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**Optimization of germination time and temperature to maximize the content of bioactive compounds
and the antioxidant activity of purple corn (*Zea mays* L.) by response surface methodology**

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KEYWORDS: Purple corn, germination, GABA, phenolic compounds, antioxidant activity.

1. Introduction

Purple corn is a pigmented variety of *Zea mays* L. that has been cultivated for centuries in the Andean Region of South America. This colored cereal is used for the preparation of traditional beverages and desserts called “Chicha morada” and “Mazamorra morada”, respectively (FAO, 2013), but also as a source of natural colorants for tissues staining (Petroni, Pilu, & Tonelli, 2014). Purple corn is a rich source of phenolic compounds, mainly anthocyanins, natural pigments that have demonstrated cardioprotective properties (Toufektsian et al., 2008), prevention of obesity (Tsuda, Horio, Uchida, Aoki, & Osawa, 2003) and diabetic nephropathy (Kang, Lim, Lee, Yeo, & Kang, 2013), and inhibition of mammary and prostate carcinogenesis (Fukamachi, Imada, Ohshima, Xu, & Tsuda, 2008; Long et al., 2013) in animal studies. Moreover, purple corn contains other non-anthocyanin phenolic compounds that also show health-promoting effects (Pedreschi & Cisneros-Zevallos, 2007), even though limited information exists on the phenolic compounds in purple corn. Therefore, in the last years, many scientific efforts have been focused on the enhancement of phenolic compounds, mainly anthocyanins, in colored vegetables.

Germination represents an inexpensive technology to increase the nutritive quality and bioactive compounds of cereal grains (Singh, Rehal, Kaur, & Jyot, 2015). During germination, endogenous seed enzymes are activated, and some reserve compounds such as storage proteins and carbohydrates are hydrolyzed into small molecules, whereas new cell constituents and phytochemical compounds are synthesized (Wu, Yang, Touré, Jin, & Xu, 2013). Recent studies have shown that germination enhances the content of γ -Aminobutyric acid (GABA) (Cáceres, Martínez-Villaluenga, Amigo, & Frias, 2014; Chung, Jang, Cho, & Lim, 2009), a compound that provides beneficial health effects by regulating blood pressure (Diana, Quílez, & Rafecas, 2014) and phenolic compounds (Chen, Yu, Wang, Gu, & Beta, 2016; Kim et al., 2012; van Hung, Maeda, Yamamoto, & Morita, 2012) in cereals. However, the extent of the changes on phytochemical compounds observed during cereal germination will depend on germination conditions. To our knowledge, there is a lack of information regarding the effect of germination conditions on the phytochemical content and antioxidant activity of purple corn. Therefore, the objective of this study was to determine optimum germination conditions to produce germinated purple corn with improved content of GABA, phenolic compounds, and antioxidant activity by using Response Surface Methodology (RSM). The phenolic composition of purple corn sprouts obtained in the optimal conditions was also evaluated.

2. Materials and methods

2.1. Chemicals

Phenolic compounds standards were purchased from Sigma-Aldrich (Madrid, Spain) and Extrasynthese (Barcelona, Spain). Methanol was of HPLC grade from Panreac (Barcelona, Spain). All other chemicals were purchased from Sigma-Aldrich (Madrid, Spain) unless otherwise specified.

2.2. Plant material

Purple corn (*Zea mays* L. var. *PMV-581*), was developed as a part of a Corn Program (La Molina, Lima, Peru). This cultivar was selected due to its high content of anthocyanins and total phenolic compounds (Quispe-Jacobo, Arroyo-Condorena, & Gorriti-Gutierrez, 2011). Seeds were cleaned and stored in darkness in polyethylene containers at 4–8°C until germination.

2.3. Germination process

Purple corn seeds were hygienised with 0.1% sodium hypochlorite (seeds: NaOCl ratio, 1:5 w/v) for 30 min and then grains were rinsed with distilled water until reaching neutral pH. Afterwards, purple corn seeds were soaked in distilled water (seed: water ratio, 1:5 w/v) at room temperature for 24 h. After draining the soaking water, hydrated seeds were placed in germination trays on wet filter paper. The trays were introduced in the 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 temperatures in the range 12–28 °C and times of 12–72 h, conditions selected according to the experimental response surface model (Table 1). Three replications were performed for each germination condition. Figure 1 shows, as an example, a purple corn sprout obtained after germination at 26°C for 63 h. Sprouts and non-germinated purple corn seeds were freeze-dried, milled and stored in vacuum bags at -20 °C until further analysis. Non germinated purple corn seeds were considered as control samples of the experiment.

2.4. Determination of GABA content

The extraction of GABA in germinated purple corn and the derivatization of the obtained extracts were performed as previously reported (Cáceres et al., 2014). Quantification of GABA 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), as described by

Caceres et al., (2014). HPLC analyses were carried out in duplicate and the results were expressed in mg GABA/100 g d.m.

2.5. Determination of total phenolic compounds (TPC) content

TPC content was determined using Folin–Ciocalteu’s phenol reagent. Briefly, 0.5 g of purple corn sprout flour was suspended in 10 mL of 80% methanol (v/v) (acidified 0.1% HCl) and the suspensions were kept for 16 h at room temperature with continuous stirring. TPC extraction and quantification was performed as described by Caceres et al. (2014). Results were expressed as mg of gallic acid equivalents (GAE)/ 100 g d.m.

2.6. HPLC-DAD-ESI/MSⁿ qualitative and quantitative determination of phenolic compounds.

Five grams of freeze-dried purple corn sprouts were macerated in 80% methanol (v/v) (acidified with 0.1% TFA) at 4 °C for 16 h. Afterwards, they were centrifuged at 4000 x g at 5 °C for 20 min (SorvallTM, U. S). The extraction process was repeated twice. The extracts were combined and concentrated at 30 °C under vacuum for methanol evaporation. The dry extracts were dissolved in 10 mL of 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 Dueñas, Pérez-Alonso, Santos-Buelga, & Escribano-Bailón, (2008).

2.6.1. Non-anthocyanin compounds. Samples were analyzed using a Hewlett–Packard 1100MS chromatographer (Agilent Technologies, Palo Alto, CA) chromatograph equipped with a quaternary pump, diode array detector (DAD) coupled to an HP Chem Station (rev.A.0504) data-processing station, as previously described (Barros et al., 2013). 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, as described by Barros et al. (2013).

The non-anthocyanin phenolic compounds were characterized according to their UV and mass spectra and 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, *p*-hydroxybenzoic acid, quercetin 3-*O*-rutinoside, isorhamnetin 3-*O*-rutinoside, isorhamnetin 3-*O*-glucoside and kaempferol 3-*O*-glucoside were obtained from Extrasynthese (France). Hexosides and quinic derivatives of vanillic, caffeic, and ferulic were quantified by the curves of the

corresponding free acids; flavonols, derivatives of kaempferol, quercetin and isorhamnetin by the curves of kaempferol-3-*O*-glucoside, quercetin-3-*O*-glucoside and isorhamnetin 3-*O*-rutinoside, respectively. Flavone, derivative of luteolin, was quantified by the curve of luteolin 6-C-glucoside. Results were expressed as µg/g d.m.

2.6.2. Anthocyanins. Samples were analyzed using an HPLC system (Phenomenex) equipped with a reverse phase C18 column (5 µm, 150 mm × 4.6 mm i.d) at 35 °C according to Alcalde-Eon, García-Estévez, Ferreras-Charro, Rivas-Gonzalo, Ferrer-Gallego & Escribano-Bailón, (2014). Detection was carried out at 520 nm. MS was performed in the same equipment described above, according to the method described by Alcalde-Eon et al., (2014). For the quantitative analysis of anthocyanins a calibration curve was obtained by injection of different concentrations of cyanidin 3-*O*-glucoside, peonidin 3-*O*-glucoside and pelargonidin 3-*O*-glucoside. The results were expressed in µg/g d.m.

2.7. Determination of antioxidant activity

The antioxidant activity was evaluated in the methanolic extracts previously obtained for TPC determination, by using oxygen radical absorbance capacity (ORAC-FL) method, as previously described (Torino et al., 2013). Determinations were carried out in triplicate and results were expressed as mg of Trolox equivalents (TE)/ g d.m.

2.8. Experimental design and statistical analysis

RSM was used for investigating the effect of time and temperature on the phytochemical content and antioxidant activity of germinated purple corn. Germination experiments were conducted according to a 2² central composite rotational design including a center point (in triplicate) and the independent variables studied were germination time (12, 21, 42, 63 and 72 h) and temperature (12, 14, 20, 26 and 28 °C). Real and coded factors levels for these variables are given in Table 1.

The following second order polynomial equation was used to express responses as a function of independent variables:

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

Where Y represents the response variable to be modelled, β_0 is a constant, β_i is the linear term coefficient, β_{ii} is the quadratic term coefficient, β_{ij} is the interaction term coefficient, and X_i and X_j are the independent variables.

The goodness-of-fit of the models obtained was evaluated by calculating the multiple determination coefficients (R^2) and the analysis of variance (ANOVA) of the regression coefficients of the fitted polynomial equations for each response variable by using Statistica 5.0 software (Statsoft, USA), where the P values indicated if the terms were significant. Three-dimensional response surface plots were drawn to illustrate the effects of the independent variables on the dependent ones.

3. Results and discussion

Germination conditions strongly influence the nutritional value and the content of phytochemical compounds of cereals (Cáceres et al., 2015; Singh et al., 2015). Therefore, optimization of germination conditions is necessary to improve the quality of germinated seeds. Optimizing each condition separately could be very time consuming. Moreover, if several factors play a role, their interaction effects on the content of phytochemical compounds in cereals would be not considered, even if they have a deep influence. The use of experimental factorial design and RSM is suitable for evaluating the role of the main factors influencing the seed germination process and their interaction effects on the content of bioactive compounds of cereals.

3.1. Influence of germination conditions on GABA content of purple corn sprouts.

Non-germinated purple corn exhibited a GABA content of 15.27 ± 0.87 mg/100 g d.m, which is higher than the levels reported in the literature for other purple corn varieties (Polthum & Ahromrit, 2014). GABA levels increased during germination to reach levels from 22.40 to 53.17 mg/100 g d.m., depending on germination conditions (Table 2). It is well known that GABA content increases during germination of cereals such as wheat, barley and rice (Cho & Lim, 2016; Singkhornart & Ryu, 2011), due to the activation of enzymes involved in GABA (e.g. glutamate decarboxylase) and glutamate synthesis (Cho & Lim, 2016). However, little is known about the effect of germination on the GABA levels of purple corn. Polthun and Ahromrit (2014) found that germination of the Thai purple corn var. KCU-KND for 12-48 h at 35°C led to GABA concentrations of 7.73-10.20 mg/100 g d.m. The differences in GABA content between both studies can be attributed to the different GABA concentration of each purple corn variety and to the variations in germination conditions.

The regression model obtained for GABA content in germinated purple corn was not significant within the time and temperature ranges studied since the determination coefficient for the model was lower than 0.75 ($R^2 = 0.58$; Adjusted $R^2=0.17$). In general, only values of R^2 higher than 0.75 indicate the goodness of the model (Peñas, Gómez, Frías, & Vidal-Valverde, 2008). However, long germination times of 63 h at 26 °C conducted to the highest GABA concentrations in purple corn sprouts, conditions that increased more than 3-fold the content of this bioactive compound in germinated purple corn in comparison to purple corn seeds (Table 2). GABA shows multiple health benefits such as blood pressure regulation, stress control (Hayakawa, Kimura, & Kamata, 2002), reduction of plasma cholesterol levels (Miura, Ito, Mizukuchi, Kise, Aoto, & Yagasaki, 2006), and inhibition of carcinogenesis (Opolski, Mazurkiewicz, Wietrzyk, Kleinrok, & Radzikowski, 2000), among others. Therefore, the production of GABA-enriched cereals has been actively pursued. However, as far as we know, this is the first study conducted to optimize germination conditions to maximize the GABA content on purple corn.

3.2. Influence of germination conditions on TPC content of purple corn sprouts

TPC content found in non-germinated purple corn was 1679.72 ± 54.98 mg GAE/100 g d.m, value within the range of those reported for several purple corn varieties (Lopez-Martinez, Oliart-Ros, Valerio-Alfaro, Lee, Parkin, & Garcia, 2009; Lopez-Martinez, Parkin, & Garcia, 2011; Monroy, Rodrigues, Sartoratto, & Cabral, 2016), and higher than those reported for a Peruvian variety of purple corn (Mrad, Debs, Saliba, Maroun, & Louka, 2014). During germination, TPC concentration decreased significantly ($P \leq 0.05$) reaching values ranging from 1042.82 to 1496.29 mg GAE/100 g d.m., depending on germination conditions. There are no literature data regarding the influence of germination on the TPC content of purple corn. However, it has been reported that germination enhances TPC levels in other grains such as wheat, barley, rye, rice and oat (Bondia-Pons, Aura, Vuorela, Kolehmainen, Mykkänen, & Poutanen, 2009; Cáceres et al., 2014; Donkor, Stojanovska, Ginn, Ashton, & Vasiljevic, 2012; Tian, Xie, Shi, Wu, Cai, Xu, et al., 2010; van Hung, Maeda, & Morita, 2015). Contrarily, a TPC-reducing trend of germination has been observed for purple corn in the present work, phenomenon that can be attributed to the activation of polyphenoloxydases, enzymes responsible for phenolic compounds degradation, during germination or also to the leaching of these compounds during the soaking period prior to germination, as it has been previously observed in grain legumes (López-Amorós, Hernández, & Estrella, 2006).

The predictive model obtained for TPC content using coded factors was the following:

$$\text{TPC (mg GAE/100g)} = 1434.03 - 38.54 X_1 + 175.03 X_1^2 + 6.13 X_2 - 68.02 X_2^2 + 8.86 X_1 X_2$$

All the terms of the second-order polynomial equation had significant influence on TPC ($P \leq 0.05$) levels in germinated purple corn, indicating that both time and temperature strongly influence the accumulation of TPC in purple corn sprouts. The value observed for determination coefficient ($R^2 = 0.94$) and the adjusted determination coefficient (Adj. $R^2 = 0.83$) demonstrated the adequacy of the quadratic model to explain the variation in the content of this phytochemical as a result of germination conditions.

A response surface plot was generated to better visualize the combined effect of the independent variables (germination time and temperature) on the content of TPC in purple corn sprouts (Figure 2A). As can be observed, TPC levels in germinated purple corn increased as germination time increased up to 63 h, decreasing with longer germination times. Regarding temperature, TPC concentration increased in purple corn sprouts up to 26 °C, decreasing afterwards. Thus, combinations of germination times ranging from 21 h to 63 h and temperatures from 14 °C to 26 °C caused high accumulation of TPC in germinated purple corn.

The more suitable conditions to maximize the TPC content of germinated purple corn corresponded exactly to the central point (0, 0), with germination time and temperature of 42 h and 20 °C, respectively. Purple corn sprouts obtained at these conditions exhibited a TPC content that was 15% lower than that of non-germinated purple corn seed.

3.3. Influence of germination conditions on antioxidant activity of purple corn sprouts

Purple corn seeds showed an antioxidant activity of 4837.06 ± 221.91 mg TE/100 g d.m., which is in agreement with that reported for a Chinese purple corn variety (Deng, Yang, Zhang, & Li, 2015) and higher than the levels previously reported for several purple and blue corn varieties (200-725 mg TE/100 g d.m.) (Del Pozo-Insfran, Brenes, Serna Saldivar, & Talcott, 2006; Mrad et al., 2014; Polthum & Ahromrit, 2014). The differences in the variety studied as well as in the method used for measuring antioxidant activity could explain the differences observed between studies. Germination brought about a reduction in the ORAC values, which ranged from 2060.83 to 4092.37 mg TE/100 g d.m, depending on germination conditions (Table 2). This decrease of antioxidant activity during purple corn germination has been previously observed by other authors (Polthum & Ahromrit, 2014). The high antioxidant activity of purple corn has been associated with its high content of anthocyanins (Lopez-Martinez et al., 2009; Yang, & Zhai, 2010), that are water-soluble

compounds. The leaching of some antioxidant soluble phenolic compounds into water during soaking could be responsible for the reduction of the antioxidant activity of purple corn sprouts, as it has been previously reported for legumes (Xu & Chang, 2008).

The antioxidant activity of germinated purple corn was fitted to the following second-order quadratic model, based on the coded values of the independent variables:

$$\text{ORAC} = 3149.64 + 79.62 X_1 + 75.26 X_1^2 + 449.72 X_2 - 249.39 X_2^2 + 543.78 X_1.X_2$$

The value of the coefficient of determination was calculated, indicating that the fitted quadratic model accounted for 83% of the variations in the experimental data. The adjusted R^2 value was 0.81, indicating the adequacy of the model. The sign and magnitude of the regression coefficients in the equation indicate the effect of each independent variable on the antioxidant activity of purple corn sprouts. Negative signs mean decrease in response when the level of the variable increases, effect only observed for the quadratic term of temperature.

The predicted response surface plot for the antioxidant activity of germinated purple corn as a function of germination time and temperature is shown in Figure 2B. As can be observed from the figure, the highest antioxidant activity in purple corn sprouts were observed with long germination times ranging from 63 h to 72 h and high temperatures from 26 °C to 28 °C. The best condition corresponded to 63 h of germination at 26 °C. It has been previously reported that temperatures around 30 °C are the most favorable for germinating purple corn, since it maximized germination rate (Deng, Yang, Zhang, & Li, 2015). Purple corn sprouts obtained in these conditions showed a reduction of 15% in the antioxidant activity as compared with non-germinated purple corn.

3.4. Model fitting

The analysis of variance (ANOVA) showed that the R^2 and adjusted R^2 values of all the parameters for TPC and antioxidant activity were above 0.75. The quadratic polynomial models generated for TPC and antioxidant activity of purple corn sprouts were highly significant with p-values of 0.0002 and 0.0005, respectively. The lack of fit statistics for these parameters were not significant ($P>0.05$), F-values ranged from 39.90 to 56.41 and the coefficients of variance were 2.64% and 5.59% for TPC and antioxidant activity models, respectively, strengthening the reliability of the models. The RSM optimization showed that the

optimal germination conditions for maximizing the content of the bioactive compounds analyzed and the antioxidant activity were temperature of 26 °C and time of 63 h (Figure 2).

3.5. Identification of phenolic compounds in purple corn seeds and sprouts.

Identification and quantification of individual phenolic compounds were performed on purple corn seeds and sprouts obtained in the optimal conditions. Data (retention time, $\lambda_{\max}$ in the visible region, pseudomolecular ions and main fragment ions observed in MS²) obtained by HPLC-DAD-ESI/MS regarding phenolic compound (anthocyanin and non-anthocyanin compounds) identification are presented in Tables 3 and 4.

3.5.1. Anthocyanins. Up to 21 anthocyanin pigments were detected in raw and germinated purple corn (Table 3). Pigments were cyanidin (Cy), pelargonidin (Pg) or peonidin (Pn) derivatives, as demonstrated for their UV-vis spectra and mass spectral data. Peaks 7, 8, 10, 12 and 15 were identified as Cy 3,5-diglucose (peak 7), Cy 3-glucoside (peak 8), Pg 3-glucoside (peak 10), Pn 3-glucoside (peak 12) and delphinidin 3-glucoside (peak 15). The identity of these compounds was elucidated by comparison of their chromatographic characteristics and absorption spectra with data in our library and confirmed by mass analysis (Table 3).

Compounds 1-6 and 9 were assigned to condensed pigments containing C-C linked anthocyanin (Cy, Pn or Pg) and (epi)catechin residues. These compounds presented bathochromic shifts in the visible region of their absorption spectra with regard to the parent anthocyanin (Table 3). Pigments with similar structural characteristics were also found in purple corn extracts by other authors (de Pascual-Teresa, Santos-Buelga & Rivas-Gonzalo, 2002; González-Paramás et al., 2006; González-Manzano et al., 2008).

Peaks 13, 14 and 16-21 corresponded to acyl derivatives of cyanidin, peonidin and pelargonidin. Peak 13, showed $\lambda_{\max}$ 516nm and spectrum molecular ion at m/z 535 releasing a unique MS² fragment at m/z 287 ([M-248]⁺, loss of malonylglycoside moiety). For peaks, 14, 16, 17 and 19, the molecular ions at m/z 535, 519 and 549, that release three and two fragments MS² at m/z 491, 475, 505 (-44 amu, loss of and carboxylic moiety), m/z 449, 463 (- 86 amu, loss a malonyl residue), and m/z 287 (cyanidin), 301(peonidin) and 271 (pelargonidin), corresponded with Cy 3-*O*-(6"-malonylhexoside), Pn 3-*O*-(6"-malonylhexoside), Pg 3-*O*-(6"-malonylhexoside), respectively. Further confirmation of the identity of these compounds were provided by comparison of their characteristics with those of the same compounds previously identified also in purple corn (de Pascual-Teresa et al., 2002). Peaks 18, 20 and 21 were 86 amu greater than peaks 14, 16 and 17 and

showed a similar fragmentation pattern, so that it can be assigned to an Cy 3-*O*-(6''-dimalonylhexoside), Pn 3-*O*-(6''-dimalonylhexoside, Pg 3-*O*-(6''-dimalonylhexoside).

3.5.2. Non-anthocyanins. Twenty-five non anthocyanin compounds were identified in purple corn belonging to hydroxybenzoic, hydroxycinnamic derivatives and flavonol phenolic classes (Table 4).

Hydroxybenzoic acids derivatives were identified as vanillic hexoside acid (peak 1) based on their UV-vis spectrum, precursor ion $[M-H]^-$ at m/z 329 and fragment ion at m/z 167, $[M-162]^-$, corresponding to the loss of an hexose moiety). Two compounds were identified as *p*-hydroxybenzoic (peak 6) and vanillic acids (peak 9) by comparison of their UV-vis spectra and retention times with commercial standards.

Peaks 2, 3, 5 were identified as quinic acid derivatives according to their UV-vis spectra, and precursors ions $[M-H]^-$ 367 and 353, all of them with a fragment ion at m/z 191, corresponding to an quinic acid residue, accompanied by a secondary fragment ion at m/z 193 (ferulic acid) and 179 (caffeic acid).

Other compounds were identified as feruloyl derivative acids (peaks 4, 21, 23-25) based on their UV-vis spectra with maximum at 328-330, and fragment ion at m/z 193 corresponding to ferulic acid unit. These compounds could not be fully identified and were tentatively identified as ferulic acid derivative.

Peaks 11-20 were identified as flavonol derivatives from kaempferol, quercetin and isorhamnetin. Compounds 13, 16-19 were positively identified as quercetin 3-*O*-rutinoside, kaempferol 3-*O*-rutinoside, isorhamnetin 3-*O*-rutinoside and quercetin 3-*O*-glucoside, kaempferol 3-*O*-glucoside, isorhamnetin 3-*O*-glucoside, by comparison with authentic standards. Compounds 11, 12, 14, 15, 20 showed a pseudomolecular ion $[M-H]^-$ at m/z 593, 463, 447, 477 and 489, and similar MS^2 fragmentation patterns releasing one fragment 285 (kaempferol), 301 (quercetin) and 315 (isorhamnetin), from the losses of rhamnoside hexoside ($[M-308]^-$), hexoside ($[M-162]^-$), acetylhexoside ($[M-42-162]^-$).

In agreement with our results, previous studies showed that ferulic acid derivatives were the most dominant non anthocyanin compound found in purple corn (Cuevas Montilla, Hillebrand, Antezana, & Winterhalter, 2011). Furthermore, the presence of kaempferol and quercetin derivatives has been previously reported in different Peruvian purple corn varieties (Pedreschi & Cisneros-Zevallos, 2007; Ramos-Escudero, Muñoz, Alvarado-Ortíz, Alvarado, & Yáñez, 2012).

3.6. *Effect of germination on phenolic profile of purple corns.*

Results showed significant differences in the phenolic profile between germinated and non-germinated purple corn (Tables 5 and 6).

Anthocyanin was the most abundant phenolic class detected in purple corn seeds and sprouts (Table 5), accounting for 92% and 89% of total phenolic compounds, respectively. The main anthocyanin found in purple corn seeds was Cy-3-*O*-glucoside that accounted for 39%, and its respective malonyl derivative. These results are in agreement with previous studies that identified Cy-3-*O*-glucoside as the major anthocyanin in purple corn, constituting 30-38% of the total anthocyanin content (Abdel-Aal, Young, & Rabalski, 2006; Pedreschi & Cisneros-Zevallos, 2007). Moreover, considerable concentrations of condensed pigments (flavonol-anthocyanins) were found in purple corn (47% of total anthocyanins). A similar anthocyanin composition has been previously reported in different varieties of purple corn (De Pascual-Teresa y col., 2002; Moreno, Sánchez, Hernández, & Lobato, 2005; Pedreschi & Cisneros-Zevallos, 2007). As it can be observed from Table 5, germination brought about a significant ($P \leq 0.05$) reduction of most of the anthocyanins present in purple corn seeds, with the exception of peonidin malonyl derivatives that significantly increased ($P \leq 0.05$) after germination. As it was observed for seeds, Cy-3-*O*-glucoside was the predominant compound found in purple corn sprouts, accounting for 26% of the total anthocyanin content, followed by Cy 3-*O*-(6"-malonylglucoside) and Pn 3-*O*-(6"-malonylglucoside), compounds that constituted 16% and 7%, respectively, of the total anthocyanins. These changes of anthocyanins observed during germination caused significant differences ($P \leq 0.05$) in the total content of these compounds between raw and germinated purple corn.

Important variations of non-anthocyanin compounds were observed during germination (Table 6). The most outstanding changes found as consequence of germination were the reduction of kaempferol-3-*O*-glucoside to not detectable levels and the sharp increase of vanillic acid and the corresponding hexoside as well as that of caffeoylquinic acid. The other phenolic compounds identified in raw corn decreased significantly ($P \leq 0.05$) during germination, with the exception of kaempferol rhamnoside hexoside and kaempferol acetylhexoside, which did not change.

There is no information in the literature on the effects of germination on the phenolic composition of purple corn. To our knowledge, this is the first study showing a detailed description of the phenolic profile of

purple corn sprouts, demonstrating that they are an excellent source of anthocyanins and some hydroxycinnamic derivatives and flavonols.

Purple corn sprouts could be consumed raw or slightly cooked in salads and sandwiches and even as decorative appetisers or as an ingredient in soups, providing health-promoting benefits to consumers.

4. Conclusions.

Purple corn sprouts constitutes an excellent source of GABA and phenolic compounds and show high antioxidant activity. However, phytochemical composition of germinated purple corn is strongly influenced by germination conditions. Temperature of 26 °C and time of 63 h were identified as the optimal conditions by RSM for enhancing the GABA and TPC content as well as the antioxidant activity of purple corn. The predicted models developed for TPC and antioxidant activity showed high coefficients of determination, demonstrating their adequacy to explain the variations in experimental data. Anthocyanins, particularly cyanidin, peonidin and pelargonidin glycosides or their acylated counterparts, were the most abundant phenolic class in these sprouts.

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1 **Figure captions**

2 **Figure 1.** Purple corn sprout obtained after germination at 26°C for 63 h.

3 **Figure 2.** Response surface plots for TPC content (A) and antioxidant activity (B), as a function of
4 germination time and temperature, in germinated purple corn.

Table 1. Coded levels for independent variables used in the experimental design.

Coded	Independent variables		Levels			
	Real	$-\alpha$	-1	0	+1	$+\alpha$
X_1	Time of germination (h)	12	21	42	63	72
X_2	Temperature of germination ($^{\circ}\text{C}$)	12	14	20	26	28

Response surfaces were obtained using $\pm\alpha = 1.41$

Table 2. Experimental conditions and the response values observed with different combinations of germination times and temperatures for purple corn.

Coded level			Response values		
Exp	X ₁	X ₂	GABA (mg/100g)	TPC (GAE mg/100g)	ORAC (mg TE/100g)
1	- 1 (21)	- 1 (14)	25.03±2.52 ^{ab}	1178.53±56.06 ^{bc}	2649.02±63.53 ^b
2	+1 (63)	- 1 (14)	47.64±4.16 ^d	1042.82±15.38 ^a	2250.45±90.90 ^a
3	- 1 (21)	+ 1 (26)	30.84±1.94 ^b	1223.39±53.41 ^{cd}	2315.80±138.55 ^a
4	+1 (63)	+ 1 (26)	53.17±4.62 ^e	1123.13±50.70 ^{ab}	4092.37±149.55 ^e
5	-α (12)	0 (20)	24.31±2.31 ^{ab}	1158.86±69.71 ^{bc}	3710.73±116.20 ^d
6	+ α (72)	0 (20)	22.45±2.07 ^a	1107.11±77.71 ^{ab}	3186.75±224.94 ^c
7	0 (42)	-α (12)	25.15±0.29 ^{ab}	1373.89±31.13 ^{ef}	2060.83±126.42 ^a
8	0 (42)	+ α (28)	40.48±1.21 ^c	1320.11±75.27 ^{de}	3538.04±156.94 ^d
9	0 (42)	0 (20)	22.40±1.13 ^a	1357.49±34.39 ^{ef}	3006.90±387.82 ^c
10	0 (42)	0 (20)	25.75±1.22 ^{ab}	1496.29±59.75 ^g	3214.09±310.02 ^c
11	0 (42)	0 (20)	23.63±2.42 ^{ab}	1448.32±16.39 ^{fg}	3227.92±189.11 ^c

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 3. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification of anthocyanins in raw and germinated purple corn.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion [M+H] ⁺ (m/z)	MS ² (m/z)	Tentative identification
1	8.93	446/528			Direct condensed
2	9.32	444/530	899	737(16), 575(5)	(Epi)catechin (4-8)-Cyanidin 3,5 diglucoside
3	12.49	432/516	883	721(35), 559(11)	(Epi)catechin (4-8)-Pelargonidin 3,5 diglucoside
4	12.87	449/528	899	-	(Epi)catechin (4-8)-Cyanidin 3,5 diglucoside
5	13.85	438/528	913	751(24), 589(18)	(Epi)catechin (4-8)-Peonidin 3,5 diglucoside
6	14.92	446/536	985	823(6), 737(9), 575(6)	(Epi)catechin (4-8)-Cyanidin 3-malonylglycoside-5 glucoside
7	18.56	518	611	-	Cyanidin 3,5 diglucoside
8	20.98	518	449	287(100)	Cyanidin 3- <i>O</i> -glucoside
9	23.56	526	883	721(36), 559*, 433*	(Epi)Catechin (4-8)-Pelargonidin 3,5 diglucoside
10	25.09	504	433	271(100)	Pelargonidin 3- <i>O</i> -glucoside
11	27.59	517	535	287(100)	Cyanidin 3- <i>O</i> -malonylhexoside
12	28.22	518	463	301(100)	Peonidin-3- <i>O</i> -glucoside
13	28.74	516	535	287(100)	Cyanidin 3- <i>O</i> -malonylhexoside
14	31.95	518	535	491(3), 449(2), 287(100)	Cyanidin 3- <i>O</i> -(6"-malonylglycoside)
15	32.6	538	465	303(100)	Delphinidin 3- <i>O</i> -glucoside
16	34.8	530	549	301(100)	Peonidin 3- <i>O</i> -(6"-malonylhexoside)
17	35.19	504	519	475(3), 271(100)	Pelargonidin 3- <i>O</i> -(6"-malonylglycoside)
18	35.87	516	621	561*, 535*, 287(100)	Cyanidin 3- <i>O</i> -(3'', 6''-dimalonylhexoside)
19	36.68	520	549	505(2), 463(2), 301(100)	Peonidin 3- <i>O</i> -(6"-malonylglycoside)
20	39.97	504	605	561(3), 519*, 271(100)	Pelargonidin 3- <i>O</i> -(3'', 6''-dimalonylhexoside)
21	40.97	520	635	591(4), 549*, 301(100)	Peonidin 3- <i>O</i> -(3'', 6''-dimalonylhexoside)

* Relative abundance $\leq$ 1%

Table 4

Table 4. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification of non-anthocyanin phenolic compounds in raw and germinated purple corn.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion [M-H] ⁻ (m/z)	MS ² (m/z)	Tentative identification
1	6.15	260,294	329	167(100)	Vanillic acid hexoside
2	7.32	314	367	193(100),191(16),173(10),134(52)	3- <i>O</i> -Feruloylquinic acid
3	7.73	324	353	191(100),179(4),161(3)	3- <i>O</i> -Caffeoylquinic acid
4	8.50	329	383	193(2),189(100)	Ferulic derivative acid
5	8.55	328	353	191(13)	Caffeoylquinic acid
6	9.07	256	-	-	<i>p</i> -Hydroxybenzoic acid
7	9.67	325	355	193(100)	Ferulic acid hexoside
8	9.97	268	372	210(4), 192(11), 164(100), 149(89)	Phenylalanine derivative
9	10.77	260,294	-	-	Vanillic acid
10	11.93	348	579	489(24), 459(16), 429(6), 369(19), 339(2)	2''- <i>O</i> -Pentoside-8- <i>C</i> -hexoside luteolin
11	16.61	346	593	285(100)	Kaempferol rhamnoside hexoside
12	18.10	356	463	301(100)	Quercetin 3- <i>O</i> -hexoside
13	18.84	346	609	301(100)	Quercetin-3- <i>O</i> -rutinoside
14	19.53	346	447	285(100)	Kaempferol <i>O</i> -hexoside
15	19.85	356	477	315(60)	Isorhamnetin- <i>O</i> -hexoside
16	20.24	352	463	301(50)	Quercetin- <i>O</i> -glucoside
17	23.39	358	623	315(100)	Isorhamnetin-3- <i>O</i> -rutinoside
18	23.59	344	447	285(100)	Kaempferol-3- <i>O</i> -glucoside
19	24.88	354	477	315(100)	Isorhamnetin-3- <i>O</i> -glucoside
20	26.06	360	489	285(100)	Kaempferol acetylhexoside
21	33.90	328	521	317(86), 193(31)	Ferulic derivative acid
22	36.53	330	777	193(2), 175(16)	Dicaffeoyl-succinoylquinic acid
23	38.92	328	819	777(7), 193(2),175(9)	Ferulic derivative acid
24	40.26	328	819	777(13), 193(2),175(16)	Ferulic derivative acid
25	43.32	330	861	819(11), 193(2),175(10)	Ferulic derivative acid

Table 5. Concentration ($\mu\text{g/g}$) of anthocyanin phenolic compounds in raw and germinated (26 °C for 63 h) purple corn.

Peak	Rt (min)	Tentative identification	Raw	Germinated
1	8.93	Direct condensed	71.69 ± 4.02^b	27.35 ± 0.52^a
2	9.32	(Epi)catechin (4-8)-Cyanidin 3,5 diglucoside	183.87 ± 18.67^b	53.94 ± 7.82^a
3	12.49	(Epi)catechin (4-8)-Pelargonidin 3,5 diglucoside	25.44 ± 1.20^b	14.17 ± 0.20^a
4	12.87	(Epi)catechin (4-8)-Cyanidin 3,5 diglucoside	29.35 ± 1.69^b	18.41 ± 0.50^a
5	13.85	(Epi)catechin (4-8)-Peonidin 3,5 diglucoside	19.76 ± 1.88^a	18.28 ± 1.52^a
6	14.92	(Epi)catechin (4-8)-Cyanidin 3-malonylglycoside-5 glucoside	60.88 ± 3.68^b	23.07 ± 1.06^a
7	18.56	Cyanidin 3,5 diglucoside	25.01 ± 1.14^b	16.25 ± 1.53^a
8	20.98	Cyanidin 3- <i>O</i> -glucoside	1406.96 ± 68.92^b	926.06 ± 40.53^a
9	23.56	(Epi)Catechin (4-8)-Pelargonidin 3,5 diglucoside	32.70 ± 1.09^b	24.29 ± 0.25^a
10	25.09	Pelargonidin 3- <i>O</i> -glucoside	111.57 ± 7.24^b	55.62 ± 2.50^a
11	27.59	Cyanidin 3- <i>O</i> -malonylhexoside	61.05 ± 1.48^b	37.09 ± 3.57^a
12	28.22	Peonidin-3- <i>O</i> -glucoside	163.75 ± 6.03^a	156.21 ± 7.60^a
13	28.74	Cyanidin 3- <i>O</i> -malonylhexoside	98.66 ± 5.23^a	100.49 ± 8.91^a
14	31.95	Cyanidin 3- <i>O</i> -(6"-malonylglycoside)	820.70 ± 30.97^b	553.24 ± 33.63^a
15	32.6	Delphinidin 3- <i>O</i> -glucoside	47.36 ± 3.74^b	nd ^a
16	34.8	Peonidin 3- <i>O</i> -(6"-malonylglycoside)	20.06 ± 1.19^a	26.58 ± 1.17^b
17	35.19	Pelargonidin 3- <i>O</i> -(6"malonylglycoside)	117.60 ± 7.17^b	88.86 ± 10.02^a
18	35.87	Cyanidin 3- <i>O</i> -(3'', 6''-dimalonylglycoside)	37.60 ± 1.27^b	28.15 ± 1.51^a
19	36.68	Peonidin 3- <i>O</i> -(6"-malonylglycoside)	208.81 ± 6.37^a	239.51 ± 20.74^b
20	39.97	Pelargonidin 3- <i>O</i> -(3'', 6''-dimalonylglycoside)	59.01 ± 2.64^a	53.19 ± 3.37^a
21	40.97	Peonidin 3- <i>O</i> -(3'', 6''-dimalonylglycoside)	34.59 ± 1.87^a	52.55 ± 3.81^b
Total			3636.41 ± 160.62^b	2443.05 ± 36.97^a

Nd: not detected

Nq: not quantified

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

Table 6. concentration ($\mu\text{g/g}$) of non-anthocyanin phenolic compounds in raw and germinated (26 °C for 63 h) purple corn.

Peak	Rt (min)	Tentative identification	Raw	Germinated
1	6.15	Vanillic acid hexoside	nd ^a	1.69 $\pm$ 0.94 ^b
2	7.32	3- <i>O</i> -Feruloylquinic acid	6.43 $\pm$ 1.35 ^a	nd ^b
3	7.73	3- <i>O</i> -Caffeoylquinic acid	2.16 $\pm$ 0.24 ^b	1.67 $\pm$ 0.10 ^a
4	8.50	Ferulic derivative acid	2.57 $\pm$ 0.42 ^b	nd ^a
5	8.55	Caffeoylquinic acid	nd ^a	28.58 $\pm$ 2.21 ^b
6	9.07	<i>p</i> -Hydroxybenzoic acid	2.50 $\pm$ 0.66 ^b	nd ^a
7	9.67	Ferulic acid hexoside	0.76 $\pm$ 0.14 ^b	nd ^a
8	10.77	Vanillic acid	4.92 $\pm$ 0.67 ^a	6.08 $\pm$ 0.07 ^b
9	11.93	2''- <i>O</i> -pentoside-8- <i>C</i> -hexoside Luteolin	12.19 $\pm$ 0.88 ^b	5.97 $\pm$ 0.80 ^a
10	16.61	Kaempferol rhamnoside hexoside	13.23 $\pm$ 0.99 ^a	12.60 $\pm$ 1.63 ^a
11	18.10	Quercetin 3- <i>O</i> -hexoside	22.83 $\pm$ 1.91 ^b	13.98 $\pm$ 1.53 ^a
12	18.84	Quercetin-3- <i>O</i> -rutinoside	35.91 $\pm$ 1.59 ^b	23.40 $\pm$ 0.66 ^a
13	19.53	Kaempferol <i>O</i> -hexoside	nd ^a	25.42 $\pm$ 2.26 ^b
14	19.85	Isorhamnetin- <i>O</i> -hexoside	12.56 $\pm$ 1.31 ^b	nd ^a
15	20.24	Quercetin- <i>O</i> -glucoside	28.09 $\pm$ 1.68 ^b	19.90 $\pm$ 1.90 ^a
16	23.39	Isorhamnetin-3- <i>O</i> -rutinoside	28.65 $\pm$ 1.98 ^b	16.76 $\pm$ 1.73 ^a
17	23.59	Kaempferol-3- <i>O</i> -glucoside	42.38 $\pm$ 0.23 ^b	nd ^a
18	24.88	Isorhamnetin-3- <i>O</i> -glucoside	41.75 $\pm$ 1.70	32.86 $\pm$ 2.10
19	26.06	Kaempferol acetylhexoside	25.68 $\pm$ 3.49 ^a	21.27 $\pm$ 0.69 ^a
20	33.90	Ferulic derivative acid	45.06 $\pm$ 2.97 ^b	28.31 $\pm$ 3.36 ^a
21	36.53	Dicaffeoyl-succinoylquinic acid	3.35 $\pm$ 0.86 ^b	1.05 $\pm$ 0.07 ^a
22	38.92	Ferulic derivative acid	13.12 $\pm$ 1.07 ^b	5.24 $\pm$ 0.61 ^a
23	40.26	Ferulic derivative acid	4.30 $\pm$ 0.15 ^b	1.89 $\pm$ 0.36 ^a
24	43.32	Ferulic derivative acid	9.87 $\pm$ 0.78 ^b	5.64 $\pm$ 1.32 ^a
Total Non-flavonoid			77.77 $\pm$ 5.19^a	124.20 $\pm$ 14.22^b
Total flavonoid			249.17 $\pm$ 12.78^b	172.39 $\pm$ 19.43^a
Total			323.93 $\pm$ 14.70^a	296.59 $\pm$ 30.64^a

nd: not detected

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

Figure 1

Figure 2

Figure 2.