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Original article

The effect of freezing at −18 °C and −70 °C with and without ascorbic acid on the stability of antioxidant in extracts of apple and orange fruits

Renata Madureira Polinati, Ana Luísa Kremer Faller & Eliane Fialho*

Departamento de Nutrição Básica e Experimental, Instituto de Nutrição Josué de Castro, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21941-590, Brasil

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Summary The influence of low temperature and storage time on the antioxidant capacity of standard solutions and apple and orange extracts was evaluated. In addition, the effects of ascorbic acid (AA) addition to the fruit extracts in terms of antioxidant capacity, AA content and soluble and hydrolyzable polyphenol contents were also analysed. Polyphenol contents in both apple and orange extracts were stable during storage period, which reflected also in the antioxidant capacity stability. Freezing at −18 °C did not result in different retention rates for polyphenols, AA and antioxidant capacity when compared to freezing at −70 °C. However, vitamin C content in orange juice, without AA addition, slightly increased along the experimental period (10 days). Thus, this study shows that it may not be necessary to measure the antioxidant capacity immediately after the preparation of fruit extracts or antioxidant standard solutions.

Keywords Antioxidant capacity, ascorbic acid, freezing, fruits, polyphenol.

Introduction

Fruits and vegetables contain many different antioxidant compounds, such as polyphenols, carotenoids, flavonoids and anthocyanins. These compounds are plant's secondary metabolites, which protect the plant against a variety of stresses and show high antioxidant activity *in vitro* (Holst & Williamson, 2008). Polyphenols, a class of phytochemicals (containing several hydroxyl groups on aromatic rings), are generally involved in defence against ultraviolet radiation, aggression by pathogens and sudden changes in temperature, among other challenges (Manach *et al*., 2004). They are the most abundant antioxidants in the human diet, and together with other dietary reducing agents, such as vitamin C, vitamin E and carotenoids, they may prevent various diseases associated with oxidative stress, such as cancers, cardiovascular diseases and inflammation (Scalbert & Williamson, 2000).

Apples contain large amounts of phenolic compounds, and the peels may contain four times the amount of these compounds compared to the pulp. Procyanidins correspond to the main group of polyphenols, followed by hydroxycinnamic acids and flavonols. These compounds are directly related to the high antioxidant capacity (AC) found in apples, particularly in the peels (Khanizadeh *et al*., 2008). On the other hand, apple juice has low amounts of vitamin C compared to citrus fruit juices, such as orange. Sacchetti *et al*. (2008) observed that antioxidant activity of apples was correlated with the total phenol concentration but not with ascorbic acid (AA) concentration.

Oranges contain high amounts of phenolic compounds, such as hydroxycinnamic acids and flavonoids; additionally, oranges are an important source of vitamin C (Klimczak *et al*., 2007). Rapisarda *et al*. (2008) showed that the AC is widely distributed among vitamin C and phenolic constituents in oranges, but vitamin C was the main antioxidant component, accounting for 87% of the total AC of these fruits.

Vitamin C is the most effective inhibitor of enzymatic browning, provided by the inhibition of oxidative enzymes present in fruits. Studies have shown that the use of additives, such as AA, is extremely important during the processing of fruit. The addition of this vitamin to beverages and its preservation under mild oxidative conditions demonstrate that phenolics in fruit juices have an ascorbate-sparing effect, when polyphenols have a higher redox potential when compared to AA (Miller & Rice-Evans, 1997). The lower AC can be observed in products derived from fruits compared to

*Correspondent: Fax: + 55 21 2280 8343;
e-mail: fialho@nutricao.ufrj.br

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fresh fruit, which is directly related to the processes of natural darkening that occur in these plants (Sacchetti *et al*., 2008). Others processes are also used to minimise the effects of natural browning of fruit. In their study, Oszmiański & Wojdyo (2008), evaluating the effect of the addition of rhubarb juice in apple purées, found that this addition increases the content of phenolic compounds and AC of purées. Already Markowski *et al*. (2009), in a study of phenolic compounds of clear apple juice using pectolytic mash enzymation, demonstrated that this method resulted in high values of phenolics when compared with commercial apple juices.

Freezing is one way to preserve the AC and bioactive components in food (Oszmiański *et al*., 2008). Several studies have shown the effects of storing fruit and their extracts at low temperatures, but few have actually examined these effects at very low temperatures, such as the temperature of liquid nitrogen. In a study with oranges, Rapisarda *et al*. (2008) noted that storage at a low temperature increased the content of some compounds, such as anthocyanins, hydroxycinnamic acids, total phenolics and vitamin C; moreover, storage at low temperatures increased the total AC of the samples. Thus, even frozen, some fruits and pulps can be excellent sources of phenolic compounds, thus maintaining their AC (Kuskoski *et al*., 2006).

Therefore, the aim of this work was to evaluate the influence of freezing at −18 °C or −70 °C and the time of storage (0–10 days) on the AC, soluble (SP) and hydrolyzable polyphenol (HP) contents and AA content in apple and orange extracts with or without added AA.

Materials and methods

Chemicals and reagents

Folin-Ciocalteu reagent, 2,6-dichlorophenolindophenol and oxalic acid were purchased from Merck. DPPH and AA were purchased from Sigma–Aldrich (St. Louis, MO, USA). Gallic acid was purchased from CQA Química (Campinas, SP, Brazil). All other chemicals and reagents were of analytical grade quality.

Fruits material

Orange (*Citrus sinensis L. Osbeck*) and apple (*Malus domestica*) fruits were acquired during harvest period at the local market in Rio de Janeiro, Brazil. Approximately 2 kg of each fruit was selected, from which damaged fruits were discarded. The fruit was then washed under running water and dried with paper. Oranges were manually peeled with a knife, removing all of the pumice and seeds. Apples with peels were cut manually into small pieces with a knife, and the seeds were removed. Fruit extracts were obtained using a commercial juice extractor (Samsom GB-9001; Greenbison Inc., Cypress, CA, USA), as it was shown in Faller & Fialho, 2009.

Sample preparation

After extraction, the fruit pulps were divided into two groups, those with 1% ascorbic acid added (with AA) and without ascorbic acid added (without AA). The samples were maintained either in a freezer (−18 °C) or in liquid nitrogen (−70 °C) for 10 days. Analyses of the AC were performed in intervals of 2 days, and the AA and polyphenol contents were analysed on days 0, 4, 6 and 10 of storage. Standard solutions of gallic acid and AA were prepared with a final concentration of 1%.

Polyphenols extraction

The extraction of SPs and HPs occurred in accordance with the protocol described by Vinson *et al*. (2001), with some modifications. The SP extraction solution consisted of deionised water and methanol (1:1 v/v). The HP extraction solution also contained hydrochloric acid at 1.2 M. Aliquots of 100 μL of the fruit extracts were added to microvials, and 500 μL of the extraction solutions were then added. The samples were placed in a water bath at 90 °C for 3 h. After this period, the samples were cooled to room temperature, and the volume was increased to 1000 μL with absolute methanol. The samples were then centrifuged at 1400 × ***g*** for 5 min. The supernatant, called polyphenol extracts (PE), was used for the SP and HP analyses.

Determination of polyphenol content

The determination of the SP and HP was performed using the Folin-Ciocalteu reagent, according to methodology described by Karou *et al*. (2005). In microvials, 75 μL of the Folin reagent (50%) was added to 30 μL of the PE, which was then maintained at room temperature for 5 min. After this period, 75 μL of sodium carbonate solution (20% anhydrous sodium carbonate) was added and maintained at room temperature for 30 min; subsequently, the absorbance of the solution was measured in a spectrophotometer at 750 nm. The content of SP and HP was expressed in mg of gallic acid equivalent (GAE)/mL.

Ascorbic acid analysis

The content of AA was determined in triplicate by titration with 2,6-dichlorophenolindophenol, according to AOAC (1984) and with the changes proposed by Benassi & Antunes (1988). The dilution was maintained in 1% oxalic acid, according to the standard protocol of Tillmans.

DPPH analysis

The AC by DPPH (1,1-Diphenyl-2-picrylhydrazyl) was measured according to method described by Kuskoski *et al*. (2006). The DPPH solution (100 μM) was prepared using 80% methanol. Then, 0.1 mL of each sample was added to 3.9 mL of the 100 μM DPPH solution. The tubes were carefully agitated and kept in the dark for 30 min before reading at 517 nm.

The AC was represented as % radical scavenging capacity (RSC) remaining after 30 min of reaction according to the following equation:

$$\%\mathrm{RSC} = (A_0 - A_t)/A_0$$

where A_0 represents the absorbance of the DPPH solution alone measured at zero time, and A_t is the absorbance for each sample 30 min after the addition of the DPPH solution. The value of A_0 was considered 100%.

Statistical analysis

Results were expressed as mean and standard deviation of two independent experiments each conducted in triplicates. The statistical analysis was performed using GraphPad Prism 5.0 for Windows (GraphPad Software Inc., La Jolla, CA, USA). A one-way ANOVA was applied, followed by Tukey's test. Trends were considered significant when the means of the compared sets differed at $P < 0.05$.

Results and discussion

Soluble and hydrolyzable polyphenol contents

Figure 1 shows the SP and HP contents in apple extracts. The HP and SP contents were higher with the addition of AA. Moreover, freezing at −18 °C and −70 °C over 10 days showed no influence on the HP or SP contents in the apple extracts with or without added AA (Fig. 1a–d).

The HP and SP contents in orange extracts are shown in Fig. 2. For the orange extracts stored at −18 °C, the HP and SP contents were higher with the addition of AA compared to the extracts without added AA up to day 10, showing the same profile described for the apple extract (Fig. 2a). For the SP content, during freezing at −18 °C, this content was significantly higher with the addition of AA in orange extracts when compared to the extracts without added AA. Only on the tenth day of freezing did this not occur (Fig. 2c). The addition of AA in samples freezed at −70 °C did not affect the HP

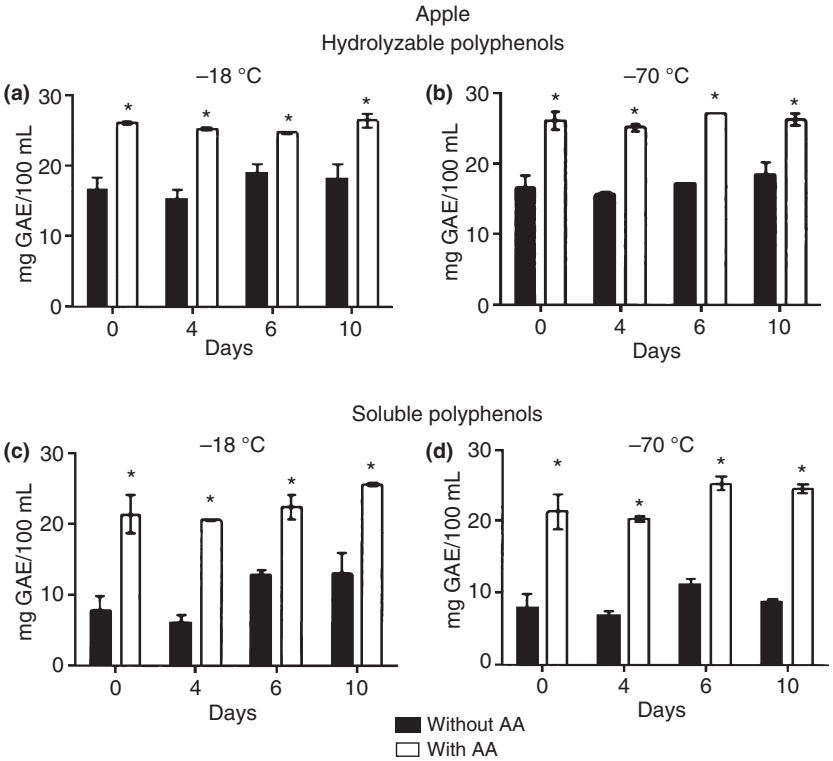

Figure 1 Hydrolyzable and soluble polyphenol contents in apple extracts without and with ascorbic acid (AA) during freezing at −18 °C and −70 °C over 10 days.

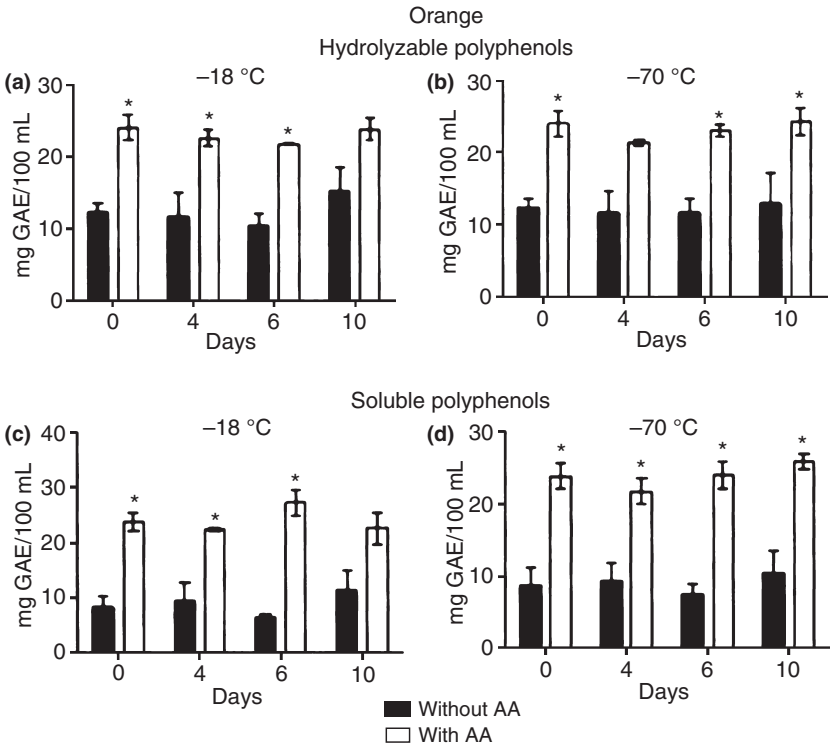

Figure 2 Hydrolyzable and soluble polyphenol contents in orange extracts, without and with ascorbic acid (AA), during freezing at −18 °C and −70 °C over 10 days.

content beyond the fourth day. On the other hand, freezing at −70 °C, the SP content was significantly higher with the addition of AA throughout the storage period (Fig. 2b,d). As with the apple extracts, freezing at −18 °C and −70 °C over ten days did not show any influence on the HP and SP contents in orange extracts with or without added AA (Fig. 2a–d).

In a study conducted with preparations of apple purée, the addition of AA also significantly increased the phenolic content (Oszmiański *et al*., 2008). This result can be explained by the protective effect caused by AA on the phenolic compounds. AA has been shown by some authors to be an excellent inhibitor of polyphenol oxidase (PPO), thus protecting the polyphenols of oxidation (Gundogmaz *et al*., 2003). On the other hand, some authors, such as Huang *et al*. (2005) and Scalbert & Williamson (2000), have suggested that the Folin-Ciocalteu reagent is not specific to phenolic components and therefore can be reduced by AA, producing false results. In this study, AA was added to the fruit extracts, but as was previously explained, during the extraction of the HP and SP; the samples were left for 3 h at 90 °C in a water bath, which should oxidise the acid and minimise its effect on the polyphenol contents.

During freezing, there are several changes that occur in the phenolic metabolism. One such change is the activation of the enzyme phenylalanine ammonia-lyase (PAL), which is an important enzyme in the phenylpropanoid pathway involved in phenolic synthesis. This activation can be related to the increase in phenolic compounds during storage at low temperatures (Padda & Picha, 2008). In this study, the content of polyphenols did not change significantly during freezing, in agreement with Tavarini *et al*. (2008), who showed no polyphenol increase in kiwi storage at low temperature for 2 months. Rapisarda *et al*. (2008), during 20 days of storage at low temperature for oranges, found an increase or stability in the total phenols content, depending on the variety of oranges. Evaluating the apples stored at 0 °C, Lejaa *et al*. (2003) found that the total phenolic content increased significantly. It is important to emphasise that in these comparative studies, the storage was for long periods, and this may have caused an increase in the activity of the PAL and, consequently, an increase in the polyphenols content. However, some authors have stated that the prolonged storage at a low temperature may result in more tissue darkening because of the oxidation of phenolics by the enzyme PPO (Padda & Picha, 2008).

Ascorbic acid content

The titrimetric method chosen for the analysis has been widely applied to foods and other samples because of the fact that it is an easy, rapid and cheap method that does not require any complex instrumen-

tation. The main drawback of the method resides in the fact that it cannot detect dehydroascorbic acid, species that can be converted back into AA and therefore is considered to exhibit vitamin C activity. Nonetheless, it is a useful method for the assessment of the vitamin C activity of orange juices, as several studies in which both species, AA and dehydroascorbic acids were quantified in such source showed that the latter accounts only for some 5% on average or even less of the vitamin C activity of the samples (Meléndez-Martínez *et al*., 2007a,b).

Table 1 shows the values of AA from apple and orange extracts. As expected, the addition of AA significantly increased the AA content in all samples regardless of the type of freezing employed. Analysing the apple extracts with or without added AA storing at −18 °C or −70 °C has no effect on AA content of apples extracts with or without added AA, even after 10 days. However, the orange juice without added AA showed some changes in the AA content independent of the freezing conditions. The AA content of the samples stored at −18 °C increased over the ten days, with significant differences for days 0 and 6 and for days 0 and 10. When stored at −70 °C, the AA content also increased significantly over the ten days (Table 1). The AA content in orange juice with added AA did not show any significant differences during the storage period.

The addition of organic substrates, rich in antioxidants, can preserve the vitamin C contents from fruit extracts against degradation (Pernice *et al*., 2008). Studies have shown that phenolic constituents found in organic extracts are capable of delaying the oxidative decomposition of vitamin C (Miller & Rice-Evans, 1997). Thus, we observed that the AA content increased with the addition of a synthetic substrate (AA) and that during freezing, the presence of both AA and polyphenols protected the extracts against the degradation of these antioxidant components.

Kalt *et al*. (1999) found that the storage of small fruits (strawberries, raspberries, highbush blueberries and lowbush blueberries) at low temperatures had minimal influence on the ascorbate content. Analysing the effect of industrial freezing at −40 °C in orange juice, Gil-Izquierdo *et al*. (2002) noted that this process does not affect the total vitamin C content. Nojavana *et al*. (2008) showed that the freezing sample preparation method

Table 1 Ascorbic acid content (mgAA/100 mL) in fruit extracts without and with ascorbic acid (AA) during freezing at −18 °C and −70 °C over 10 days

	Apple				Orange			
	−18 °C		−70 °C		−18 °C		−70 °C	
Days	Without AA	With AA	Without AA	With AA	Without AA	With AA	Without AA	With AA
0	2.1 ± 0.0	795.7 ± 53.8*	2.1 ± 0.0	795.7 ± 53.8*	38.1 ± 0.6[a]	1028.4 ± 51.1*	38.1 ± 0.6[a]	1028.4 ± 51.1*
4	1.6 ± 0.5	763.4 ± 10.8*	2.1 ± 0.0	860.2 ± 53.7*	40.3 ± 0.6	1170.4 ± 56.8*	39.8 ± 0.0[b]	1102.2 ± 45.4*
6	2.7 ± 0.5	790.3 ± 5.4*	2.7 ± 0.5	871.0 ± 10.8*	41.5 ± 0.6[b]	1119.3 ± 5.7*	40.9 ± 0.0[b]	1232.9 ± 5.6*
10	1.6 ± 0.5	741.9 ± 30.4*	1.6 ± 0.5	876.3 ± 48.4*	42.6 ± 0.6[b]	1176.1 ± 96.6*	43.2 ± 0.0[c]	1210.2 ± 28.4*

*Significant difference between groups (without and with AA): $P < 0.05$.
[a,b,c]Significant difference between days in the same group: $P < 0.05$.

Table 2 Antioxidant capacity percentage of fruit extracts without and with ascorbic acid (AA) during freezing at −18 °C and −70 °C over 10 days

	Apple				Orange			
	−18 °C		−70 °C		−18 °C		−70 °C	
Days	Without AA	With AA	Without AA	With AA	Without AA	With AA	Without AA	With AA
0	87.7 ± 1.6	94.2 ± 0.3*	87.7 ± 1.6	94.2 ± 0.3*	87.6 ± 3.0	89.7 ± 1.7	87.6 ± 3.0	89.7 ± 1.7
2	86.6 ± 2.6	94.3 ± 1.2*	90.9 ± 0.9	94.4 ± 1.2	88.1 ± 0.4	89.3 ± 0.9	89.1 ± 1.0	88.3 ± 1.8
4	88.1 ± 1.1	94.9 ± 1.0*	88.2 ± 1.6	95.1 ± 1.2*	90.1 ± 1.9	91.6 ± 0.7	88.9 ± 0.8	90.3 ± 2.4
6	88.0 ± 0.3	94.4 ± 0.5*	88.8 ± 1.5	94.6 ± 0.7*	89.9 ± 2.4	91.1 ± 1.4	88.5 ± 2.8	89.1 ± 2.6
8	86.3 ± 1.1	93.2 ± 0.7*	86.4 ± 1.1	93.3 ± 0.7*	89.9 ± 0.2	90.4 ± 0.0	88.0 ± 0.1	89.5 ± 0.7
10	89.1 ± 0.9	94.0 ± 1.1	88.6 ± 1.3	94.1 ± 0.9*	91.1 ± 0.6	91.3 ± 0.9	87.9 ± 2.7	90.8 ± 0.4

*Significant difference between groups (without and with AA): $P < 0.05$.
No significant difference was found between days in the same group: $P < 0.05$.

prevents oxidation and degradation of AA, in addition to providing a better extraction of AA in dog rose and orange samples. The relative stability of ascorbate among the fruits may also be related to the intracellular compartmentalisation of ascorbate and phenolic components (Kalt *et al*., 1999).

Oranges are rich sources of vitamin C, while apples, in contrast, have a low vitamin C concentration. Some authors have stated that when the vitamin C content is greater than the content of other compounds, such as phenolic compounds, it acts as the first line of antioxidant defence in plant tissue (Miller *et al*., 1997, Klimczak *et al*., 2007 and Tavarini *et al*., 2008). We showed that during freezing, the AA present in orange extracts without added AA is more unstable than in apple extracts with the same characteristics. The AA content in orange extracts without added AA increased gradually during storage at −18 °C and −70 °C over 10 days in all samples. This might be related to the previously stated idea that freezing may lead to a disruption of cells and, thus, the release of compounds present in the intracellular region to the extracellular regions. AA is among the compounds released, which may have caused the largest detection of this compound in orange extracts, a fruit rich in vitamin C (Kalt *et al*., 1999 and Viña & Chaves, 2006).

Antioxidant capacity

The AC of the samples is presented in Tables 2 and 3. The AC of the fruit juices from both apples and oranges did not vary significantly at −18 °C or −70 °C (Table 2). The same stability was found for the standards, gallic acid and AA, over 10 days (Table 3). The apple juice with added AA frozen at −18 °C and −70 °C showed significant differences for AC up to day 8 and day 10, respectively (Table 2). For orange juice, the AC did not show significant differences in either type of storage, as well as with or without AA addition. This indicated that for this fruit, the addition of AA does not influence the AC of samples recently extracted (0 day) or frozen over ten days. In addition, the percentage of AC was increased for most samples with added AA.

Shivashankara *et al*. (2004) showed that the AC of mangos remained constant for up to 20 days of storage at low temperature, but after this period, this capacity decreased. This suggested that an increase in AC during low-temperature storage may be possible only in fruit for which the contribution of total phenolics is greater than that of AA. Rapisarda *et al*. (2008) suggested that during storage at low temperatures, there is an increase in AC because of the production of various bioactive compounds, such as anthocyanins, flavanones, hydrocinnamics acids and total phenolics, such as that in oranges.

Cryogenic freezing at −30 °C induces large cracks and cellular collapse. This kind of study should be performed to assess whether the freezing processes, as well as the frozen storage period, result in the release of radical species and antioxidant compounds from injured plant tissues and cells, which could lead to an increase in AC (Olivera *et al*., 2008).

The stability of the polyphenol content during freezing, as well as the stability of the AA content (except in orange without added AA where there was an increase in content), also reflected the stability of the AC in all samples analysed during ten days of freezing. It should be noted that the addition of AA in orange extracts did not increase the AC of the samples, which supports the hypothesis that oranges, as a fruit rich in vitamin C, were already saturated with AA, meaning that additional AA would cause no increase in AC.

Table 3 Antioxidant capacity percentage of gallic acid (GA) and ascorbic acid (AA) standards during freezing at −18 °C and −70 °C over 10 days

	−18 °C		−70 °C	
Days	**Gallic acid**	**Ascorbic acid**	**Gallic acid**	**Ascorbic acid**
0	95.9 ± 0.5	96.3 ± 0.0	95.9 ± 0.5	96.3 ± 0.0
2	97.0 ± 1.0	98.0 ± 0.9	97.3 ± 1.0	97.5 ± 0.7
4	95.1 ± 0.6	95.9 ± 0.7	95.2 ± 0.7	95.9 ± 0.7
6	96.4 ± 1.1	95.8 ± 0.0	96.8 ± 1.6	95.4 ± 0.5
8	93.7 ± 1.4	94.7 ± 1.5	93.6 ± 1.4	94.5 ± 1.5
10	96.5 ± 1.5	97.0 ± 1.4	96.5 ± 1.5	97.1 ± 1.4

No significant difference was found between groups (without and with AA): $P < 0.05$.

No significant difference was found between days in the same group: $P < 0.05$.

Conclusions

This study shows that the presence of AA is not able to increase the AC of orange extracts (a fruit rich in vitamin C), but it could increase the AC of most apple extracts (a fruit low in vitamin C content). We conclude that freezing at low temperatures for up to 10 days of storage can maintain the HP and SP contents, AA and AC of these fruit extracts.

The results suggest that there is no need for immediate analysis of AC in plant extracts and standard antioxidants within the period evaluated in this study. Additionally, the presence of AA and other bioactive compounds may be responsible for the AC and stability observed in the plant samples, independent of whether the fruit is a source of AA.

Therefore, we believe that several more studies like this, involving the temperature and time of storage, should be performed for more accurate conclusions about the effects of these parameters on vegetable extracts.

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