Antioxidant capacity, phenolic and vitamin C contents of quinoa (*Chenopodium quinoa* Willd.) as affected by sprouting and storage conditions

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Abstract

Antioxidant capacity (AC) of quinoa (*Chenopodium quinoa* Willd. cv. Real) seeds and sprouts obtained after 4 days of seed germination at 20°C and 70% humidity was evaluated using trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC) assays, able to highlight reducing activity and peroxyl radical scavenging capacity, respectively; phenolic content (PC) was also measured. Both TEAC and ORAC assays revealed a significantly higher (about 2- and 2.8-fold, respectively) AC of 4-day-old sprouts compared to seeds; consistently, also PC values of sprouts resulted about 2.6 times higher than seeds. In order to investigate the influence of storage on AC and PC, as well as on vitamin C content (VCC), 4-day-old sprouts were subjected for 7 days at 5°C to three different conditions of controlled atmosphere storage (CAS) compared with air. Interestingly, whatever the CAS conditions, storage of quinoa sprouts up to 7 days induced an increase of AC evaluated in terms of reducing activity by TEAC assay. Consistently, an increase of PC and VCC was measured during storage, positively correlated to TEAC values. Moreover, a decrease of peroxyl radical scavenging activity, measured by ORAC, was observed after 7 days of storage, in accordance with a shift of AC towards the reducing activity component. Overall, these findings indicate that sprouting approach using quinoa may provide highly antioxidant-enriched seedlings that may improve nutritional quality of diet or of functional foods. Interestingly, antioxidant properties of quinoa sprouts may be deeply influenced by storage, able to increase reducing activity by increasing phenols and vitamin C.

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Introduction

Quinoa (*Chenopodium quinoa* Willd.), a pseudo-cereal of Andean region, has received an increasing scientific and commercial attention in recent years, due to exceptional nutritional value of its seeds, associated to high protein quality and content, good balanced amino acid composition, high level of polyunsaturated fatty acids, dietary fibres, minerals and vitamins (Abugoch, 2009; Vega-Gálvez *et al.*, 2010). Quinoa seeds are also gluten-free, thus their use has expanded as healthy alternative to gluten-containing grains. Moreover, seeds contain significant amounts of bioactive compounds, including polyphenols (mainly phenolic acids, including vanillic acid, ferulic acid and their derivatives, as well as flavonoids, including quercetin, kaempferol and their glycosides) and tocopherols (vitamin E), tocotrienols and carotenoids (Tang *et al.*, 2015). In particular, quinoa seeds may represent an excellent source of natural freely soluble and highly accessible antioxidant compounds (Laus *et al.*, 2012a). Interestingly, it has been recently shown that quinoa may be well adapted also to Southern Italy environment (De Santis *et al.*, 2016).

In recent years, a new trend in nutrition is the consumption of germinated seeds. Sprouting is an important method for increasing nutritional (Ghorpade and Kadam, 1988; Augustin and Klein, 1989) and functional (Deshpande *et al.*, 2000) value of seeds. In fact, during germination reserves within the storage tissues of seeds are mobilised to support seedling growth and when the seed breaks dormancy, protective responses emerge through the increase of vitamins, phenols and other bioactive compounds showing antioxidant activity (Cevallos-Casals and Cisneros-Zevallos, 2010). In particular, accumulation of polyphenols during germination depends on biosynthesis of new compounds by induction of L-phenylalanine and L-tyrosine ammonia-lyases, representing key enzymes in the phenylpropanoid metabolism (Świeca, 2016), as well as on action of germination-induced endogenous esterases able to release cell wall bound phenolic compounds (Carciochi *et al.*, 2016). As for vitamin C, accumulation during germination was due to its *de novo* synthesis via activation of L-galactono-γ-lactone dehydrogenase, playing a key role

in the ascorbic acid biosynthetic pathway (Xu *et al.*, 2005); this ascorbic acid increase represents a specific metabolic process directly implicated in the modulation of initial embryo germination and plant growth, by protection of other biological substances from oxidative damage (Carciochi *et al.*, 2016). The knowledge about the mechanisms and enzymes involved in the accumulation of bioactive compounds during germination may provide information useful to develop genetic or agronomic management approach aimed at modifying the composition of bioactive compounds in both seeds and sprouts. However, the effect of germination process on the final nutritional quality of the sprouts greatly depends on the variety of the seeds, the environmental conditions such as temperature, humidity, light, *etc*., as well as the length of the process (Savelkoul *et al.*, 1992).

Some recently published papers regard the study of typical sprouts such as buckwheat, broccoli, mung bean and soybean, which are already easily available on the market (Alvarez-Jubete *et al.*, 2010; Pająk *et al.*, 2014). Some investigations have been also performed on sprouts from other edible seed species (Paśko *et al.*, 2009; Cevallos-Casals and Cisneros-Zevallos, 2010; Pająk *et al.*, 2014; Perales-Sánchez *et al.*, 2014). Most of these studies are focused on the evaluation of phenolic content (PC) and *in vitro* antioxidant capacity (AC) of sprouts obtained from different seed species in different germination conditions. To our best knowledge, only few reports investigated AC and PC of quinoa sprouts (Paśko *et al.*, 2009; Alvarez-Jubete *et al.*, 2010; Carciochi *et al.*, 2016; Świeca, 2016).

Two main objectives were pursued in the present study. Firstly, AC and PC of germinated quinoa seeds were assessed, with particular interest to the comparison between seeds and sprouts. Secondly, the postharvest performance of quinoa sprouts was studied with respect to both PC and AC changes during short-term storage under air and three different controlled atmosphere conditions; vitamin C content (VCC) was also evaluated. In particular, to obtain a comprehensive evaluation of AC, this was determined using two different methods: trolox equivalent antioxidant capacity (TEAC) (Re *et al.*, 1999) and oxygen radical absorbance capacity (ORAC) (Ou *et al.*, 2001). These methods highlight two different components of AC: TEAC mainly measures the reducing activity of antioxidants, while ORAC evaluates the peroxyl radical scavenging activity.

Materials and methods

Chemicals

Chemicals and solvents at analytical grade purity were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Plant material and growth conditions

Plant material consisted of organic quinoa seeds of the variety Real, chosen for its wide commercial use (Ecor, Schio, Italy). For AC and PC measurements on whole flour, the seeds were milled using a Cryo Mill cryogenic grinder (Retsch, Bergamo, Italy).

Sprouting was carried out as described by Paśko *et al.* (2009) with slight adjustments. After immersion in tap water for 3 h, seeds were germinated in a plant growth chamber (Piardi) for 4 days at constant temperature of 20±2°C and 70% humidity with a photoperiod of 24 h [values of 10,000 lux, 2.7 par (μE/m²×s) and 46.3 irradiance (watt/m²)]. A system that allows keeping seeds constantly moist in growth chamber was used. Seed germination rate (%) was 50±7.7 [standard error (SE)] and the bud from the 4-day-germinated seeds ranged from 0.7 to 1.2 cm.

After 4 days, sprouts were stored in glass containers (5 L) for 7 days at 5°C, continuously fluxed with a humidified flow of air or controlled atmosphere. The flow rate was defined on the basis of preliminary experiments based on respiration rate. Three conditions of controlled atmosphere storage (CAS) were compared with air: 5% O_2; 5% O_2 + 10% CO_2; 5% O_2 + 20% CO_2. Before storage (4-day-old sprouts) and after 2, 4 and 7 days, sprouts were harvested and extracted immediately for AC, PC and VCC measurements.

Extraction procedure for antioxidant capacity and phenolic content determination

Flour (1 g fresh weight, fw) was extracted with 40 mL of ice-cold absolute ethanol for 30 min under continuous stirring. Sprouts (1.5 g fw) were homogenised in ice-cold ethanol (30 mL) for 2 min at 13,000 rpm using an Ultra-Turrax homogeniser; then, the homogenate was kept on ice for 20 min, vigorously stirred at 5-10 min intervals. Both flour and sprout extracts were centrifuged at 10,000x*g* for 15 min at 4°C. The supernatants were recovered, filtered and evaporated to dryness under vacuum at 40°C. The dry residues were stored at -20°C; before use, they were reconstituted in ethanol.

Determination of antioxidant capacity by means of oxygen radical absorbance capacity and trolox equivalent antioxidant capacity methods

ORAC assay was performed as reported in Ou *et al.* (2001), modified as described in Soccio *et al.* (2016), by using a CLARIOstar microplate reader (BMG Labtech, Ortenberg, Germany) and 96-well plates. The TEAC protocol described in Re *et al.* (1999) and modified as in Laus *et al.* (2013, 2015) was applied. For both methods, three or four different amounts of sample were analysed in triplicate. AC was determined using a dose-response curve obtained using (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) as standard antioxidant.

Determination of phenolic content and vitamin C content

PC was determined using the colorimetric Folin-Ciocalteu method described in Singleton *et al.* (1999) and modified as reported in Pastore *et al.* (2009) and Laus *et al.* (2012b). Briefly, 62.5 μL of an appropriate dilution in ethanol of extract were mixed with 750 μL of water and 62.5 μL of Folin-Ciocalteu reagent; after 6 min, 625 μL of a 7.5% (w/v) sodium carbonate solution were added. The samples were mixed, incubated at room temperature for 90 min, and then centrifuged at 15,000x*g* for 2 min. Absorbance of the supernatant was measured at 760 nm. Three different amounts of sample were analysed in triplicate and PC values were calculated by means of a proper calibration curve prepared using gallic acid. Extraction and measurement of VCC were performed as reported in Amodio *et al.* (2015); VCC was calculated as sum of ascorbic and dehydroascorbic acid contents.

Statistical analysis

Statistical analysis was performed by using the statistical package Statistica, version 7.1 (StatSoft, Tulsa, OK, USA). Normal distribution of data and homogeneity of variances were verified by Shapiro-Wilk and Bartlett's tests, respectively. All data were subjected to analysis of variance (ANOVA) using a completely randomised design. In particular, data of Figure 1 were submitted to a *one-factor* ANOVA model considering the matrix (seeds and sprouts) as a single independent variable. Data of Figure 2 were

submitted to a *two-factor* ANOVA model analysing interactive effects of two independent variables [the two variables being *storage time* (2, 4, 7 days) and *storage condition* (air and controlled atmosphere, *i.e.* 5% O_2, 5% O_2 + 10% CO_2, 5% O_2 + 20% CO_2)]. The significant differences were assessed by Duncan's test at 0.05 P level of significance. In Tables 1 and 2, the F significance levels of ANOVAs are reported.

Results

AC values, determined by TEAC and ORAC assays, as well as PC values of extracts from quinoa seeds are reported in Figure 1, in comparison with those obtained from sprouts after 4 days of germination. In Table 1, results of one-way ANOVA of data of Figure 1 are reported, showing significant effect of matrix on all parameters under study. In particular, the reducing activity (TEAC) and peroxyl radical scavenging activities (ORAC) of 4-day-old quinoa sprouts resulted about 2- and 2.8-fold significantly higher than whole flour, respectively (Figure 1A and B). Accordingly, also PC values of sprouts were about 2.6 times higher than seeds (Figure 1C).

In another experiment, the effect of different storage times (ST) and storage conditions (SC) on AC, PC and VCC of 4-day-old sprouts was evaluated. In Table 2, outputs from two-way ANOVA, taking into account ST, SC and their interaction (ST×SC), are reported. Interestingly, ST significantly affected all examined variables, while SC revealed significant effect on AC and VCC, but not on PC; moreover, no significant ST×SC interaction was found only for TEAC. Values of TEAC, ORAC, PC and VCC of differently stored sprouts are shown in Figure 2. As for TEAC (Figure 2A), the largest significant differences among the applied CAS treatments were observed at the 7th day of storage, at which air induced the highest AC value compared to the other storage conditions. Interestingly, in all storage treatments, a statistically significant increase of TEAC values with increasing storage time up to a maximum at the 7th day was observed; in particular, TEAC value increased by about 3 times, from 2.4 to 6.9 µmol Trolox eq./g dry weight (dw), measured in seeds and sprouts air-stored for 7 days, respectively. ORAC protocol pointed out the highest differences among CAS conditions after 2 days of treatments (Figure 2B). Unlike TEAC measurements, a strong decrease of ORAC values was observed during storage, from 98 µmol Trolox eq./g dw, registered before storage, to a minimum of 22 µmol Trolox eq./g dw, measured in sprouts stored under 5% O_2 for 7 days. Moreover, unlike TEAC, a general decreasing trend of ORAC values with increasing storage time was observed in all tested conditions. Regarding PC, no statistically significant differ-

Table 1. Statistical significance of F values from one-way analysis of variance for the effect of the matrix (seeds or sprouts) on trolox equivalent antioxidant capacity, oxygen radical absorbance capacity and phenolic content.

Source of variance	DF	TEAC	ORAC	PC
Matrix	1	**	*	*
Error	3	-	-	-

DF, degree of freedom; TEAC, trolox equivalent antioxidant capacity; ORAC, oxygen radical absorbance capacity; PC, phenolic content. *P≤0.05; **P≤0.01.

Table 2. Statistical significance of F values from two-way analysis of variance for trolox equivalent antioxidant capacity, oxygen radical absorbance capacity, phenolic content and vitamin C content as affected by storage time, storage condition and their interaction.

Source of variance	DF	TEAC	ORAC	PC	VCC
ST	2	***	***	**	***
SC	3	***	***	ns	***
ST×SC	6	ns	***	*	***
Error	24				

DF, degree of freedom; TEAC, trolox equivalent antioxidant capacity; ORAC, oxygen radical absorbance capacity; PC, phenolic content; VCC, vitamin C content; ST, storage time; SC, storage condition. *P≤0.05; **P≤0.01; ***P≤0.001; ns, not significant.

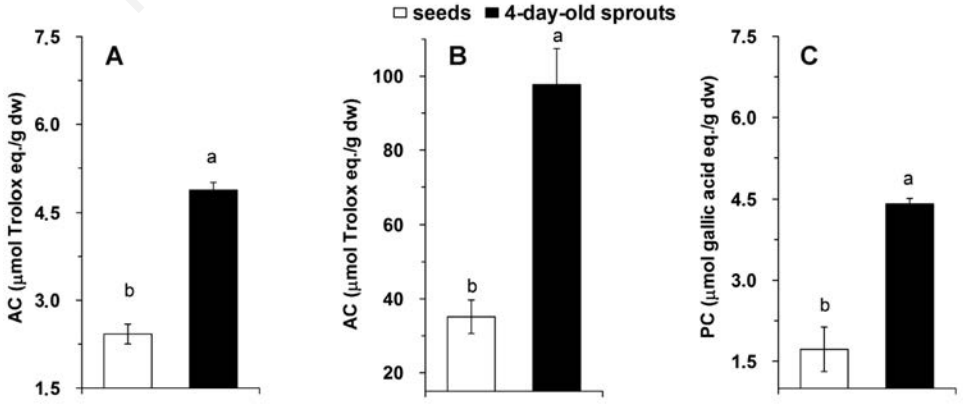

Figure 1. Antioxidant capacity (AC), evaluated by (A) trolox equivalent antioxidant capacity and (B) oxygen radical absorbance capacity methods, and (C) phenolic content (PC) of extracts obtained from quinoa seeds and 4-day-old sprouts. Extract preparation and determination of AC and PC were performed as reported in *Materials and methods* section. Data are reported as mean value±standard error (n=3). Different letters indicate significant differences at P≤0.05, according to Duncan's test.

ences among the different CAS treatments were obtained (Figure 2C), while a general increasing trend over storage time was registered, similarly to TEAC. As for VCC (Figure 2D), like TEAC assay, the highest statistically significant differences among storage conditions were observed after 7 days. Moreover, a significant increase was observed for all treatments during the first 4 days of storage, equal, on average, to about 1.5 times the VCC value (28 mg/100 g dw) measured before storage; interestingly, under air treatment, VCC further increased up to 52 mg/100 g dw at 7 days.

The correlation analysis of AC, PC and VCC values of stored quinoa sprouts is reported in Table 3. A highly statistically significant positive correlation was found between TEAC and PC values; this resulted higher when obtained with the fw values compared to the dw ones (0.575*** and 0.321*, respectively). Interestingly, a significant positive correlation was also obtained between TEAC and VCC values on both fw and dw basis (0.570* and 0.583*,

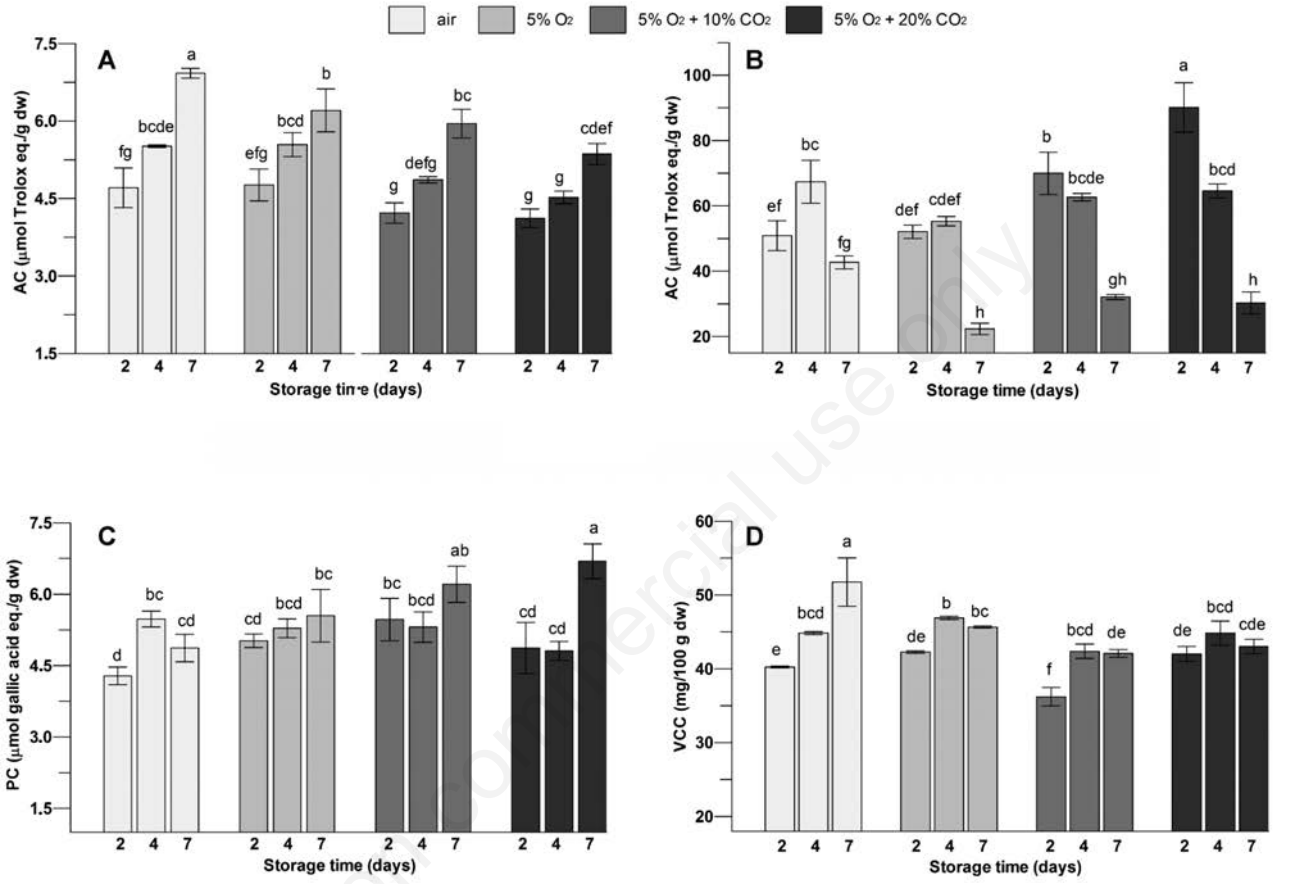

Figure 2. Antioxidant capacity (AC), evaluated by (A) trolox equivalent antioxidant capacity and (B) oxygen radical absorbance capacity methods, (C) phenolic content (PC) and (D) vitamin C content (VCC), of extracts obtained from 4-day-old quinoa sprouts submitted to 7-day storage under different conditions. Air and different CAS conditions (5% O_2, 5% O_2+10% CO_2, 5% O_2+20% CO_2) are described in *Materials and Methods* section. The same section reports how extract preparation and determination of AC, PC and VCC were performed. Data are reported as mean value±standard error (n=3). Different letters indicate significant differences at P≤0.05, according to Duncan's test.

Table 3. Correlation coefficients (r) among phenolic content, vitamin C content and antioxidant capacity, evaluated by trolox equivalent antioxidant capacity and oxygen radical absorbance capacity methods, on quinoa sprout extracts.

Traits	PC_{fw}	PC_{dw}	VCC_{fw}	VCC_{dw}	$AC_{TEAC\ fw}$	$AC_{TEAC\ dw}$	$AC_{ORAC\ fw}$
PC_{dw}	0.904***						
VCC_{fw}	0.264	0.062					
VCC_{dw}	0.431	0.231	0.952***				
$AC_{TEAC\ fw}$	0.575***	0.235	0.570*	0.579*			
$AC_{TEAC\ dw}$	0.595***	0.321*	0.588*	0.583*	0.975***		
$AC_{ORAC\ fw}$	-0.587***	-0.449**	-0.411	-0.558*	-0.522***	-0.513***	
$AC_{ORAC\ dw}$	-0.597***	-0.418**	-0.458	-0.588*	-0.599***	-0.582***	0.993***

PC, phenolic content; VCC, vitamin C content; AC, antioxidant capacity; TEAC, trolox equivalent antioxidant capacity; ORAC, oxygen radical absorbance capacity; fw, fresh weight; dw, dry weight. Correlation analysis was performed using PC, VCC and AC values expressed as both fresh weight (fw) and dry weight (dw). *P≤0.05; **P≤0.01; ***P≤0.001.

respectively). On the contrary, ORAC values resulted inversely related to PC values, with a correlation higher in the case of fw values compared to the dw ones (-0.587*** and -0.418**, respectively), as well as to VCC values on dw basis (-0.588*). Accordingly, a highly significant negative correlation between both fw and dw ORAC and TEAC values was obtained (-0.522*** and -0.582***, respectively).

Discussion

The first goal of this study was the assessment of AC and PC of quinoa seeds and sprouts obtained after 4 days of seed germination. Results of AC, evaluated by the widely used TEAC and ORAC methods, highlighted high antioxidant properties of quinoa flour. Data fit well our previous results and confirm superiority of the freely soluble antioxidant fraction of quinoa seeds with respect to that of some cereals traditionally used for human foods (Laus *et al.*, 2012a). As for germinated seeds, our results showed that 4-day-old sprouts have a significantly higher AC than seeds, associated to a strong PC increase. These results are in accordance with some literature data, showing a remarkable increase of both AC and PC (in particular, protocatechuic, vanillic and caffeic acids, as well as kaempferol and quercetin glycosides) after germination of seeds of quinoa (Paśko *et al.*, 2009; Alvarez-Jubete *et al.*, 2010; Carciochi *et al.*, 2016) and of other species (Cevallos-Casals and Cisneros-Zevallos, 2010; Pająk *et al.*, 2014; Perales-Sánchez *et al.*, 2014).

The second aim of this study was focused on evaluation of AC, PC and VCC of 4-day-old quinoa sprouts during storage. In order to explore the possible effect of different times and modes of storage, air and three different CAS treatments were applied for 7 days. Under our experimental conditions, the different tested storage conditions did not significantly influence all examined parameters. Nevertheless, results obtained in this study allow identifying the air as the best storage condition able to guarantee, over a period of 7 days, the maximum value of VCC and of the related reducing activity evaluated by TEAC assay, together with a satisfactory level of PC. On the contrary, the interest may be to preserve the peroxyl radical scavenging activity, having potential physiological relevance (Huang *et al.*, 2005); in this case, the best condition of storage is the controlled atmosphere consisting of 5% O_2 + 20% CO_2 for 2 days, able to maximise the ORAC value. Further studies will be necessary to assess which of the two modes of antioxidant action may affect more deeply pro-healthy properties of quinoa sprouts.

On the other hand, storage time revealed significant effect on all variables under study. In fact, independently from the applied CAS treatment, a general increasing trend over storage time of AC values was observed, as evaluated by TEAC assay, as well as of PC and VCC values. This behaviour is in agreement with the similar chemical principles of TEAC and total phenols assay by Folin-Ciocalteu reagent, which are based mainly on single electron transfer reactions (Huang *et al.*, 2005), thus measuring reducing capacity of antioxidants. Vitamin C may also act as reducing agent. On the other hand, storage time induced a general decrease of ORAC values. The opposite behaviour of ORAC in respect to TEAC, PC and VCC may be explained in the light of different chemistry behind ORAC assay, able to quantify peroxyl radical scavenging capacity according to a hydrogen atom transfer-based mechanism (Huang *et al.*, 2005).

Overall, the picture emerging from AC dissection is that an increase of reducing activity of quinoa sprouts occurs during time, whatever the storage conditions, dependent on accumulation of phenolic compounds and vitamin C, which are able to act as reducing agents. Interestingly, by a physiological point of view, the concurrent loss of peroxyl radical scavenging activity suggests an interconversion of the two kinds of antioxidant modes.

Conclusions

Germination process may be an effective strategy to improve the antioxidant properties and phenolic content of quinoa seeds, thus increasing interest for utilisation of quinoa sprouts as an excellent source of natural antioxidant compounds in ready-to-eat fresh products or in the formulation of new healthy functional foods. Further studies will be necessary to elucidate the genetic and environmental influence on content and composition of sprout bioactive compounds. Finally, quinoa seed germination could also serve as a model for applying similar nutraceutical enhancement strategies to other crops.

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