹⁰ In order to validate our leaching technique as a method to prepare LC-UV-MS samples for quantification of total PE and FG from quinoa seeds, we compared PE and FG levels determined via our leaching method to

those determined using 5 modified extraction protocols with the same seed source (AlterEco Red). Seeds were macerated to powder and extracted with 70% ethanol or 70% methanol at room temperature (25 °C) or with heat (80 °C), with or without solvent acidification using hydrochloric acid or acetic acid (Supp. Table 2).

LC-UV-MS Analysis

Identification and quantification of PE and FG were performed using LC-UV-MS as described earlier.¹⁰ Briefly, 20HE (Fig. 1A) was quantified from a calibration curve of a pure 20HE standard with peak areas of UV absorbance measured at 247 nm. 20HE analogs (minor PE) were quantified as 20HE equivalents (concentrations of individual molecules were estimated using the 20HE standard). Concentrations of FG were simultaneously calculated from peak areas at the same wavelength (247 nm) corresponding to the calibration curve obtained with quercetin 3-*O*-(2,6-di- α -L-rhamno-pyranosyl)- β -D-galactopyranoside (Fig. 1B), the major FG isolated from AlterEco Red quinoa seeds.¹⁰ Because the most abundant FG in quinoa have consistently been reported to be quercetin trisaccharides,^{9, 10, 38} total FG concentrations among all quinoa samples were expressed as quercetin 3-*O*-(2,6-di- α -L-rhamno-pyranosyl)- β -D-galactopyranoside equivalents.

The LC-UV-MS system was composed of a Phenomenex® C8 reverse phase column, photodiode array UV detector, and triple quadrupole mass spectrometer with electrospray ionization as described earlier.¹⁰ The mobile phase consisted of two components: solvent A (0.5% acetic acid in water, pH 3.0 – 3.5), and solvent B (100% acetonitrile). The mobile phase flow was 0.2 mL/min and a gradient mode was used as follows: initial concentrations were 93% A and 7% B; the proportion reached 73% A and 27% B over 40 min; solvent B reached 100% in the next 5 min and was maintained for 2 min to wash the column; the column was re-equilibrated to initial conditions for 13 min. The total run time was 60 min.

Statistical Analysis

Data from this study were reported as the mean $\pm$ SD ($n=3$) for each sample. Results of the 20HE, total PE, and FG content among all 17 quinoa sources and 9 desaponification samples were subjected to Tukey's multiple comparison one-way analysis of variance (ANOVA). Using the mean 20HE, total PE, and FG values for each Chilean variety, t-tests were performed to compare phytochemical content among the 4 Northern varieties versus 4 Central-Southern varieties. Pearson correlation analysis and Principle Component Analysis (PCA) were performed among genotype, seed color, mean 100-seed weight, and the mean contents of protein, oil, 20HE, total PE, and FG for each quinoa source. ANOVA, t-tests, and Pearson correlation analyses were run on GraphPad Prism 6.0 software (GraphPad, Inc., La Jolla, CA). PCA was performed using InfoStat software (Universidad Nacional de Córdoba, Argentina). $P < 0.05$ was considered significant for all analyses.

RESULTS AND DISCUSSION

Physicochemical Diversity Among Quinoa Sources

As shown in Table 2, the quinoa sources employed in our study varied significantly in physicochemical characteristics, including seed color (white, red, or black), 100-seed weight

(2.18-fold difference; $P < 0.0001$), protein content (2.11-fold difference; $P < 0.0001$), and oil content (1.92-fold difference; $P = 0.0017$) (Tukey's multiple comparison one-way ANOVA). AlterEco Pearl (Q1) had the highest 100-seed weight (441 ± 15 mg), while Kalustyan's Black (Q6) had the lowest (202 ± 7 mg). Wegman's Red (Q5) had the highest protein content (157.3 ± 11.7 mg/g), while Kalustyan's Black (Q6) had the lowest (74.7 ± 5.5 mg/g). Highest oil content was found in Ancovinto Roja (C2; 56.2 ± 4.4 mg/g), while Gramolino (Q8) contained the lowest (29.3 ± 2.6 mg/g). With the exception of some quinoa sources that showed lower protein content than expected, the protein and oil levels observed in our study generally corroborate previous reports that these macronutrients vary within a range of 111 – 162 mg/g protein²⁸ and 18 – 95 mg/g oil.⁶

LC-UV-MS Analytical Method and Compound Recovery

We previously developed and optimized a leaching procedure to efficiently harvest all 20HE present in quinoa seeds using 70% ethanol and heat.¹⁰ Here, we compared the efficiency of harvesting total PE and FG using our leaching procedure versus traditional extraction of macerated seed material with methanol or ethanol combined with heat or solvent acidification. Significant differences were observed for 20HE ($P = 0.0027$) and total PE ($P = 0.009$) content between phytochemical harvesting methods, with highest 20HE and PE content occurring in the samples obtained using our leaching procedure (Supp. Table 2). No significant differences were observed in total FG content between samples ($P = 0.3191$). Acid hydrolysis was not used in our sample preparation because previous studies have shown that FG mainly exist in quinoa in solvent-extractable (free) form, unlike quinoa-derived phenolic acids, which are often bound to cell wall polysaccharides and require liberation via acid hydrolysis prior to analysis.^{21, 38}

The phytochemical leaching procedure and LC-UV-MS protocol used in this study accomplished the rapid and simultaneous quantification of up to 8 PE and 9 FG in triplicate samples of 17 different quinoa sources. Recently, private and public breeding programs have recognized the need for extraction and analysis protocols that facilitate rapid, accurate, and reliable comparative screening for a large number of samples. The methods employed in our study were simpler, faster, and more efficient than previously described methods.

In our study, we compared the PE and FG contents of unprocessed Chilean quinoa varieties (containing saponin-rich pericarps) with that of desaponified commercial sources. However, 20HE, total PE, and FG contents were not significantly different between initial (saponin-containing) seeds (S1) versus processed seeds after one or two rounds of desaponification (S2, S6, S5, S9) (Supp. Table 3). This data is corroborated by previous reports that PE content was not significantly affected by quinoa desaponification,⁸ and FG content was affected only minimally (16.7% decrease after a high level of desaponification).³⁰ Together, these data indicate that the quantification of PE and FG content among unprocessed Chilean quinoa versus commercially available sources provides a meaningful comparison by which conclusions can be drawn about the sources containing the highest and lowest phytochemical concentrations. Interestingly, quinoa desaponification has a much lower impact on phenolic content than the milling of wheat or barley, which has been reported to reduce total phenolic content 42.5 – 72.5% and 69.7 – 90.7% in these grains, respectively.³⁰

Phytoecdysteroid (PE) Content among Quinoa Sources

20HE and PE content varied significantly across Chilean varieties and commercial quinoa sources ($P < 0.0001$; Tukey's multiple comparison one-way ANOVA), with >4-fold difference observed between highest and lowest source (Fig. 3A). Among Chilean varieties, Villarrica (C8) had the highest PE content ($570 \pm 124 \mu\text{g/g}$), and Cancosa (C3) contained the lowest ($224 \pm 103 \mu\text{g/g}$). Among commercial sources, AlterEco Red (Q2) had the highest PE content ($568 \pm 14 \mu\text{g/g}$), while Arrowhead Mills (Q9) contained the lowest ($138 \pm 11 \mu\text{g/g}$). 20HE constituted 71.6 – 90.0% of total PE among all 17 sources, corroborating earlier reports.^{8, 39} The level of variation we observed in 20HE content among quinoa sources was similar to a previous report ($184 - 491 \mu\text{g/g}$).⁸

We found no significant differences in 20HE or total PE content between Northern versus Central-Southern Chilean quinoa varieties ($P = 0.8949$ for 20HE content, $P = 0.6635$ for total PE content; 2-tailed, unpaired t-test). PE have been hypothesized to play a role in plant defense from insects, and their production has been shown to increase in spinach following mechanical damage or the application of methyl jasmonate, a plant-defense signaling molecule.²⁹ Therefore, environmental factors may have a greater influence on PE accumulation in quinoa than genotype. In future studies, it would be interesting to investigate the environmental factors that influence PE content in quinoa, and to determine whether PE content correlates with saponin content since both are triterpenoid compounds biosynthesized via the mevalonate pathway.⁴⁰

Flavonoid Glycoside (FG) Content among Quinoa Sources

FG content varied significantly across Chilean varieties and commercial quinoa sources ($P < 0.0001$; Tukey's multiple comparison one-way ANOVA), with > 4-fold difference between the highest and lowest source (Fig. 3B). Among Chilean varieties, Ancovinto Roja (C2) showed the highest FG content ($804 \pm 91 \mu\text{g/g}$) and Regalona (C7) contained the lowest ($192 \pm 24 \mu\text{g/g}$). Among commercial sources, AlterEco Pearl (Q1) had the highest FG content ($674 \pm 79 \mu\text{g/g}$), while Eden Red (Q4) contained the lowest ($196 \pm 48 \mu\text{g/g}$).

Mean FG content was significantly higher (2.6-fold) in Northern Chilean varieties ($583.6 \pm 148.9 \mu\text{g/g}$) compared with Central-Southern varieties ($228.2 \pm 63.1 \mu\text{g/g}$) ($P = 0.0046$; 2-tailed, unpaired t-test). Northern Chilean varieties are genetically and morphologically distinct from Central-Southern ecotypes.^{7, 27, 28} Taken together, these data are the first to suggest that FG content may be genotypically regulated in quinoa. Since FG function as protective plant compounds that increase under water stress,^{21, 22} it is plausible that Northern Chilean varieties have evolved genetic mechanisms to promote FG accumulation as an adaptive strategy to survive extreme environmental conditions.

Correlations Between PE/FG Content and Physicochemical Parameters

We conducted Pearson correlation analysis among latitude of origin, seed color, 100-seed weight, and contents of protein, oil, 20HE, total PE, and FG (Table 3). Chilean varieties and commercial sources were analyzed separately and together (all quinoa sources). No link was identified between seed color intensity and any other parameter, contrary to similar studies in rice, in which positive, significant correlations were found between seed color intensity

and total polyphenol, flavonoid, and antioxidant content.^{41–43} In rice, purple and red colors are attributable to two polyphenolic groups, anthocyanins and proanthocyanins, respectively.⁴¹ However, in quinoa, red - black coloration is attributable to the presence of betalains, non-phenolic nitrogen-containing pigments exclusive to the order Caryophyllales.^{44, 45}

In corroboration with our previous statistical analysis of FG content in Northern versus Central-Southern varieties (Fig. 3B), FG content was highly correlated to latitude of origin according to Pearson correlation analysis ($r = -0.963$, $P = 0.0001$). Seed weight was also significantly correlated to latitude of origin (Northern varieties displayed higher seed weights than Central-Southern varieties; $r = -0.874$, $P = 0.0046$). Lowland varieties typically produce smaller seeds than highland varieties within their respective regions of origin.⁴⁶ However, previous studies have also demonstrated that lowland varieties produce smaller, lighter seeds when grown in the highlands compared the lowlands,⁴⁷ possibly because photoperiod, altitude, and arid climate accelerate flowering and seed filling, thereby inhibiting seed growth.⁴⁸ In our field experiment, Central-Southern varieties reached maturity several weeks before their Northern counterparts (Supp. Table 1). Therefore, it is likely that both genotypic and environmental factors influenced the smaller seed weights of Central-Southern varieties compared to Northern varieties in our study. FG content and seed weight may function as two independent characteristics that are each strongly dependent on genotype, thereby resulting in their statistically significant correlation ($r = 0.834$, $P = 0.010$).

Among commercial quinoa sources, 100-seed weight was positively and significantly correlated with protein content ($r = 0.731$, $P = 0.025$) and oil content ($r = 0.717$, $P = 0.030$). Since protein and oil content account for a major portion of seed weight, this correlation is to be expected, especially among commercially produced seeds in which agricultural practices are optimized for high yield and high nutritional quality.

As expected, positive and highly significant correlations were observed between 20HE and total PE content across all analyses ($r > 0.960$, $P < 0.001$). Interestingly, 20HE and total PE content were both positively and significantly correlated with oil content across all analyses ($r > 0.664$, $P < 0.05$), except for the correlation of 20HE content to oil content among Chilean varieties, which was still very high ($r = 0.661$, $P = 0.0742$). This correlation may be a consequence of the biosynthetic pathway of phytoecdysteroids; phytoecdysteroids originate from the precursor cholesterol,⁴⁹ the content of which, in turn, is strongly correlated with lipid content.⁵⁰

Principle Component Analysis (PCA)

PCA analysis was performed for the 8 investigated parameters among all quinoa sources in order to visualize groupings of samples and identify meaningful underlying variables (Fig. 4, Supp. Table 4). PCA analysis transforms the number of investigated parameters into a smaller number of uncorrelated variables termed “principle components (PCs)”.⁵¹ The output from PCA consisted of a scores plot (Fig. 4A) to visualize the relationship of quinoa sources to one another, and a loading plot (Fig. 4B) to visualize the relationship of the investigated parameters.

The results indicated that the first two components (PC1 and PC2), which both had eigenvalues >1.0 , accounted for majority (61%) of the variance (Fig. 4A). The first principal component (PC1) shows that there is a positive correlation between oil, 20HE and total PE contents (Fig. 4B), which indicates that seeds containing higher lipid content also contain a higher content of phytoecdysteroids. The second principal component (PC2) shows that there is a positive correlation among latitude, 100-seed weight, and FG content (Fig. 5B), which indicates that seeds with greater mass contain higher FG content, and correspond to seeds originating from lower latitudes. These relationships corroborate those determined by Pearson correlation analysis above.

Figure 4A shows the spatial relationships of the various quinoa sources, in which three clusters of quinoa sources were revealed. The Central-Southern Chilean varieties formed a distinct group to the lower middle area of the scatter plot. These varieties all originate from lower latitudes, have low seed weight (<288 mg/100 seeds) and low FG content (<322 $\mu\text{g/g}$). Meanwhile, Northern Chilean varieties grouped together to the upper middle of the scatter plot. These varieties originate from higher latitudes, have high seed weight (>389 mg/100 seeds), and high FG content (>389 $\mu\text{g/g}$). This bifurcated grouping pattern corroborates previous genetic analyses which indicate that Central-Southern Chilean quinoa varieties group separately from Northern Chilean varieties.²⁷

Among commercial quinoa sources, Q3, Q4, Q6, Q8, and Q9 grouped together in the upper left quadrant, while Q1, Q2, Q5, and Q7 grouped with the Northern Chilean varieties in the upper right quadrant. The quinoa sources visualized to the upper left of the scatter plot share low PE content (<314 $\mu\text{g/g}$) and low oil content (29.3 – 39.3 mg/g), while those to the upper right share high PE content (>383 $\mu\text{g/g}$) and high oil content (29.3 – 45.4 mg/g). Based on the variables analyzed in our study, our data indicates that most commercial quinoa sources are highly related to Northern Chilean varieties, and likely share common ancestors.

Localization of phytoecdysteroids in quinoa seeds

Previous research has suggested that 20HE may be concentrated in the oil-rich embryos of quinoa seeds. The embryos, representing 30% of total seed weight, contained 50 – 60% of total 20HE, and the concentration of total PE in the embryos was 2 – 3 times higher than in the whole seeds.⁸ In our study, we confirmed that PE are concentrated in quinoa embryos by dissecting and chemically analyzing the three major seed parts (embryo+endosperm, seed coat, perisperm). We found that the seed embryo+endosperm contained the highest concentration of 20HE (618.3 ± 5.1 $\mu\text{g/g}$) compared with the seed coat (284.1 ± 9.6 $\mu\text{g/g}$) and perisperm (85.6 ± 11.7 $\mu\text{g/g}$) (Supp. Table 5).

These data, taken together with the results of our correlation and PCA analyses, indicate that PE content may be improved in quinoa through mechanisms aimed at increasing oil content. Possible strategies include enlargement of relative embryo size or upregulation of oil biosynthesis via breeding or genetic manipulation^{52, 53} and agricultural techniques such as enhanced fertilization.⁵⁴ Since vegetable oil is of high demand⁵² and quinoa is a rich source of health-beneficial unsaturated fatty acids,^{2, 28} the improvement of oil content in quinoa has vast implications for agriculture and human health. Though oil content has been successfully

improved in several cereal crops through conventional breeding techniques,⁵³ to our knowledge, no work has yet been performed to this effect in quinoa.

Implications for Crop Improvement and Development

In response to the rising consumer demand for foods with functional value, agricultural and industrial programs have begun to target improved phytoactive content in crops through genetic and environmental means.^{55, 56} Quinoa has recently received worldwide attention for its potential to promote human health,¹ while PE and FG are under rigorous investigation for their anti-diabetic, weight reducing, cardioprotective, and anticarcinogenic properties.^{10, 11, 22, 24} Quinoa is especially notable in phytoactive content due to the presence of PE, which are not found in any Poaceae cereal grains, and are only reported to be present in a few other edible crops such as spinach, though in lower concentrations.^{10, 15} Therefore, enhancement of PE and FG content in quinoa may represent a new frontier for crop improvement and functional food development.

This study is the first to determine PE and FG content among a diverse set of quinoa sources while exploring their relationship to other physicochemical seed parameters, including morphological and nutritional characteristics. We recommend assessment of phytochemical variation among additional quinoa genotypes, identification of possible correlations to saponin and betalain content, and investigation into mechanisms to improve quinoa oil content. Findings from our work and future studies can be useful for plant breeders, commercial producers, industrial processors, and local communities to promote the pharmacological value of quinoa varieties and products. Furthermore, the knowledge or enhancement of phytoactive content among quinoa varieties may promote the sustainability and livelihoods of quinoa-producing communities in South America via increased nutritional value, market price, and exportation potential of locally grown food.^{1, 57, 58}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Representative structures of quinoa-derived phytoecdysteroid (PE) and flavonoid glycoside (FG), and a schematic diagram of a quinoa seed in which these phytochemicals are found

A: 20-Hydroxyecdysone (20HE), the most abundant phytoecdysteroid (PE) in quinoa. **B:** Quercetin 3-O-(2,6-di-α-L-rhamno-pyranosyl)-β-D-galactopyranoside, one of the most abundant flavonoid glycosides (FG) previously identified in quinoa. **C:** Longitudinal section of a quinoa seed, adapted from Prego *et al.* (1998) by permission of Oxford University Press on behalf of The Annals of Botany company.³⁴ Quinoa seeds are covered in a saponin-rich pericarp that is removed by mechanical processing (desaponification) prior to commercial distribution and consumption, as depicted in Supp. Fig. 1. Desaponified seeds are covered by a 2-cell layered seed coat containing starch grains. The embryo is large and circular. The endosperm, a 1–2 cell layer comprised of protein and lipids, envelopes the hypocotyl-radicle axis of the embryo. Together, the endosperm and embryo contain the protein and lipid reserves of the seed. The central perisperm is primarily composed of starch grains.^{34, 35}

Fig. 2. Description of local Chilean quinoa sources (C1 – C8) and global commercial sources (Q1 – Q9) employed in the study

A: Map of locations from which each source originated or was cultivated. **B:** Photographs of each quinoa source. Codes (C1 – C8, Q1 – Q9) correspond with those in Table 1, Table 2, and Fig. 3.

Fig. 3. Variation in (A) phytoecdysteroid (PE) and (B) flavonoid glycoside (FG) content among local Chilean and commercial sources of quinoa seeds

Codes for each quinoa source (C1 – C8, Q1 – Q9) correspond with those in Table 1, Table 2, and Fig. 2. PE and FG were leached from quinoa seeds in 70% ethanol for 4h at 80 °C. 20-Hydroxyecdysone (20HE) content was quantified via HPLC alongside an external standard of 20HE and reported as µg/g seed. Minor phytoecdysteroids (Minor PE) were quantified as 20HE equivalents. FG were quantified as quercetin 3-*O*-(2,6-di- α -L-rhamnosyl)- β -D-galactopyranoside equivalents, and reported as µg/g seed. Data are the mean $\pm$ SD ($n=3$). Significant difference between groups is signified by letters a – e; different letters indicate significant difference ($P < 0.05$) between groups, while the same letter indicates no difference, as determined by Tukey's multiple comparison 1-way ANOVA test. FG content was significantly higher in Northern Chilean varieties compared with Central-Southern varieties (** $P < 0.01$; 2-tailed, unpaired t-test).

Fig. 4. Principle Component Analysis (PCA) of 8 phytochemical and physicochemical parameters investigated among 17 distinct quinoa sources, including 4 Northern Chilean varieties, 4 Central-Southern varieties, and 9 commercial sources

A: Scores plot showing relationship between quinoa sources. **B:** Loading plot showing relationship between investigated parameters, where FG = flavonoid glycoside content, 20HE = 20-hydroxyecdysone content, oil = oil content. PE = total phytoecdysteroid content, and protein = protein content.

Table 1

List of local Chilean quinoa varieties and global commercial sources investigated for their phytochemical content, along with the biogeographic region from which they originated.

Code ^a	Quinoa source	Biogeographic region of origin	GPS coordinates
<i>Chilean varieties^b</i>			
C1	Ancovinto Blanco	Northern Chile (Region I) ^c	−19.39, −68.58
C2	Ancovinto Roja	Northern Chile (Region I) ^c	−19.39, −68.58
C3	Cancosa	Northern Chile (Region I) ^c	−19.85, −68.60
C4	Socaire	Northern Chile (Region II) ^c	−23.59, −67.89
C5	Cáhuil	Central Chile (Region VI) ^c	−34.65, −71.90
C6	Faro	Central Chile (Region VI / VII) ^c	−36.36, −72.06
C7	Regalona	Southern Chile (Region IX) ^c	−38.39, −72.28
C8	Villarrica	Southern Chile (Region IX) ^c	−39.30, −72.23
<i>Commercial sources</i>			
Q1	AlterEco Pearl	Bolivia (ANAPQUI)	−18.58, −66.55 ^d
Q2	AlterEco Red	Bolivia (ANAPQUI)	−18.58, −66.55 ^d
Q3	Trader Joe's	Bolivia (salt flats)	−18.58, −66.55 ^d
Q4	Eden Red	Bolivia (Andean plateau)	−18.58, −66.55 ^d
Q5	Wegman's Red	Bolivia/Peru	−18.58, −66.55/−15.82, −70.23 ^d
Q6	Kalustyan's Black	Peru	−15.82, −70.23 ^d
Q7	Mas Corona	Ecuador (Ambato)	−1.43, −78.65 ^d
Q8	Gramolino	Ecuador	−1.43, −78.65 ^d
Q9	Arrowhead Mills	USA (Colorado)	37.57, −105.83 ^d

^a Code for each quinoa source corresponds with those in Table 2, Fig. 2, and Fig. 3.

^b Chilean varieties were all grown under similar environmental conditions in 2010 and 2011 in the same region of Chile (Chiu Chiu, Calama, Region II, Northern Chile; GPS coordinates: −22.34, −68.65).

^c Chile is comprised of 12 regions. Among those relevant to our study: Region I = Tarapacá, Región II = Antofagasta, Region VI = O'Higgins, Region VII = Maule, Region IX = Araucanía.

^d GPS coordinates for commercial sources were estimated based on the limited information available from suppliers as to the sites of quinoa cultivation.

Table 2
Physicochemical characteristics of local Chilean and global commercial sources of quinoa seeds, including seed color, 100-seed weight, oil content, and protein content

Data are the mean $\pm$ SD (n=3).

Code ^e	Seed color (1 – 3) ^f	100-seed weight (mg/100 seeds)	Protein content (mg/g)	Oil content (mg/g)
<i>Chilean varieties</i>				
C1	1	439 $\pm$ 6 a	102.0 $\pm$ 6.2 defg	35.6 $\pm$ 0.9 bc
C2	1	393 $\pm$ 3 c	134.3 $\pm$ 12.2 ab	56.2 $\pm$ 4.4 ab
C3	1	397 $\pm$ 5 bc	117.0 $\pm$ 6.0 bcd	33.9 $\pm$ 2.2 bc
C4	1	389 $\pm$ 20 c	130.0 $\pm$ 9.0 bc	36.7 $\pm$ 1.7 bc
C5	1	248 $\pm$ 4 d	89.7 $\pm$ 7.5 efg	45.5 $\pm$ 13.3 ab
C6	1	280 $\pm$ 5 d	79.3 $\pm$ 5.1 fgh	34.7 $\pm$ 4.9 bc
C7	1	288 $\pm$ 6 d	128.7 $\pm$ 5.1 bc	41.5 $\pm$ 2.3 bc
C8	1	244 $\pm$ 14 d	106.3 $\pm$ 10.2 cdef	47.8 $\pm$ 8.8 ab
<i>Commercial sources</i>				
Q1	1	441 $\pm$ 15 a	107.7 $\pm$ 3.1 cde	42.2 $\pm$ 4.1 bc
Q2	2	415 $\pm$ 11 abc	111.7 $\pm$ 8.5 bcde	43.4 $\pm$ 2.2 bc
Q3	1	385 $\pm$ 4 c	106.7 $\pm$ 3.5 cde	36.6 $\pm$ 1.3 bc
Q4	2	433 $\pm$ 8 ab	127.3 $\pm$ 9.5 bc	39.3 $\pm$ 6.7 bc
Q5	2	432 $\pm$ 31 ab	157.3 $\pm$ 11.7 a	39.3 $\pm$ 4.2 bc
Q6	3	202 $\pm$ 7 e	74.7 $\pm$ 5.5 h	35.0 $\pm$ 2.4 bc
Q7	1	412 $\pm$ 1 abc	100.7 $\pm$ 3.1 defg	45.4 $\pm$ 2.2 ab
Q8	1	278 $\pm$ 7 d	77.7 $\pm$ 5.5 gh	29.3 $\pm$ 2.6 c
Q9	1	302 $\pm$ 12 d	110.3 $\pm$ 13.6 bcde	37.1 $\pm$ 0.7 bc

Significant difference between groups is signified by letters a – h; different letters indicate significant difference ($P < 0.05$) between groups, while the same letter indicates no difference, as determined by Tukey's multiple comparison 1-way ANOVA test.

^e Code for each quinoa source corresponds with those in Table 1, Fig. 2, and Fig. 3.

^f Seed color intensity was rated on a scale of 1 – 3, where 1 = white - lightly colored, 2 = red, 3 = black.

Table 3

Pearson correlation coefficients between seed color, 100-seed weight, protein content, oil content, 20HE content, total PE content, and FG content among local Chilean and global commercial sources of quinoa.

	Latitude of origin	Seed color	100-seed weight	Protein content	Oil content	20HE content	Total PE content
<i>Chilean varieties</i>							
100-seed weight	-0.953***						
Protein content	-0.417		0.460				
Oil content	0.396		-0.525	0.158			
20HE content	0.101		-0.077	0.220	0.661 [†]		
Total PE content	0.352		-0.329	0.155	0.761*	0.960***	
FG content	-0.883**		0.834*	0.490	-0.174	0.172	-0.066
<i>Commercial sources</i>							
Seed color	-0.246						
100-seed weight	0.280	-0.301					
Protein content	0.513	0.032	0.731*				
Oil content	0.164	-0.042	0.717*	0.429			
20HE content	-0.059	0.335	0.577	0.459	0.709*		
Total PE content	-0.099	0.344	0.576	0.436	0.712*	0.998****	
FG content	0.247	-0.250	-0.003	-0.123	0.363	0.283	0.269
<i>All quinoa sources</i>							
Seed color	-0.422						
100-seed weight	-0.267	-0.097					
Protein content	0.187	-0.009	0.597*				
Oil content	0.303	-0.102	0.078	0.306			
20HE content	0.225	0.031	0.228	0.373	0.664**		
Total PE content	0.276	0.023	0.131	0.335	0.707**	0.988****	
FG content	-0.346	-0.043	0.464 [†]	0.177	0.004	0.125	0.017

[†] $P < 0.1$,

P<0.00000001

 $P<0.001$,

$$P < 0.01,$$
$$*P<0.05,$$

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