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Title: Optimizing germination conditions to enhance the accumulation of bioactive compounds and the antioxidant activity of kiwicha (*Amaranthus caudatus*) using response surface methodology

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Abstract: Controlled grain germination is a method of emerging interest to naturally improve nutritional and nutraceutical profile of grains. The aim of this study was to optimize germination temperature and time using response surface methodology to maximize the concentration of γ -amminobutyric acid (GABA), phenolic compounds (PC) and the antioxidant activity of kiwicha (*Amaranthus caudatus*) grains. The optimal germination temperature and time were 26°C and 63h, respectively, in which GABA, PC and antioxidant activity increased 29.1, 4.0 and 6.4-fold, respectively. Individual phenolic composition of kiwicha sprouts obtained in the optimal conditions was analyzed by HPLC-DAD-ESI/MS. Sprouting in the optimal germination conditions enhances mainly the concentrations of 4-O-caffeoylquinic, 4-O-feruloylquinic acids and quercetin-3-O-rutinoside. Predicted models were significant ($P < 0.05$) for all the parameters studied with high regression coefficients ranging from 0.965 to 0.989. The predicted values from the developed quadratic polynomial equation were in close agreement with the experimental values. In conclusion, germination at 26 °C for 63 h offers a practical opportunity for enhancing the nutraceutical quality of kiwicha grains supporting their preferential use over non-germinated grains for improving health outcomes associated to lifestyle-associated diseases.

HIGHLIGHTS

- Germination increased phytochemicals and antioxidant activity of kiwicha
- Optimal conditions (26 °C and 63 h) were identified by response surface methodology
- Hydroxycinnamic quinic acid derivatives were the most abundant phenolics
- Prediction models showed good correlation with experimental data

Optimizing germination conditions to enhance the accumulation of bioactive compounds and the antioxidant activity of kiwicha (*Amaranthus caudatus*) using response surface methodology

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ABSTRACT

Controlled grain germination is a method of emerging interest to naturally improve nutritional and nutraceutical profile of grains. The aim of this study was to optimize germination temperature and time using response surface methodology to maximize the concentration of γ -aminobutyric acid (GABA), phenolic compounds (PC) and the antioxidant activity of kiwicha (*Amaranthus caudatus*) grains. The optimal germination temperature and time were 26°C and 63h, respectively, in which GABA, PC and antioxidant activity increased 29.1, 4.0 and 6.4-fold, respectively. Individual phenolic composition of kiwicha sprouts obtained in the optimal conditions was analyzed by HPLC-DAD-ESI/MS. Sprouting in the optimal germination conditions enhances mainly the concentrations of 4-O-caffeoylquinic, 4-O-feruloylquinic acids and quercetin-3-O-rutinoside. Predicted models were significant ($P < 0.05$) for all the parameters studied with high regression coefficients ranging from 0.965 to 0.989. The predicted values from the developed quadratic polynomial equation were in close agreement with the experimental values. In conclusion, germination at 26 °C for 63 h offers a practical opportunity for enhancing the nutraceutical quality of kiwicha grains supporting their preferential use over non-germinated grains for improving health outcomes associated to lifestyle-associated diseases.

KEYWORDS: *Amaranthus caudatus*, germination, γ -aminobutyric acid, antioxidant activity, phenolic compounds.

24 1. Introduction

25 Amaranth, a pseudocereal that belongs to *Amaranthaceae* family, has more than 80 species.
26 *Amaranthus caudatus*, *Amaranthus hypocondriacus* and *Amaranthus cruentus* are the three main species
27 that produce grains. *A. caudatus*, also known as kiwicha in the Andean region, is native of Peru.
28 Peruvian annual production of kiwicha was 2,732 Tn in 2012 (MINAG, 2016). Recently, interest has
29 increased considerably in pseudocereals for use in the formulation of high quality, healthy gluten-free
30 products such as bread and pasta (Alvarez-Jubete, Arendt, & Gallagher, 2010). Kiwicha is a good source
31 of starch (58–66%), proteins (13–19%) with an adequate balance of amino acids, fiber high in pectic
32 substances and xyloglucans (14–16%), lipids containing significant amounts of squalene (5–13%),
33 minerals (Ca, Fe, Mg, Mn, K, P, S and Na), and vitamins of B complex (Lamothe, Srichuwong, Reuhs,
34 & Hamaker, 2015; Nascimento et al., 2014). Besides its nutritional quality, kiwicha contains health-
35 promoting compounds including bioactive peptides, phenolic compounds and betacyanins, the latter
36 only found in low amounts in pink varieties (Montoya-Rodriguez, Gómez-Favela, Reyes-Moreno,
37 Milán-Carrillo, González de Mejía, 2015; Repo-Carrasco-Valencia, Hellström, Pihlava, & Mattila,
38 2010). Scientific data evidence the potential of phenolic compounds as chemopreventive agents against
39 chronic diseases associated to oxidative stress through an array of actions including anti-oxidant, anti-
40 inflammatory, anti-hypertensive and anti-atherosclerotic activities (Del Rio, Rodriguez-Mateos,
41 Spencer, Tognolini, Borges, & Crozier, 2013). Phenolic acids (16.8–59.7 mg/100g) are the most
42 abundant phenolic compounds found in kiwicha that may occur as free and bound forms. Soluble forms
43 of phenolics acids may vary from 7% to 61% of the total amount of phenolic compounds in kiwicha
44 (Repo-Carrasco-Valencia, Hellström, Pihlava, & Mattila, 2010).

45 There is an emerging interest in the application of controlled grain germination for improving
46 nutritional and phytochemical properties of grains (Nelson, Stojanovska, Vasiljevic, & Mathai, 2013).

47 During grain germination, extensive changes are reported in the composition of a variety of grains,
48 reflecting the dynamic and complex flux of nutrients and phytochemicals including remobilization,
49 degradation and accumulation. For this reason, increased, decreased or unchanged values could be found
50 for nutrients and bioactive compounds within the same type of grain or across different grains.
51 Therefore, optimization of germination conditions for each grain variety is of crucial relevance to
52 maximize the phytochemical composition and consequently the health-promoting potential of
53 germinated grains. Recent studies have observed that specific germination conditions increase the
54 content of proteins, total dietary fiber, free and bound phenolic compounds as well as the free radical
55 scavenging activity of *A. hyponcondriacus* and *A. cruentus* (Paško, Bartoń, Zagrodzki, Gorinstein, Fołta,
56 & Zachwieja, 2009; Perales-Sánchez, Reyes-Moreno, Gómez-Favela, Milán-Carrillo, Cuevas-
57 Rodríguez, Valdez-Ortiz, et al., 2014). Other studies have demonstrated that germinated cereal and
58 pseudocereal grains such as quinoa **have increased levels of γ -aminobutyric acid (GABA)** (Morita, Park,
59 & Maeda, 2013; Zhang, Xiang, Zhang, Zhu, Evers, van der Werf, et al., 2014), a non-protein amino acid
60 that may exert beneficial physiological effects *in vivo* such as regulation of blood pressure and heart
61 rate, alleviation of pain, anxiety and sleeplessness (Diana, Quílez, & Rafecas, 2014). From these studies
62 it can be concluded that germination offers new opportunities for naturally enhancing the nutritional
63 quality and health-promoting properties of grains.

64 In this study germination temperature and time were optimized to maximize the phytochemical
65 content (γ -amminobutyric acid and phenolic compounds) as well as the antioxidant activity of kiwicha
66 grains using response surface methodology (RSM). The identification and quantification of individual
67 phenolic compounds of kiwicha sprouts obtained in the optimal conditions was also carried out.

68 2. Materials and Methods

69 2.1. Materials

70 Kiwicha (*Amaranthus caudatus* var. Centenario) grains were provided by Cereals and Native
71 Grains Program (Peru). Seeds were cleaned and stored in darkness in polyethylene containers at 4–8°C.

72 2.2. Procedure of germination

73 Kiwicha seeds (30 g) were rinsed first with distilled water and subsequently with 0.1% sodium
74 hypochlorite at 1:5 ratio (w/v) for 30 min. Afterwards, sodium hypochlorite was drained and kiwicha
75 grains were rinsed with sterile distilled water to reach pH 7. Seeds were then placed in deionized water
76 (seed:water ratio, 1:5, w/v) and soaked at room temperature for 24 h. Soaking water was drained and
77 seeds were placed on a drilled grille over moist filter. Seeds were covered by moist filter paper and grille
78 was placed in germination trays containing tap water. Germination trays containing hydrated kiwicha
79 seeds were introduced in a germination cabinet (model EC00-065, Snijders Scientific, Netherlands)
80 provided with a water circulating system to keep 90% air humidity. Germination was carried out in
81 darkness at different temperatures (12–28 °C) and times (12–72 h) based on our previous experience on
82 edible seeds germination. Germinated samples were freeze-dried (Virtis Company, INC Gardiner, NY,
83 USA), and milled by using a ball mill (Glen Creston Ltd., Stanmore, UK). Powdered samples were
84 stored in plastic bags, under vacuum, in darkness at 4 °C until further analysis. Three replicates were
85 performed for each germination condition.

86 2.3. Determination of γ -aminobutyric acid.

87 γ -Aminobutyric acid (GABA) was extracted and analysed by RP-HPLC as described previously
88 (Cáceres, Martínez-Villaluenga, Amigo, & Frias, 2014). Aliquots of 50 μ L of concentrated water-
89 soluble extract containing 10 μ L of internal standard (allyl-L-glycine, Sigma-Aldrich) were derivatized
90 with 30 μ L phenyl isothiocyanate (PITC 99%, Sigma-Aldrich). An Alliance Separation Module 2695
91 (Waters, Milford, USA), a photodiode array detector 2996 (Waters) and Empower II chromatographic
92 software (Waters) were used as the chromatographic system. Twenty microliters of sample were injected

into a C18 Alltima 250 × 4.6 mm internal diameter, 5 µm size (Alltech) column equipped with a C18 guard column (Alltech), both thermostatted at 30 °C. The chromatogram was developed at a flow rate of 1.0 ml/min by eluting the sample in gradient mode using mobile phase A (0.1 M ammonium acetate, pH 6.5) and mobile phase B (0.1 M ammonium acetate, acetonitrile, methanol, 44:46:10, v/v/v, pH 6.5). Analysis were performed in duplicate and results are expressed as mg GABA/100 g of dry weight (dw).

2.4. Determination of phenolic compounds by Folin Ciocaltau.

Phenolic compound content (PC) were determined using Folin-Ciocalteu's phenol reagent. Briefly, 0.5 g of sample were extracted in 10 mL of methanol:HCl:water (80:0.1:19.9, v/v) by continuous magnetic stirring at room temperature for 16 h. Two independent extractions were performed for each replicate. Extracts were centrifuged at 5000 rpm 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 (BioTek Instruments, Winooski, VT, USA) controlled by the Gene 5™ Version 1.1 software (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)/100g of dry weight.

2.5. Determination of antioxidant activity.

Antioxidant activity was evaluated by the oxygen radical absorbance capacity (ORAC-FL) method as described in a previous study (Caceres et al., 2014). Briefly, 0.5 g of sample were extracted in 10 mL of methanol:HCl:water (80:0.1:19.9, v/v) by continuous magnetic stirring at room temperature for 16 h. Two independent extractions were performed for each replicate. Extracts were centrifuged at 5000 rpm at 5 °C for 5 min. ORAC values of non-germinated and germinated kiwicha extracts were

determined in triplicate. Fluorescence was measured in a microplate reader (Synergy HT, Biotek, USA) at 528 and 485 nm for emission and excitation wavelengths, respectively. Different concentrations of Trolox (4–160 μ M) were used to prepare a standard curve. Results were expressed as mg Trolox equivalents (TE)/100 g dw.

2.6. Experimental design and data analysis

Polynomial regression equations were developed to describe the effects of two independent processing parameters such as germination temperature (X_1 , $^{\circ}$ C) and time (X_2 , h) on GABA and TPC content as well as antioxidant activity as measured by ORAC were analyzed using Response Surface Methodology (RSM) with a 2^2 central composite rotational design. Independent variables of germination temperature (12, 14, 20, 26 and 28 $^{\circ}$ C) and time (12, 21, 42, 63 and 72 h) were varied to investigate the effects on the dependent variables mentioned above. A list of independent variables with their symbols and coded factor levels is given in Table 1.

The following second order polynomial regression model employed in this study is presented in equation (1). By using this equation, linear (X_1 , X_2), quadratic (X_1^2 , X_2^2) and interactive (X_1X_2) effects of independent variables germination temperature (X_1) and time (X_2) on dependent variable (Y) were determined:

$$Y = \beta_0 + \beta_i X_i + \beta_{ii} X_i^2 + \beta_{ij} X_i X_j \quad (1)$$

Where Y is the predicted response variable, β_0 the constant (intercept), β_i is the linear coefficient, β_{ii} the quadratic **coefficients** and β_{ij} is the cross product coefficient. X_i and X_j are independent variables. The response surface regression was used to analyze the experimental data using Statistica Version 5.0 software (Statsoft. USA). Two dimensional contour plots were developed. All processing trials were conducted in triplicate.

138 The predictive performance of the developed models describing the combined effect of
139 temperature and time on independent variables (GABA, TPC and ORAC) of kiwicha sprouts were
140 validated with optimal extraction conditions as predicted by the design. The criterion used to
141 characterize the fitting efficiency of the data to the model was the multiple correlation coefficients (R^2),
142 the analysis of variance (ANOVA) of the regression coefficients of the fitted polynomial equations, and
143 the significance level at $P < 0.05$.

144 2.7. HPLC–DAD–ESI/MSⁿ qualitative and quantitative analyses of phenolic compounds

145 Freeze-dried samples (5 g) were macerated in methanol:TFA:water (80:0.1:20, v/v) at 4°C for 16
146 h. Subsequently, they were centrifuged at 4000 *g* and 5°C for 20 min in a super-speed centrifuge (Sorval
147 RC 5B). The extraction process was repeated twice. The extracts were combined and concentrated at 30
148 °C under vacuum for methanol evaporation.

149 For phenolic analysis, the dry extracts were dissolved in 10 mL of water. For purification, an
150 aliquot (4 mL) was passed through a C18 Sep-Pak cartridge (Waters, Milford, MA, USA), previously
151 activated with methanol followed by water (Dueñas, Pérez-Alonso, Santos-Buelga, & Escribano-Bailón,
152 2008).

153 Samples were analyzed using Hewlett–Packard 1100MS (Agilent Technologies, Palo Alto, CA)
154 chromatograph equipped with a quaternary pump, diode array detector (DAD) coupled to an HP Chem
155 Station (rev.A.0504) data-processing station, following a previous method (Barros, Dueñas, Carvalho,
156 Ferreira, & Santos-Buelga, 2012). Solvents used were 0.1% formic acid in water (solvent A), and 100%
157 acetonitrile (solvent B). The elution gradient established was 15% B for 5 min, 15–20% B for 5 min, 20–
158 25% B for 10 min, 25–35% B for 10 min, 35–50% B for 10 min, and re-equilibration of the column. The
159 separation of phenolic compounds was performed in a Spherisorb S3 ODS-2 C8 column (Waters,
160 Millford, USA) (3 μ m, 150 mm×4.6 mm i.d.) operating at 35 °C and a flow rate of 0.5 mL min⁻¹.

Double online detection was carried out in the DAD using 280 nm and 370 nm as preferred wavelengths. Mass spectrometer (MS) connected to the 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 an earlier method (Barros, Alves, Dueñas, Silva, Oliveira, Carvalho, et al., 2013).

The phenolic compounds were characterized according to their UV and mass spectra, retention times and compared with authentic standards when available. Calibration curves were prepared by injection of known concentrations of different standard compounds. The standards, vanillic, *trans-p*-coumaric acids and quercetin 3-*O*-rutinoside were obtained from Extrasynthese (France). Hexosides and quinic derivatives of protocatechuic, vanillic, caffeic, ferulic and *p*-coumaric acids were quantified by the curves of the corresponding free acids. Results were expressed as µg/g dw.

3. Results and Discussion

3.1. Effect of germination temperature and time on GABA content of kiwicha.

Germination temperature and time had a significant ($P < 0.05$) positive effect on GABA content (Figure 1A; Tables 1 and 2). Temperature showed a higher effect than time on GABA content, however these two factors did not show significant quadratic effects ($P > 0.05$) (Table 2). The interaction of temperature and time also showed a non-significant effect on GABA accumulation in kiwicha sprouts ($P > 0.05$). Our results agree with earlier studies reporting a time-dependent GABA accumulation during germination of rice and quinoa grains (Cáceres, Martínez-Villaluenga, Amigo, & Frias, 2014; Morita, Park, & Maeda, 2013). GABA accumulation begins in the soaking process and continues during germination due to the increased activity of glutamate decarboxylase (GAD), enzyme involved in GABA synthesis (Scott-Taggart, Van Cauwenberghe, McLean, & Shelp, 1999). The highest GABA concentrations observed at higher temperatures in kiwicha sprouts are consistent with studies showing

that GAD activity in cereal grains increase with increasing temperatures from 20 to 40 °C (Yang, Yin, Guo, & Gu, 2013). The regression model and the contour plot for GABA is shown in equation (2) and Figure 1A, respectively.

$$\text{GABA (mg/100g dw)} = 43.26 + 9.21 X_1 + 23.26 X_2 \quad (2)$$

Among the treatments generated by RSM, the highest GABA content was observed in kiwicha sprouts obtained at 26°C for 63 h (75.7 mg/100g dw) and 28 °C for 42 h (74.4 mg/100g dw) (Table 1). These values were 29-fold higher than those present in non-germinated kiwicha grains (Table 3). GABA content in kiwicha sprouts obtained in the optimal conditions was similar to those of GABA enriched brown rice sprouts (70.8–83.1 mg/100g dw) (Cáceres, Martínez-Villaluenga, Amigo, & Frias, 2014).

3.2. Effect of germination temperature and time on phenolic content of kiwicha

The PC of kiwicha sprouts varied greatly from 86.22 to 385.43 (mg GAE/100g dw) depending on germination conditions (Table 1, Figure 1B). Germination temperature had a higher effect on PC of kiwicha sprouts than time and their interaction ($P < 0.05$); however these factors had no significant effects at the quadratic level ($P > 0.05$) (Table 2). The positive effect of germination time and temperature on PC of kiwicha sprouts was in agreement with Perales-Sanchez et al. (2014) who found that free, bound and total phenolics content of *Amaranthus hyponcondriacus* increase with temperature and time up to 30 °C and 70–80h. Pasko et al. (2009) reported a similar effect of time on PC of *Amarathus cruentus*. These effects have been directly related to the induction of enzymes involved in the phenylpropanoid pathway and in the degradation of the cell wall polysaccharides and proteins that cause the release of bound phenolics (He, Han, Yao, Shen, & Yang, 2011). The regression model and the contour plot for PC is shown in equation (3) and Figure 1B, respectively.

$$\text{PC (mg GAE/100g dw)} = 150.45 + 70.86 X_1 + 80.04 X_2 + 65.5 X_1 X_2 \quad (3)$$

206 Among the germination conditions assayed, the highest PC was observed in kiwicha sprouts
207 produced at 26 °C for 63 h (385.4 mg GAE/100 g dw) (Table 1). These germination conditions led to a
208 4-fold increase of PC compared to non-germinated kiwicha grains (Table 3). PC content in kiwicha
209 sprouts obtained in the optimal conditions was higher than reported values for germinated brown rice
210 (207.61–306.65 mg GAE/100g dw) and *A. hypocondriacus* and *A. cruentus* sprouts produced in optimal
211 germination conditions (247–300 mg GAE/100g dw) (Paško, Bartoń, Zagrodzki, Gorinstein, Fołta, &
212 Zachwieja, 2009; Perales-Sánchez, et al., 2014).

213 3.3. Effect of germination temperature and time on antioxidant activity of kiwicha

214 Antioxidant activity was positively affected by germination time and temperature, being the
215 effect of temperature higher than time. ORAC values ranged from 369.46 to 2525.58 (mg TE/100g dw)
216 in kiwicha sprouts depending on germination conditions (Table 1; Figure 1C). Temperature and time
217 had significant quadratic and interaction effects on antioxidant activity of kiwicha sprouts ($P < 0.05$,
218 Table 2).

219 An increase in antioxidant activity with higher germination temperature and time has also been reported
220 in other species of amaranth (Paško, Bartoń, Zagrodzki, Gorinstein, Fołta, & Zachwieja, 2009; Perales-
221 Sánchez, et al., 2014) and brown rice (Cáceres, Martínez-Villaluenga, Amigo, & Frias, 2014). The
222 results of our study could be attributed to the release of bound phenolics and the biosynthesis of
223 antioxidants to maintain a balance in redox homeostasis during germination (He, Han, Yao, Shen, &
224 Yang, 2011). For instance, biosynthesis of vitamin C has been reported to occur during the sprouting of
225 wheat, quinoa and buckwheat (Lintschinger, Fuchs, Moser, Jäger, Hlebeina, Markolin, Gössler, 1997).
226 PC was positively correlated with ORAC ($r = 0.894$, $P < 0.05$) which supports this hypothesis. Several
227 studies have demonstrated that phenolic compounds may protect from oxidative stress acting as radical

scavengers by hydrogen and electron donation or activating the antioxidant defense through upregulation of antioxidant enzymes expression (Martins, Barros, & Ferreira, 2016).

The regression model and the contour plot for ORAC is shown in equation (4) and Figure 1C, respectively.

$$\text{ORAC} = 580.55 + 397.90 X_1 + 202.38 X_1^2 + 497.22 X_2 + 221.17 X_2^2 + 394.82 X_1 X_2 \quad (4)$$

The highest ORAC value was found when germination was performed at 26 °C and 63 h (2525 mg TE/100g dw) which was approximately 6.4 times higher than that observed in non-germinated kiwicha grains. As compared with other grains germinated in optimal conditions, kiwicha sprouts had better ORAC values than brown rice (686.8–1054.7 mg TE/100g dw) (Cáceres, Martínez-Villaluenga, Amigo, & Frias, 2014) and comparable to other amaranth species (Perales-Sánchez, et al., 2014).

3.4. Model fitting, optimization and model validation

The analysis of variance showed that the R-squared statistic of all the parameters was in the range of 0.965–0.989 (Table 2). The predicted values obtained by the quadratic polynomial equations showed strong correlation with experimental values with Pearson coefficients (r) from 0.0001 to 0.0557 (Table 2). The quadratic polynomial models generated were highly significant with p-values ranging from < 0.0005–0.001. The lack of fit statistics of all the parameters were not significant (P>0.05) and high degree of F-value range 26.96–111.64 further strengthened the reliability of the models (Table 2). The RSM guided optimization demonstrated that the optimum germination conditions for maximizing the GABA content, PC and antioxidant activity measured as ORAC were 26 °C (temperature) and 63 h (time). The development of radicle and cotyledons of the germinated kiwicha grain in the optimal conditions is shown in Figure 2. All response variables in kiwicha sprouts at optimal conditions were found significantly higher than non-germinated grains (P <0.05). The predicted optimal germination conditions for all parameters combined was also 26 °C and 63 h which predicted GABA, PC and ORAC

values of 85.7 mg/100g dw, 406.3 mg GAE/100g dw and 2294.04 mg TE/100g dw, respectively (Table 3). The predicted values were close to experimental values (Table 3).

3.5. Effect of germination in optimal conditions on phenolic profile of kiwicha

Identification and quantification of individual phenolic compounds of kiwicha (*Amaranthus caudatus* var. *centenario*) seeds and sprouts were performed by HPLC–DAD–MSⁿ analysis have been carried out. The retention time, λ_{max} , molecular ion and their fragment ion pattern allowed the identification of 14 phenolic compounds belonging to hydroxybenzoic, hydroxycinnamic derivatives and flavonol phenolic classes (Table 4).

Hydroxycinnamic acids derivatives were the major group of phenolic compounds identified. Six compounds (peaks 6, 8–11) were detected as quinic acid derivatives according to their UV–vis spectra (λ_{max} 302–330 nm) and precursors ions $[M-H]^-$ 337, 353 and 367, all of them with a fragment ion at m/z 191, corresponding to an quinic acid residue. The esterification of the quinic acid moiety was assigned at the position 4 in these hydroxycinnamoylquinic acids distinguished from its base peak at m/z 175 ($[quinic\ acid-H-H_2O]^-$), accompanied by a secondary fragment ion at m/z 179 (caffeic acid), 163 (*p*-coumaric acid) and 193 (ferulic acid), according to the fragmentation patterns described previously (Clifford, Johnston, Knight, & Kuhnert, 2003; Clifford, Knight, & Kuhnert, 2005). Peaks 9 and 10 were identified as the *cis* and *trans* isomers of 4-*p*-coumaroylquinic acid based on their fragmentation.

Other compounds were identified as feruloyl hexoside (peaks 4 and 5) based on their UV–vis spectra with maximum at 328–330 nm, precursor ions $[M-H]^-$ (at m/z 355) and fragment ions at m/z 193 corresponding to the loss of an hexose unit. One peak (peak 12) showed an UV–vis spectrum similar to caffeic acid with λ_{max} at 330 nm, but eluted at different retention time. It presented a fragment at m/z 179, due to caffeic acid. This compound could not be fully identified and was tentatively identified as caffeic acid derivative.

Other hydroxybenzoic acid derivatives were identified as protocatechuic hexoside acid (peak 1) based on their UV-vis spectrum, precursor ion $[M-H]^-$ at m/z 315 and fragment ion at m/z 153, ($[M-162]^-$, corresponding to the loss of an hexose moiety). Two compounds were identified as vanillic hexoside (peak 2) and pentoside (peak 3) acids based on their UV-vis spectra, precursor ions $[M-H]^-$ (at m/z 329 and 299, respectively) and fragment ions at m/z 167, corresponding to the loss of an hexose unit $[M-162]^-$ and pentoside moiety $[M-132]^-$.

Vanillic acid (peak 7), *trans-p*-coumaric acid (peak 12) and quercetin 3-rutinoside (peak 14) were identified by comparison of their UV-vis spectra and retention times with commercial standards.

According to our knowledge, hydroxybenzoic and hydroxycinnamic acids linked to hexoside and quinic acid have not been described in kiwicha by other authors, since some studies have reported the presence of free hydroxybenzoic and hydroxycinnamic acids (protocatechuic, vanillic, caffeic, *p*-coumaric and ferulic acids) (Barba de la Rosa, Fomsgaard, Laursen, Mortensen, Olvera-Martínez, Silva-Sánchez, et al., 2009; Klimczak, Małecka, & Pacholek, 2002), which could be due to the different varieties studied. However, the presence of quercetin-3-rutinoside was also reported in other varieties of *A. caudatus* (Barba de la Rosa, et al., 2009).

Notable qualitative and quantitative differences in the identified phenolic compounds were observed between the raw and germinated kiwicha grains (Table 5). Non flavonoid compounds (hydroxybenzoic and hydroxycinnamic acids) were the most abundant phenolic class detected in kiwicha grain and sprouts accounting between 85% and 99%, respectively, of the total phenolic content. However, kiwicha grains presented higher content of hydroxybenzoic acids, including free forms (vanillic acid) and those linked to sugars (pentose and hexose), accounting for approximately 51% of the total phenolic content, whereas hydroxycinnamic acids linked to hydroxyacids, quinic acid, represented 34% of the total phenolic content. Germination process significantly increased the concentration of

hydroxycinnamic compounds ($P < 0.05$), representing 97% of the total content, being 4-*O*-caffeoylquinic and 4-*O*-feruloyl quinic acids the main compounds of this group in sprouts, which was not detected in kiwicha grains. Thus, germination caused a 50% increase in the content of total hydroxybenzoic compounds, being vanillic acid the only hydroxybenzoic compound detected in kiwicha sprouts.

Flavonol, quercetin 3-*O*-rutinoside, was the only group of flavonoids in kiwicha grain and sprouts. This compound represented higher percentage, approximately 15%, of total content in non-germinated kiwicha whereas it only represented 1% of the total content in kiwicha sprouts. Quercetin 3-*O*-rutinoside significantly increased during germination process ($P < 0.05$) in comparison to control (84%).

Germination process in optimized conditions significantly enhanced the concentration of total phenolic compounds in kiwicha (Table 5). According to our knowledge, no previous studies have been reported about the phenolic composition in kiwicha sprouts. However, few studies concerning the phenolic composition in kiwicha grains using HPLC-DAD-ESI/MS. Barba de la Rosa et al. (2009) also reported that amaranth seed flour presented three flavonols (rutin, isoquercitrin and nicotiflorin). Rutin (quercetin 3-*O*-rutinoside) was present at higher concentrations (10.1–4.0 $\mu\text{g/g}$ flour) in different varieties to the studied in the present work. Lower content of hydroxybenzoic acids (4-hydroxybenzoic, syringic and vanillic acids) were reported in previous studies for other kiwicha varieties (1.5–1.8 $\mu\text{g/g}$ dw). These differences in phenolic content of amaranth grains could be attributable to genetic factors and environmental conditions during plant growth. Hydroxybenzoic and hydroxycinnamic acids were also identified by Klimczak *et al.* (2002), although compounds linked to hydroxyacids were not detected. *p*-Coumaric acid content reported by these authors was 5.20 $\mu\text{g/g}$ dw in *A. caudatus*, although variety was not indicated in the study.

4. Conclusions

320 Germination is an effective process to maximize the phytochemical content and antioxidant
321 activity of kiwicha. Optimal germination conditions such as temperature and time were identified by
322 RSM. Results demonstrated that germination at 26 °C for 63 h was optimal for maximizing the content
323 of GABA and phenolic compounds as well as the antioxidant activity of kiwicha. These sprouts
324 presented mainly non-flavonoid compounds, being hydroxycinnamic quinic acid derivatives (4-O-
325 caffeoylquinic, 4-O-feruloylquinic acids) as the most abundant compounds in sprouts. Results from this
326 study support the possible application of kiwicha germinated grains as ready to eat sprouts or as raw
327 material to obtain flours with improved nutritional, phytochemical content and antioxidant activity that
328 could be used as ingredients in bakery products.

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and 63 h).

Figure 3. Phenolic profiles of germinated kiwicha extract recorded at 280 nm (A) and 370 nm (B)

Table 1

Combinations of temperature and time with their coded terms obtained from RSM and the respective values of GABA, PC, ORAC.

Run	Temperature (°C)	Time (h)	GABA (mg/100g dw)	TPC (mg GAE/100g dw)	ORAC (mg TE/100g dw)
1	14 (−1)	21 (−1)	9.47±0.73 ^g	86.22±4.43 ^h	369.46±38.93 ^g
2	14 (−1)	63 (+1)	20.69±1.56 ^{gt}	96.42±2.07 ^{gh}	573.28±47.15 ^{egdt}
3	26 (+1)	21 (−1)	58.17±3.85 ^{cb}	113.23±3.04 [†]	742.48±96.88 ^d
4	26 (+1)	63 (+1)	75.69±4.18 ^a	385.43±11.73 ^a	2525.58±223.58 ^a
5	20 (0)	12 (− α)	29.57±1.82 ^{et}	95.68±1.30 ^{gh}	513.77±41.30 ^{egt}
6	20 (0)	72 (+ α)	61.34±4.17 ^b	296.83±10.43 ^c	1359.69±86.74 ^c
7	12 (− α)	42 (0)	16.14±1.47 ^g	107.89±7.79 ^{tg}	390.07±49.41 ^{gt}
8	28 (+α)	42 (0)	74.39±14.59 ^a	337.23±11.54 ^b	1558.57±114.01 ^b
9	20 (0)	42 (0)	38.40±3.11 ^{ed}	141.17±12.59 ^e	519.81±60.05 ^{egt}
10	20 (0)	42 (0)	44.88±2.54 ^d	152.14±4.00 ^{ed}	650.77±47.40 ^{ed}
11	20 (0)	42 (0)	47.21±2.16 ^{cd}	158.05±4.31 ^d	571.08±42.01 ^{edt}

Data are the mean ± standard deviation of three replicates analyzed in duplicate. Different lower case letters indicate statistical differences among response values at different germination conditions (P<0.05, Duncan test)

Table 2

Analysis of the variance of the regression coefficients to the fitter polynomial equation for GABA (mg/100 g dw), PC (mg GAE/ 100g dw) and ORAC (mg TE/100 g dw)

Coefficients	GABA	PC	ORAC
β_0 (intercept)	43.26**	150.45**	580.55*
Linear			
β_1 (Temperature)	23.26**	80.04**	497.22**
β_2 (Time)	9.21*	70.86**	397.90*
Quadratic			
β_{11}	-0.11	13.13	202.38*
β_{22}	-0.20	26.28	221.17*
Interaction			
β_{12}	1.58	65.5*	394.82*
R^2	0.967	0.979	0.989
Predicted R^2	0.965	0.972	0.963
CV	5.85	7.3	13.58
p	0.0005	0.0005	0.0010
F-value	111.64	35.34	26.96

*Regression coefficient values are statistically significant at $P < 0.05$.

**Regression coefficient values are statistically significant at $P < 0.001$

Table 3

Predicted and experimental values of the parameters tested at optimal germination conditions in comparison to average mean deviation between predicted and experimental values of optimal germination conditions

Parameter	Optimum germination condition for all the parameters combined	Predicted values at optimal conditions	Experimental optimal cor
GABA (mg/100 g dw), PC (mg GAE/100 g dw) ORAC (mg TE/100g dw)	26 °C and 63 h	85.73 406.26 2294.04	75.69±4 385.43±1 2525.58±2

Data are the mean ± standard deviation of three replicates analyzed in duplicate. Different lower case letters indicate statistical differences between kiwicha at optimal conditions (P<0.05, Duncan test)

Table 4

Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification of compounds isolated from germinated kiwicha grains.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion [M-H] ⁻ (m/z)	MS ² (m/z)	Tentative identification
1	5.13	258, 290	315	153(100)	Protocatechuic hexoside acid
2	5.42	264, 296	329	167(67)	Vanillic hexoside acid
3	7.89	265, 297	299	167(100)	Vanillic pentoside acid
4	8.54	312	355	193(100), 179(25), 149(36)	<i>trans</i> -Feruloylhexoside acid (I)
5	9.53	330	355	193(68), 175(100), 161(70), 149(12)	<i>trans</i> -Feruloylhexoside acid (II)
6	10.22	330	353	191(100), 179(11), 173(94), 155(42)	4- <i>O</i> -Caffeoylquinic acid
7	10.64	264, 296	167	152 (93)	Vanillic acid
8	12.25	330	353	191(53), 179 (19), 173(85), 155 (40), 135 (17)	Caffeoylquinic acid
9	15.04	314	337	173(98), 163(17), 155(100)	<i>cis</i> -4- <i>O</i> - <i>p</i> -Coumaroylquinic acid
10	15.33	306	337	191(29), 173(100), 163(4), 155(74)	<i>trans</i> -4- <i>O</i> - <i>p</i> -Coumaroylquinic acid
11	16.73	330	367	193(13), 191(21), 173(100), 155(96)	4- <i>O</i> -Feruloylquinic acid
12	17.23	308	163	–	<i>trans</i> - <i>p</i> -coumaric acid
13	19.07	330	–	179(22), 135(100)	Caffeic acid derivative
14	19.09	356	609	301(17)	Quercetin 3- <i>O</i> -rutinoside

Table 5

Quantification of individual phenolic compounds ($\mu\text{g/g dw}$) in non-germinated and germinated (26 °C for 63 h) kiwicha grains.

Compounds	Raw	Germinated
Protocatechuic hexoside acid	0.56 ± 0.08^b	nd ^a
Vanillic hexoside acid	nd	nd ^a
Vanillic pentoside acid	3.03 ± 0.08^b	nd ^a
Feruloylquinic acid	0.25 ± 0.04^b	nd ^a
<i>trans</i> -Feruloyl hexoside acid (I)	1.30 ± 0.12^a	1.74 ± 0.16^b
<i>trans</i> -Feruloyl hexoside acid (II)	nd ^a	15.84 ± 0.94^b
4- <i>O</i> -Caffeoylquinic acid	nd ^a	879.92 ± 51.28^b
Vanillic acid	3.79 ± 0.84^a	14.88 ± 1.85^b
Caffeoylquinic acid	2.32 ± 0.19^b	nd ^a
4- <i>O-p</i> -coumaroyl quinic acid	nd ^a	30.31 ± 1.47^b
<i>p</i> -coumaroyl quinic acid	nd ^a	1.42 ± 0.22^b
4- <i>O</i> -Feruloylquinic acid	nd ^a	112.13 ± 4.06^b
<i>trans-p</i> -coumaric acid	0.32 ± 0.04^b	nd ^a
Caffeic acid derivative	0.73 ± 0.00^a	11.73 ± 2.26^b
Quercetin 3- <i>O</i> -rutinoside	2.15 ± 0.09^a	13.74 ± 0.64^b
Non-Flavonoid	12.3 ± 1.35^a	1067.97 ± 60.28^b
Flavonoid	2.15 ± 0.09^a	13.74 ± 0.64^b
Total	14.45 ± 0.90^a	1081.71 ± 60.67^b

Data are the mean $\pm$ standard deviation of three replicates. Different lowercase letters indicate statistical differences between values within a row ($P < 0.05$)

Figure 1

A

B

C

Figure 2.

Figure 3