**Response surface optimization of germination conditions to improve the accumulation of
bioactive compounds and the antioxidant activity in quinoa**

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Running title: Bioactive compounds in quinoa sprouts

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Abstract:

Germination has been proposed as an economic approach to improve the content of bioactive compounds in pseudocereals. In this work, response surface methodology (RSM) was employed to investigate the impact of germination conditions on the phytochemical content and antioxidant activity of quinoa. The use of desirability methodology showed that the optimum conditions to maximize the content of total phenolic content (TPC) and antioxidant activity in sprouted quinoa were 20 °C for 42 h. Sprouts produced under these conditions exhibited increases of 80% and 30% in TPC and antioxidant activity, respectively, compared to un-germinated seeds, and contained high γ -aminobutyric acid (GABA) concentration. The non-significant lack-of-fit and high determination coefficients obtained confirmed the suitability of the predictive models developed for TPC and antioxidant activity, whilst the one obtained for GABA was not significant ($R^2 < 0.75$) within the conditions studied. Sprouting under optimum conditions enhanced the content of both flavonoid and non-flavonoid compounds, being the increase of flavonoids more pronounced. Kaempferol-*O*-dirhamnosyl-galactopyranose and quercetin-*O*-glucuronide were the compounds that experienced the most noticeable increase in quinoa after germination. In conclusion, this study provides useful information on the optimum germination conditions to improve the levels of health-promoting compounds in quinoa.

KEYWORDS: Quinoa, germination, phenolic compounds, antioxidant activity, GABA, Response Surface Methodology

1. Introduction

A large number of epidemiological data have established a link between some chronic diseases (obesity, type 2 diabetes, cardiovascular diseases) and nutritional habits (WHO, 2003). Therefore, during the last decades a lot of research efforts have been invested in the development of more nutritive and healthier foods.

Quinoa (*Chenopodium quinoa* Willd.), a pseudocereal indigenous to the Andean region, has been singled out by FAO as a healthy food with an excellent nutritional value (FAO & CIRAD, 2015). Quinoa seed has a carbohydrate content comparable to that of barley and rice (62.3-74.0 %) and a remarkable high quality protein concentration (16.5-23.9%), attributed to its balanced amino acid composition, similar to milk and close to the ideal balance recommended by FAO (Ferreira *et al.*, 2015). This pseudocereal is also a good source of beneficial unsaturated fatty acids and provides adequate amounts of minerals and vitamins (Tang *et al.*, 2015). Quinoa provides desirable health benefits beyond its excellent nutritional characteristics, since it is a good source of phenolic compounds such as phenolic acids and flavonoids (Abderrahim *et al.*, 2015; Repo-Carrasco-Valencia *et al.*, 2010). These compounds have recognized antioxidant and anti-inflammatory activities (Cicero & Colletti, 2015), and hence, quinoa consumption may contribute to reduce the incidence of chronic diseases.

Quinoa has been traditionally consumed as cooked seeds or seed flour in bakery products, but also as extrudates. Technological innovation in quinoa processing can be a valuable approach to promote quinoa consumption and to diversify the quinoa market worldwide. Germination of quinoa can achieve these purposes since sprouts are considered novel and healthy foods highly appreciated by vegetarians and people interested in improving their health status by changing dietary habits (Vega-Gálvez *et al.*, 2010). Quinoa sprouts are also suitable for gluten-free diets since they lack prolamins, the toxic proteins for coeliac people (Peñas *et al.*, 2014). Germination has been proposed as an economic and feasible approach to enhance the nutritive value and phytochemical content of cereals and

pseudocereals. This process causes an increase of protein and starch digestibility, improves the total phenolic content and the bioavailability of some minerals and decreases some non-nutritive factors in quinoa and other pseudocereals (Omary, Fong, Rothschild, & Finney, 2012; Gan *et al.*, 2017). Several studies have shown that germination of cereals and pseudocereals also enhanced the content of γ -aminobutyric acid (GABA) (Cáceres *et al.*, 2014; Gan *et al.*, 2017; Paucar-Menacho *et al.*, 2017), a compound with desirable health benefits since it is involved in blood pressure regulation (Diana *et al.*, 2014). Germination of quinoa could represent an attractive strategy to improve the nutritive value and health promoting properties of quinoa. However, the accumulation of nutrients and bioactive compounds in sprouts varies greatly depending on germination conditions. Therefore, it is mandatory to optimize germination conditions to maximize the phytochemical content and functional properties of this pseudocereal. So far, the influence of germination conditions on quinoa phytochemicals has not been fully explored. Therefore, the aim of this study was to optimize germination conditions (time and temperature) by Response Surface Methodology (RMS) to maximize the content of GABA and total phenolic compounds as well as the antioxidant activity of quinoa.

2. Materials and Methods

2.1. Plant material.

Quinoa (*Chenopodium quinoa* Willd var. INIA-415 Pasankalla) seeds were provided by Cereals and Native Grains Program of National Institute of Agricultural Innovation (INIA) of Peru. This cultivar was selected due to its high protein content. Seeds were cleaned and stored in polyethylene containers in darkness at 4-8 °C.

2.2. Germination procedure.

Quinoa seeds (30 g) were soaked in 0.1% sodium hypochlorite solution (1:5, w/v) for 30 min at room temperature. Then, sodium hypochlorite was drained and quinoa seeds were

washed with distilled water until they reached neutral pH. Afterwards, quinoa seeds were steeped in distilled water (1:5, w/v) for an additional 6 h and shaken every 30 min. Distilled water was drained and finally hydrated seeds were placed in trays on a wet filter paper. Germination was carried out in a germination chamber (G-120 model, ASL Snijders International S. L., The Netherlands), with a water circulating system to keep 90% air humidity. Germination was performed in darkness at different temperatures (12-28 °C) and times (12-72 h) according to the experimental response surface model (Table 1). Every germination experiment was performed in triplicate. Quinoa seeds and sprouts were freeze-dried, milled by using a ball mill (Glen Creston Ltd, Stanmore, UK), packed in plastic bags under vacuum and stored at -20 °C until further analysis.

2.3. Determination of GABA content

The extraction of GABA in quinoa grains and derivatization of the resultant extracts was carried out as previously described (Cáceres *et al.*, 2014). GABA quantification was performed by Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) in an Alliance Separation Module 2695 (Waters, Milford, USA) equipped with a photodiode array detector 2996 (Waters) and Empower chromatographic software (Waters), as reported by Cáceres *et al.*, (2014). Determinations were performed in duplicate and the results were expressed as mg GABA/100 g dry weight (dw).

2.4. Determination of total phenolic content (TPC)

The TPC concentration was determined using the Folin-Ciocalteu's phenol reagent (Peñas *et al.*, 2015). Briefly, 0.5 g of sample were extracted in 10 mL of methanol-HCl (1⁰/₀₀₀)–water (80:20 v/v) by continuous stirring at 4 °C for 16 h. Two independent extractions were performed for each replicate. Extracts were centrifuged at 2,990 rcf at 5 °C for 5 min. An aliquot of 100 µL of diluted extract was mixed with 625 µL distilled water, 250 µL 7.5% (w/v) sodium carbonate and 25 µL of 2 N Folin-Ciocalteu's phenol reagent. Reaction mixtures were vortexed and incubated in darkness at room temperature for 2 h. The

absorbance was measured at 739 nm in triplicate using a microplate reader (Synergy HT, BioTek Instruments, VT, USA) controlled by the Gene 5TM software version 1.1. (BioTek Instruments). A gallic acid standard curve with a linear range (0-225 µg gallic acid/mL) was prepared from a freshly made 1 mg/mL gallic acid stock solution. Results were expressed as mg of gallic acid equivalents (GAE)/100 g dw.

2.5. HPLC-DAD-ESI/MSⁿ analysis of phenolic compounds

Raw and germinated quinoa samples (5 g) were macerated in methanol:TFA:water (80:0.1:20, v/v/v) at 4 °C for 16 h. Subsequently, they were centrifuged at 4,000 g and 5 °C for 20 min in a super-speed centrifuge (SorvallTM RC 5B, USA). The extraction process was repeated twice. The extracts were combined and concentrated at 30 °C under vacuum for methanol evaporation. For phenolic analysis, the extracts previously obtained were adjusted to a volume of 10 mL with water. For purification, an aliquot (4 mL) was passed through a C18 Sep-Pak cartridge (Waters, Milford, MA, USA), previously activated with methanol followed by water, according to supplier instructions.

Samples were analyzed using Hewlett–Packard 1100MS (Agilent Technologies, Palo Alto, CA) chromatograph equipped with a quaternary pump and diode array detector (DAD) coupled to an HP Chem Station (rev.A.0504) data-processing station. A Waters Spherisorb S3 ODS-2 C8, 3 µm (4.6 mm×150 mm) column at 35°C was used. Solvents were (A) 0.1% formic acid in water, and (B) 100% HPLC grade acetonitrile. The elution gradient established was isocratic 15% for 5 min, 15-20% B for 5 min, 20-25% B for 10 min, 25-35% B for 10 min, 35-50% B for 10 min, and re-equilibration of the column. The flow-rate was 0.5 mL min⁻¹. Double online detection was carried out in the DAD using 280 nm and 370 nm as preferred wavelengths. For identification, a mass spectrometer (MS) connected to a HPLC system via the DAD cell outlet was used and detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source, triple quadrupole-ion trap mass analyzer and controlled by the Analyst 5.1 software, following the method

described by Dueñas *et al.*, (2015). The settings were: zero grade air as the nebulizer gas (30 psi), turbo gas for solvent drying (400°C, 40 psi), nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupols were set at unit resolution. The ion spray voltage was set at -4500V in the negative mode. The MS detector was programmed to perform a series of two consecutive modes: enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to show full scan spectra, to give an overview of all the ions in sample. Settings used were: declustering potential (DP) -45 V, entrance potential (EP) -6V, collision energy (CE) -10V. Spectra were recorded in negative ion mode between m/z 100 and 1000. EPI mode was further performed with the aim to obtain the fragmentation pattern of the parent ion(s) of the previous experiment using the following parameters: DP: -50V, EP: -6V, CE: -25V, and collision energy spread (CES) 0V.

The phenolic compounds were characterized according to their UV-VIS, mass spectra, retention times, and comparison with authentic standards when available. For quantitative analysis, calibration curves were prepared by injection of known concentrations of different standard compounds. The standards, vanillic, *trans-p*-coumaric acid, quercetin 3-*O*-rutinoside, quercetin 3-*O*-glucoside and kaempferol 3-*O*-rutinoside were obtained from Extrasynthese (France). Hexosides derivatives of vanillic, ferulic, *p*-coumaric and sinapic acids were quantified using standard curves of the corresponding free acids; derivatives of quercetin and kaempferol were quantified using standard curves of kaempferol 3-*O*-glucoside and quercetin 3-*O*-glucoside. Results were expressed as $\mu\text{g/g}$ o dw.

2.6. Determination of antioxidant activity

Antioxidant activity was determined in the methanolic extracts previously obtained for TPC evaluation, by using oxygen radical absorbance capacity (ORAC-FL) method, as previously described (Cáceres *et al.*, 2014). ORAC of non-germinated and sprouted quinoa extracts were determined in triplicate. Fluorescence was measured in a microplate reader (Synergy HT, Biotek, VT, USA) at 528 and 485 nm for emission and excitation wavelengths,

respectively. A standard curve prepared by using different concentrations of Trolox (4-160 μ M) was used. Results were expressed as mg Trolox equivalents (TE)/100 g dw.

2.7. Experimental design

RSM was applied for determining the optimal germination conditions to maximize the phytochemical content and antioxidant activity of quinoa sprouts. Germination was optimized using a 2^2 central composite rotational design with two independent variables: germination time (X_1) and germination temperature (X_2) at three different levels, indicated as -1, 0 and +1, and two axial points ($-\alpha$ and $+\alpha$), as it is shown in Table 1. The values between parentheses correspond to the real level values for independent variables, whilst the values outside parentheses indicate the coded levels of these variables. The content of GABA, total phenolic compounds and antioxidant activity were considered as the response variables. The experimental design consisted of 11 trials performed in duplicate with three replications of the central point (Table 1). Experimental data were fitted to the following second order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^2 \beta_i X_i + \sum_{i=1}^2 \beta_{ii} X_i^2 + \sum_{i=1}^2 \sum_{j=1}^2 \beta_{ij} X_i X_j$$

where Y is the predicted response, β_0 , β_i , β_{ii} , β_{ij} represent the model coefficients for intercept, linear, quadratic and interaction terms, respectively, and X_i and X_j the independent variables.

2.8. Statistical analysis

The adequacy of the models was evaluated by calculating the multiple determination coefficient (R^2), the adjusted determination coefficient (R^2 -adj), the lack of fit and the F- and p-values obtained from the analysis of variance (ANOVA) by using Statistica 5.0 software (Statsoft, USA). The validation of the obtained models was performed by direct comparison of predicted and experimental values obtained for the different responses in the optimal conditions. The optimal conditions were determined by applying the Derringer's desirability

function, that identifies a combination of the variable levels that maximize a set of dependent variables, using Statistica 5.0 software (Statsoft, USA).

3. Results and Discussion

3.1. Impact of germination conditions on GABA content of quinoa sprouts

Quinoa grains showed a GABA concentration of 22.41 ± 2.64 mg/100 g dw. This content increased markedly during germination up to levels ranging from 17.97 to 122.32 mg/100 g dw, depending on sprouting conditions (Table 1).

The coefficient of determination determined for the model was $R^2=0.62$. This coefficient illustrates the quality-of-fit of the predictive model and generally only values higher than 0.75 indicate that the estimated model fits the experimental data with good prediction (Peñas *et al.*, 2008). The low value obtained for R^2 indicated that the quadratic polynomial equation obtained is not suitable, in the ranges of time and temperature studied, to predict the variation in the experimental data. Nevertheless, according to the experimental data obtained (Table 1), the conditions that maximize the GABA content in quinoa sprouts were 63 h at 26°C, which caused an increase of more than 5-fold in comparison to quinoa seeds. To our knowledge, there is no information on the influence of germination conditions on GABA concentration in quinoa sprouts. However, we have previously found that the highest GABA content in other pseudocereals such as kiwicha (*Amaranthus caudatus*), is also obtained at 26 °C for 63 h (Paucar-Menacho *et al.*, 2017). GABA is primarily synthesized from glutamic acid by glutamate decarboxylase (GAD), but it can be also formed from polyamines via γ -aminobutyraldehyde intermediate, being diamine oxidase (DAO) and aminoaldehyde dehydrogenase (AMADH) the enzymes involved in the process. Several studies have demonstrated that the activity of GAD and DAO is significantly increased during germination in cereals and legumes (Xu & Hu, 2014; Xu *et al.*, 2010; Yang *et al.*, 2011; Gan *et al.*, 2017), phenomenon that would explain the accumulation of GABA in quinoa sprouts. In fact, it has been reported that the suitable temperature for improving GAD activity and

enhances GABA content is 30 °C (Guo *et al.*, 2011; Xu & Hu, 2014), findings that support the results of the present study.

3.2. Impact of germination conditions on TPC content of quinoa sprouts

Quinoa seeds exhibited a TPC concentration of 270.99 mg GAE/100 g dw. This content was positively affected by germination. Quinoa sprouts showed TPC levels from 184.40 mg GAE/100 g dw to 499.24 mg GAE/100 g dw (Table 1), depending on the germination conditions. The quadratic term of time was the most dominant factor that influences the phenolic content in germinated quinoa, but the linear term of time and temperature and the quadratic term of the latter factor also showed significant effects (Table 2). The interactive effect between germination time and temperature, however, was not found significant ($P>0.05$). The non-significant factors were removed from the mathematical model, obtaining the following reduced regression model:

$$\text{TPC} = 489.78 + 61.78 X_1 - 94.57 X_1^2 + 59.45 X_2 - 75.29 X_2^2$$

The values of the coefficient of determination ($R^2=0.996$) and adjusted coefficient of determination ($R^2\text{-adj}=0.961$) indicated that the mathematical model adequately fits the experimental data, since only 0.4% of the variability in the responses could not be explained by the model. Furthermore, the model F-value (41.85) and the associated p-value (0.0001) implied that the model was very significant. The low value of the CV (2.84%) evidenced a high degree of correlation between the experimental and predicted data.

The response surface plot of the model was generated to better illustrate the impact of germination time and temperature on the TPC concentration of quinoa sprouts (Figure 1 A). As it can be observed from the figure, TPC levels increased as temperature and time increased up to 20-22 °C and 42-44 h, respectively, decreasing afterwards. The highest phenolic concentration in quinoa sprouts was obtained at 20 °C and 42 h of germination, conditions that increased almost 2-fold the TPC compared to quinoa seeds. These findings are supported by those previously reported by other authors, who also observed 2-fold increase in phenolic

compounds of quinoa sprouts obtained after 72-82 h of germination at 10-20 °C (Alvarez-Jubete *et al.*, 2010; Carciochi *et al.*, 2014). The enhancement observed in phenolic levels during quinoa germination can be attributed to their *novo* synthesis during this process (Kim *et al.*, 2016). However, quinoa seeds exhibit a high content of insoluble phenolics that are covalently found to cell wall structural components and rod-shaped structural proteins (Tang *et al.*, 2016). It has been reported that bound phenolics are released during early stages of germination due to the degradation of proteins and carbohydrates of cell wall, but, with the germination time increase, new soluble phenolic compounds are synthesized and secreted to cell wall to form new bound phenolics (Gan *et al.*, 2017). Therefore, the content of bound phenolics is dependent on their release and conjugation rate, and the conversion of some bound phenolic compounds into extractable free phenolic forms could be contributing to the increase of phenolic levels observed in quinoa sprouts in the present study.

3.3. Impact of germination conditions on antioxidant activity of quinoa sprouts

Quinoa seeds exhibited an ORAC value of 1085.75 mg TE/100 g dw, concentration that was modified during germination. Sprouts exhibited ORAC values in the range of 315.8 to 1410.42 mg TE/100 g dw, depending on germination time and temperature used for their production (Table 1). Analysis of the RSM model clearly indicated that antioxidant activity of sprouted quinoa was highly dependent on germination time, which showed both linear and quadratic effects, whilst temperature only showed a linear impact. The interaction between time and temperature had also a significant negative influence on antioxidant activity (Table 2). The non-significant terms were omitted for the model, obtaining the following polynomial equation:

$$\text{ORAC} = 1410.42 + 228.66 X_1 - 250.51 X_1^2 + 109.99 X_2 - 142.85 X_1.X_2$$

The goodness of the reduced model was evaluated by calculating the coefficient of determination and the adjusted coefficient of determination. The high values of these coefficients ($R^2=0.85$ and $R^2\text{-adj}=0.77$) illustrated the validity of the model. The non-

significant value of the lack-of-fit, along with the F-value (37.35) and the low p-value (0.0005), confirmed the suitability of the model (Table 2).

The predicted response surface plot obtained (Figure 1 B) indicates that the better germination conditions were at 20°C for 42 h, that caused an increase of 130% in antioxidant activity of sprouted quinoa in comparison to quinoa seeds. The enhancement of antioxidant activity of quinoa during germination has been reported in previous studies, where increases of 2-fold were noticed after germination at 20 °C for 48-72 h (Carciochi *et al.*, 2014, 2016). Similarly, Pasko *et al.* (2009) found that antioxidant activity measured by DPPH radical scavenging assay was maximal after germination of quinoa at 20 °C for 144 h. Moreover, an increase of antioxidant activity with higher germination temperature and time has been also observed in other pseudocereals such as amaranth (Paucar-Menacho *et al.*, 2017; Perales-Sánchez *et al.*, 2014). The increase of antioxidant activity in sprouted quinoa can be attributed to the accumulation of phenolic compounds and to the release of bound phenolics during germination, as it has been reported for cereals and legumes (Tang *et al.*, 2014). In fact, a positive correlation was observed between TPC and ORAC values ($r=0.76$) in quinoa sprouts, suggesting the strong contribution of phenolics compounds to the antioxidant activity of sprouted quinoa. However, the influence of other antioxidant compounds, such as vitamin C, which has been found to increase during germination (Gan *et al.*, 2016), cannot be ruled out.

3.4. Determination of optimal germination conditions and verification of the predictive RSM models.

The optimal germination conditions for enhancing TPC concentration and antioxidant activity in quinoa sprouts were determined by maximizing the desirability of both responses. These conditions were found to be temperature of 20 °C and time of 42 h. The overall desirability value at these conditions was 0.89. The accuracy and suitability of model quadratic equations developed were further validated by comparison of the experimental and predicted values under the optimal conditions (Table 3). As it can be observed from the table,

the theoretical responses at optimal conditions were in good agreement with the experimental ones, and no significant differences ($P \leq 0.05$) between them were found, indicating that the models developed adequately predict the levels of TPC and the antioxidant activity in sprouted quinoa. However, further validation of the model at pilot plant scale is necessary to translate the results from this model system.

3.5. Identification of phenolic compounds in raw and sprouted quinoa.

The identification and quantification of individual phenolic compounds in raw and quinoa germinated in optimal conditions (at 20 °C for 42 h) were carried out by HPLC-DAD-ESI/MS analysis. The retention time, $\lambda_{\max}$ in the UV-visible region, pseudomolecular ions and main fragments ions observed are summarized in Table 4. This information was also compared with data reported for different quinoa varieties (Gómez-Caravaca *et al.*, 2011). Twenty-two phenolic compounds were identified, belonging to hydroxybenzoic and hydroxycinnamic free and derivative forms, and flavonols (quercetin, kaempferol and isorhamnetin glycosides) phenolic classes.

Vanillic, *trans-p*-coumaric acids and quercetin 3-rutinoside, quercetin 3-glucoside and kaempferol 3-rutinoside (peaks 8, 13, 19, and 20) were tentatively identified according to their retention mass and UV-vis characteristics by comparison with commercial standards. Peaks 1 and 2 presented similar UV spectra to vanillic acid, suggesting that may derive from vanillic acid. These peaks showed a fragment ion at m/z 167, corresponding to vanillic acid they were tentatively identified as vanillic hexoside (peak 1) and vanillic acid derivative (peak 2).

Other compounds were found only in quinoa sprouts and identified as hydroxycinnamic hexosides (peak 3-6), based on their UV-vis spectra with maximum at 316, 324, 328 and 318 nm, precursor ions $[M-H]^-$ (at m/z 325, 355 and 385, respectively) and fragment ions at m/z 163, 193 and 223, corresponding to the loss of an hexose unit.

Quercetin derivatives (peaks 7, 9, 11, 14, and 18) presented similar UV spectra to quercetin glycoside. Quercetin dirhamnopyranosyl-galactopyranoside (peak 7) was found in seeds and sprouts, which showed a UV-vis spectra with a maximum wavelength of absorption at 354 nm, a precursor ion $[M-H]^-$ at m/z 755 and two fragments at m/z 609 due to the loss of an rhamnose unit and m/z 301 corresponding to quercetin. Peak 14 was identified as quercetin apiofuranosyl galactopyranoside according to the UV-vis spectra and precursor ion $[M-H]^-$ at m/z 595, with a fragment ions at m/z 463 and 301, corresponding to the loss of a pentose unit and pentosyl + hexose residues, respectively. These two flavonols (peaks 7 and 14) have been also identified in different quinoa varieties by Gómez-Caravaca *et al.* (2011, 2012).

Quercetin rhamnosyl glucuronide (peak 9), quercetin rhamnosyl glucoside (peak 11), quercetin glucuronide (peak 18) and quercetin 3-glucoside were identified according to $[M-H]^-$ at m/z 623, 609, 477 and 477, respectively. Fragment ion at m/z 301 corresponded to quercetin.

Peak 10 (kaempferol apiofuranosyl rhamnopyranosyl-galactoside) and peak 12 (kaempferol dirhamnosyl-galactopyranose) were identified according to mass and UV-vis characteristics by comparison with those proposed by Gómez-Caravaca (2011, 2012).

Kaempferol derivatives (peaks 17 and 22) showed precursor ions $[M-H]^-$ at m/z 607 and 447, and unique fragment ions at m/z 285 (corresponding to isorhamnetin) due to the loss of pentosyl + glucuronyl residues and glucuronide unit, respectively, which allowed their identification as kaempferol rhamnosyl glucuronide (peak 17) and kaempferol glucuronide (peak 22).

Isorhamnetin rhamnosyl hexoside (peak 21) was observed according to its UV-vis and mass spectra with fragment ion at m/z 315.

Hydroxybenzoic and hydroxycinnamic free forms and derivatives have been previously described in different quinoa varieties by other authors (Alvarez-Jubete *et al.*, 2010; Carciochi *et al.*, 2014; Gómez-Caravaca *et al.*, 2011, 2012; Repo-Carrasco-Valencia *et al.*, 2010). These

authors identified vanillic glucoside and feruloyl glucoside as hydroxybenzoic and hydroxycinnamic acids, vanillic, *p*-coumaric and ferulic acids as bound acids and flavonols such as kaempferol and quercetin 3-*O*-apifuranosyl-galactopyranoside, kaempferol and quercetin 3-*O*-dirhamnosyl-galactopyranoside, quercetin glucuronide and others.

3.6. Impact of germination on phenolic profile of quinoa.

Results showed important differences in the composition of phenolic compounds between raw and sprouted quinoa (Table 5). The concentration of total phenolic compounds was positively affected by germination ($P<0.05$), causing an increase of 42% with respect to quinoa seeds. Non-flavonoid compounds (hydroxybenzoic and hydroxycinnamic acids) concentration in raw quinoa accounted for approximately 22% of the total phenolic compounds. Germination enhanced the concentration of non-flavonoid compounds by 32%, which may be due to the formation of *trans-p*-coumaroylhexoside acid, *trans*-feruloylhexoside acid isomers and sinapoylhexoside acid, which were not identified in quinoa seeds. Other authors also found an enhancement of phenolic acids during quinoa germination (Carciochi *et al.*, 2014, 2016; Repo-Carrasco-Valencia *et al.*, 2010), that can be attributed to the activation of the phenylpropanoid pathway involved in their *novo* synthesis. Quinoa sprouts, however, exhibited lower concentrations of some hydroxybenzoic acids (vanillic hexoside acid, vanillic derivative and vanillic acid) and a ferulic acid derivative (II) than seeds.

The most outstanding variations found as consequence of germination were those occurred in the flavonoid group. In general, total flavonol concentration increased by 44% in quinoa sprouts with the exception of quercetin rhamnosyl glucuronide, quercetin rutinoid and quercetin apiofuranosyl galactopyranoside, which experienced a reduction of approximately 26-38% in the sprouts. Interestingly, the most noticeable increase during germination was found in kaempferol dirhamnosyl-galactopyranose and quercetin glucuronide, which rose 1600% and 328%, respectively. Our results are in agreement with

those reported by Carciochi *et al.* (2014) and Alvarez-Jubete *et al.* (2010), who also reported moderate rises of flavonols, especially quercetin and kaempferol glycosides, during quinoa germination.

4. Conclusions

In this study, RSM was successfully used to determine the optimal germination conditions that maximize the content of bioactive compounds and the antioxidant activity of quinoa sprouts. The quadratic models developed for TPC and antioxidant activity showed non-significant lack-of-fit and satisfactory determinant coefficients, illustrating that they fit with good prediction to the experimental data. The optimum conditions for maximizing both responses were estimated to be temperature of 20 °C and time of 42 h. Quinoa sprouts obtained at these conditions exhibited also higher GABA content than seeds and were good sources of flavonoid compounds, mainly flavonols and phenolic acids. This work provides useful information on the application of germination under controlled conditions as a sustainable and economical process to improve the levels of health-promoting compounds in quinoa. The production of quinoa sprouts would fulfill the industry and consumer demands of fresh and healthy foods.

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515 **Figure Captions**

516 **Figure 1.** Response surface plots for TPC concentration (A) and antioxidant activity (B) as a
517 function of germination time and temperature in sprouted quinoa