

EFFECT OF TEMPERATURE, LIGHT, BUTAYLATED HYDROXY ANISOLE AND METHODS OF ANALYSIS ON THE ASCORBIC ACID CONTENT OF UN-PASTEURIZED IRANIAN SOUR ORANGE (*CITRUS AURANTIUM*, L.) JUICE DURING STORAGE

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Abstract

Sour orange juice has long been known to be an excellent source of Ascorbic acid (vitamin C) in Iran and is a product desired by many consumers who are interested in maintaining a healthy diet. Presence of light, time and temperature affect the ascorbic acid retention in sour orange juice. In this study sour orange juice was prepared by hand-squeezing fresh fruit. It was filtered, and then poured into clear and dark glass bottles also into polyethylene. Bottles were stored at three different temperature storage [room temperature (28 ± 2) °C, refrigerator (4 ± 1) °C and freezer (-18 ± 1) °C], in the presence or not of an antioxidant additive Butylated Hydroxy Anisole (BHA) for 45 days. Vitamin C contents of the juice in all samples were determined by HPLC and 2,6-DICHLOROPHENOL INDOPHENOL METHODS. Acid degradation in sour orange juice during 15, 20 and 45 days storage at 28, 4 and -18 °C were investigated, respectively. Ascorbic acid content of the juice in all samples was reduced during the storage time. The major losses of ascorbic acid occurred in clear glass bottles in the room temperature. No statistically significant differences were observed between samples with or without Butylated Hydroxy Anisole (BHA). There was statistically significant difference between the two methods that were applied for determination of ascorbic acid. Storage condition specially temperature and time predominates as a deteriorative/retention factor.

Keywords: Ascorbic acid, Temperature, Light, BHA, Sour orange (*Citrus aurantium*, L.), Iran

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1. INTRODUCTION

The sour orange (*Citrus aurantium*, L.), is one of the most popular fruits in Iran, especially in southern provinces. It is available between mid-October to March and inexpensive. Sour orange is mainly consumed fresh. They are also often processed into juice to extend shelf life of the product. The necessity of ascorbic acid in the human diet has been known at least since 1700, and possibly earlier, It's well known that ascorbic acid and carotenoids are abundant in some citrus fruits. Ascorbic acid is very popular for its antioxidant properties. Consumption of fruit and vegetables can prevent certain diseases such as cancer and cardiovascular diseases, as these foods are rich in antioxidant vitamins, such as vitamins C and E, phenolic compounds, and carotenes. Some fruits like sour orange can be kept under appropriate conditions for several months. Sour orange juice is a high-quality source of

ascorbic acid which may be lost during storage and processes such as pasteurization (Fennema, 2007; Harold, 1957; Pardio Sedas, 1994). Ascorbic acid is highly sensitive to various modes of deterioration. The main factors that can affect ascorbic acid loss include temperature, salt and sugar concentration, pH, oxygen, light, metal catalysts, initial concentration of ascorbic acid, the ratio of ascorbic acid to dehydroascorbic acid, microbial load and protection by the container (Zerdin et al, 2003).

Abbasi and Niakousari (2008) stored fresh lemon at refrigerated condition in dark, as well as room condition with and without presence of normal daylight for a period of 12 weeks and they have found that during storage, the ascorbic acid content of citrus juice decreased at a rate depending on storage conditions (Abbasi and Niakousari, 2008). Uddin, Hawlader, Ding and Mujumdar (2001) studied

during storage. With the increase of water activity and temperature from 0.43 to 0.97 and 30–50 °C, respectively, the rate constant of ascorbic acid deterioration was found to increase (Uddin et al, 2002). Kabasakalis, Siopidou and Moshatou (2000) reported that loss of ascorbic acid from different commercial fruit juices stored in closed containers for a period of 4 months at room temperature ranged between 29 and 41%. When the containers are opened for consumption and then stored in the refrigerator for 31 days, commercial orange juice can lose up to 60–67% of its ascorbic acid whereas under the same conditions, ascorbic acid losses from fresh orange juice are much lower (7–13%). When open containers of commercial orange juice were kept outside the refrigerator for 10 days, ascorbic acid losses were as high as 12.5%, decreasing to about 9% if the containers were refrigerated for the same period (Kabasakalis et al, 2000).

Many researches have pointed out that both heating method and temperature had significant effect on the destruction of ascorbic acid in fruit juices. For examples Villota and Hawkes (1992) have reviewed for kinetics of ascorbic acid destruction in several fruits, vegetables and cereals during thermal processing and storage (Villota and Hawkes, 1992). In Iran, especially in southern provinces, when the fresh fruit is not available, pasteurized and un-pasteurized sour orange juices are consumed regardless of storing conditions. In majority of cases, consumers store un-pasteurized freshly squeezed juice at various conditions of temperature and light for consumption throughout the year. This fact is of great importance to the consumer who must know how to store the juice containers and when to consume them in order to get the maximum benefit of their vitamin C content. The objective of this study was to determine degradation of ascorbic acid in un-pasteurized sour orange juice by two method of analysis (HPLC and 2,6-DICHLOROPHENOL INDOPHENOL) AT different storage conditions.

2. MATERIAL AND METHODS

2.1.PREPARATION OF sour orange JUICE

Sour oranges (*Citrus aurantium*, L.) were purchased from a local market (Iran, Shiraz). The fruits were washed with tap water and cut into two pieces. Fresh juice was squeezed using a household citrus juice extractor and filtered using a whatman No. 4 filter paper to remove pulp. Orange juice extraction and filtration were performed in three type bottles with lids and were stored in conditions stated in Table 1 for 45 days. Ascorbic acid examined on 2, 4, 6, 8, 10 and 12 day for bottles that were stored at room temperature and on 2,4,9,16 and 20 day FOR bottles that were stored at refrigerator temperature. Because of mould GROWTH IN THESE SAMPLES, WE COULD NOT continue determination of ascorbic acid FOR 45 DAYS. Ascorbic acid in the frozen samples were determined every week. The analysis was carried out in two replicates.

TABLE 1: Various conditions in storing sour orange juice

Light glass bottles, Room, BHA(LRB)	Room natural day light (15 hrs), temperature (28±2) °C, presence of antioxidant
Light glass bottles, Room, Non BHA(LRN)	Room natural day light (15 hrs), temperature (28±2) °C, without presence of antioxidant
Dark glass bottles, Room, BHA(DRB)	Room natural day light (15 hrs), temperature (28±2) °C, presence of antioxidant
Dark glass bottles, Room, Non BHA(DRN)	Room natural day light (15 hrs), temperature (28±2) °C, without presence of antioxidant
Polyethylene bottles, Room, BHA(PRB)	Room natural day light (15 hrs), temperature (28±2) °C, presence of antioxidant
Polyethylene bottles, Room, Non BHA(PRN)	Room natural day light (15 hrs), temperature (28±2) °C, without presence of antioxidant
Dark glass bottles, Cold, BHA(DCB)	In the refrigerator, temperature (4±1) °C, presence of antioxidant
Dark glass bottles, Cold, Non BHA(DCN)	In the refrigerator, temperature (4±1) °C, without presence of antioxidant

Polyethylene bottles, Cold, BHA(PCB)	In the refrigerator, temperature (4±1) °C, presence of antioxidant
Polyethylene bottles, Cold, Non BHA(PCN)	In the refrigerator, temperature (4±1) °C, without presence of antioxidant
Dark glass bottles, Freezed, BHA(DFB)	In the freezer, temperature (-18 ± 1) °C, presence of antioxidant
Dark glass bottles, Freezed, Non BHA(DFN)	In the freezer, temperature (-18 ± 1) °C, without presence of antioxidant
Polyethylene bottles, Freezed, BHA(PFB)	In the freezer, temperature (-18 ± 1) °C, presence of antioxidant
Polyethylene bottles, Freezed, Non BHA(PFN)	In the freezer, temperature(-18 ± 1) °C, without presence of antioxidant

2.2. DETERMINATION OF ascorbic acid by titration method

Ascorbic acid was determined by titration method using 2,6-dichlorophenol indophenol (DCIP) as described in Official Methods of Analysis, AOAC (2010). This method is based on the reduction of DCIP with ascorbic acid in acidic solution. The content of ascorbic acid was expressed in mg/100ml (AOAC, 2010).

2.3. DETERMINATION OF ascorbic acid by HPLC

Ascorbic acid content was determined following the HPLC (Shimadzu Model no: C-R6A, CHROMATOPAC, Japan). 15 ml of juice samples were centrifuged (Eppendorf, 5417R) for 5 min at 6000g and 4 °C; 5 ml of the supernatant was filtered through 0.22µm membrane filter (Millipore, GS) and 20 µl of SAMPLES WAS THEN INJECTED INTO THE C18 HPLC COLUMN. The mobile phase was 25 mM KH₂PO₄ (adjusted to pH 3.0 with phosphoric acid) with a flow rate of 1 ml/min. Eluate was monitored by UV-VIS detection at 245 nm. Results were reported as mg/100 ml of sour orange juice.

2.4. Statistical analysis

The analysis of variance were done using a general linear models procedure of SPSS software (version 11.5). The differences between mean values were established using

Duncan's multiple range test and $p < 0.05$ was considered as a level of significance

3. RESULTS AND DISCUSSION

3.1. Storage temperature effects

Ascorbic acid content had a negative relationship with storage temperature (Figure 1). Comparison of average ascorbic acid contents at a different storage temperatures showed significant differences between the refrigerator and freezing with room temperatures (Table 2). It is well-known that ascorbic acid is heat labile and could be destroyed during heat treatment.

Table 2: Comparison of storage temperature effects on ascorbic acid (ppm) contents

Analysis method	Storage temperature(°C)		
	(28 ± 2) °C	(4 ± 1) °C	(-18 ± 1) °C
Titration	0.37±0.12a	0.45±0.06b	0.48±0.05b
HPLC	0.29±0.1a	0.35±0.08b	0.35±0.03b

Different letters in one row (a–b) show significant differences by statistical programme

Esteve, Farre and Frogola (1996) studied the stability of ascorbic acid in fresh orange juice and commercial orange juices maintained at 4 and 10 °C, finding that at 4 °C the loss of ascorbic acid was less than 10% after 7 days of storage (Esteve et al, 1996). Choi, Kim and Lee (2002) found that, for pasteurized juice (90 °C, 90 s), during refrigerated storage (4.5 °C) more than 50% of the ascorbic acid was lost within 3 weeks of storage, and it was completely degraded after 5 weeks of storage [8]. However, Kavousi's (1997) results on pasteurized lemon juice indicate lesser dependency of ascorbic acid retention/loss on temperature range 5-25 °C (Kavousi Chahak, 1997).

Amiri and Niakousari (2008) studied the ascorbic acid content in un-pasteurized sour orange juice which was stored at room temperature [(28 ± 2) °C], in the refrigerator [(4 ± 1) °C] and in the freezer [(-12 ± 1) °C] for 12 weeks. They found that the initial ascorbic acid content after 2 weeks, was reduced by

nearly 50% for all unfrozen samples. The final concentration of ascorbic acid in the juice was reduced by nearly 85%, regardless of storage conditions (Amiri et al, 2008).

3.2.Storage time Effects

Comparison of average ascorbic acid contents during the storage time showed significant decreases of 68.6%, 38.4% and 9.9% in ascorbic acid at $(28 \pm 2) ^\circ\text{C}$, $(4 \pm 1) ^\circ\text{C}$ and $(-18 \pm 1) ^\circ\text{C}$, respectively (Tables 3 and 4). The major loss of ascorbic acid occurred during the first days of storage at any temperature (Fig 1).

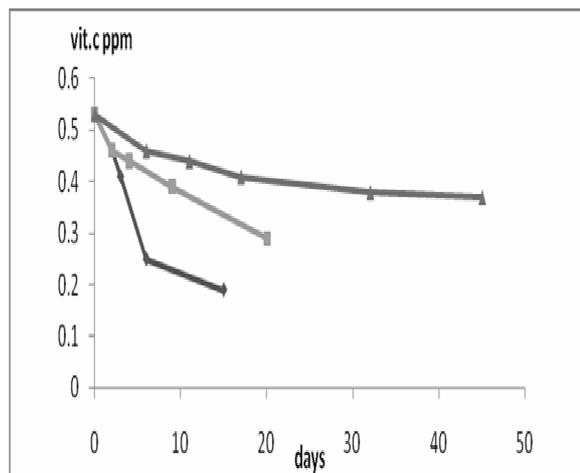


Figure 1.Changes in mean ascorbic acid content of sour orange juice during storage at $(4 \pm 1) ^\circ\text{C}$, $(-18 \pm 1) ^\circ\text{C}$ and (28 ± 2)

Table3: Comparison of storage time effects by HPLC method on ascorbic acid (ppm) contents

Storage	Storage time (day)											
	2	3	4	6	9	11	15	17	20	32	45	
$(28 \pm 2) ^\circ\text{C}$	0.42 ± 0.03a	0.36±0.01b		0.21±0.02c			0.16±0.01d					
$(4 \pm 1) ^\circ\text{C}$	0.43 ± 0.008a		0.40±0.007b		0.35±0.02c				0.22±0.04d			
$(-18 \pm 1) ^\circ\text{C}$	0.40 ± 0.01a					0.36±0.01b		0.36±0.01b		0.33±0.01c	0.32±0.02c	

Different letters in one row (a–d) show significant differences by statistical programme

Table4: Comparison of storage time effects by titration method on ascorbic acid (ppm) contents

Storage tempera	Storage time (day)											
	2	3	4	6	9	11	15	17	20	32	45	
$(28 \pm 2) ^\circ\text{C}$	0.50±0.02a	0.47±0.03a		0.29±0.06b			0.23±0.07c					

(4 ± 1) °C	0.52 ± 0.03a		0.48 ± 0.02b		0.43 ± 0.009c			0.36 ± 0.03d		
(-18 ± 1) °C	0.53 ± 0.02a					0.52 ± 0.02a		0.47 ± 0.04b		0.44 ± 0.043bc
										0.42 ± 0.03c

This result confirms those of Ibanez, Foin, Cornillon and Reid (1996), that showed the sharpest decrease of ascorbic acid occurred in the first 15 days of storage. No significant differences, between different temperatures and some days showed, however, decrease of ascorbic acid was significant (Ibanez et al, 1996). These results were similar with those of Abbasi and Niakousari, who reported a quick decreasing trend of ascorbic acid content of un-pasteurized lemon juice in different temperatures at the first two weeks (Abbasi and Niakousari, 2008). This rapid loss is due to the oxidation of residual air layer trapped within the bottles. The incorporation of air in the juice during extraction, clarification and bottling have long been recognized. Two main reasons of ascorbic acid reduction are oxidative reactions by aerobic enzymes such as cytochrome oxidase, ascorbic acid oxidase and peroxidase found in citrus fruits and nonenzymatic anaerobic reactions (Nagy, 1980). However, it is thought that the main factor in the long-term excessive degradation of ascorbic acid will be the un-pasteurized nature of the samples (Abbasi and Niakousari, 2008).

During storage, the vitamin C content gradually decreases at a rate depending on the processing and storage temperature. The more rapid decrease of ascorbic acid concentration at the beginning of the storage can be attributed to the immediate reaction of an amount of ascorbic acid with the dissolved oxygen (Burdurly et al, 2006; Zerdin et al, 2003). Oxidation of ascorbic acid occurs mainly during the processing, whereas, anaerobic degradation of vitamin C mainly appears

during storage, which is especially observed in thermally preserved juices (Polydera et al, 2003).

Recent developments in oxygen scavenging packaging now provide an opportunity to further reduce the exposure of packaged juice products to oxygen (Brody, 2001; Gontard, 2000; Rooney, 1995). However, the rate of oxygen scavenging packaging for this oxygen is likely to be important because the depletion of oxygen present at the time of packaging can also occur through reaction with the ascorbic acid. Zerdin, Rooney and Vermuë (2003) studied the stability of ascorbic acid of orange juice packed in oxygen scavenging (OS) film and oxygen barrier film, finding that the rapid removal of oxygen, to be an important factor in sustaining a higher concentration of ascorbic acid over long storage times (Zerdin, 2003).

3.3. Light effects

It found that different light transmission has a decreasing and non significant effect on ascorbic acid content of bottles in room condition. This evidence includes reports that the significant differences between ascorbic acid contents in the frozen/refrigerated and room temperature samples may be proportional to the amount of light received during storage. These results in agreement with Solomon, Svanberg and Sahlström (1995) that studied the effect of oxygen and light exposure and the combination of these two parameters on the retention of ascorbic acid, in orange juice stored at 8 °C for 52 days. They stored samples in glass containers, covered on the sides with aluminum foil and sealed on the top with

packaging materials, i.e. glass, polyethylene and paper carton, having different light transmission and oxygen permeation characteristics. They found the ascorbic acid content was significantly affected by the level of dissolved oxygen in the juice, but no effect of light could be observed under the conditions of that study (Solomon et al ,1995)

Abbasi and Niakousari reported that a significant ($P < 0.05$) effect of light on ascorbic acid loss. Their data for the 9 weeks showed somewhat higher retention for the lemon juice samples in dark condition (about 60% as compared to 53% for samples in light condition (Abbasi and Niakousari, 2008). It seems likely that, light presence or not is important. But when light slightly penetrates in the sample, it can decrease ascorbic acid. Therefore, they aren't seen significant difference between dishes with different light transmission.

3.4. Presence or not of an antioxidant additive (BHA) effects

In all samples there were no significant differences in ascorbic acid contents between presence or not of BHA samples (Table 5). No previous study has directly compared decreases of ascorbic acid, with respect to presence of BHA during storage. We observed that the presence of BHA (100ppm) in samples has a major effect on growth inhibition of mold.

Table 5: Comparison of presence or not of an antioxidant additive (BHA) effects on ascorbic acid contents:

Analysis method		Storage temperature(°C)		
		(28 ± 2) °C	(4 ± 1) °C	(-18 ± 1) °C
Titration	BHA	0.33±0.12	0.38±0.09	0.37±0.06
	Non BHA	0.33±0.12	0.38±0.09	0.38±0.05
HPLC	BHA	0.42±0.14	0.47±0.08	0.41±0.06
	Non BHA	0.41±0.13	0.48±0.07	0.5±0.06

This is consistent with evidence from other investigators. This result is like to those of Baohua and Chengxiang (1993). These investigators have found that 10% K-sorbate +

1% BHA dip in bacon could reduce the growth of mold and resist fat oxidation significantly (Baohua and Chengxiang, 1993).

This result also is in agreement with the findings of Ahmad and Branen (2006) for BHA treatment of processed cheese spread. Their results showed that spread direct addition of 400 ppm of BHA inhibited growth of *Aspergillus flavus* or *Penicillium expansum* in processed cheese (Ahmad and Branen, 2006).

3.5. Material of bottles effects

Ascorbic acid contents appeared not to be significantly influenced by material of bottles during storage time (Table 6). No previous study has directly compared decreases of ascorbic acid, with respect to material of sour orange juice bottles.

Table 6: Comparison of material of bottles effects on ascorbic acid (ppm)contents:

Analysis method	Material of bottles		
	Light glass bottles	Dark glass bottles	polyethylene bottles
Titration	0.46±0.12	0.46±0.1	0.46±0.09
HPLC	0.36±0.09	0.36±0.11	0.36±0.1

3.6. Analysis method effects

In this study, results showed significant difference between the concentrations of ascorbic acid measured by the HPLC and titration methods (HPLC: 0.36 ± 0.1 ppm, titration: 0.47 ± 0.12 ppm). HPLC method data were lower than titration method but Tran and Farid (2002), in ultraviolet treatment of orange juice, reported no significant difference between the concentrations of ascorbic acid measured by the HPLC and titration methods (Tran and Farid, 2004)

HPLC constitutes an interesting alternative to correct the lack of selectivity and sensitivity of classical methods. Although the Association of Official Analytical Chemists (AOAC) recommends the volumetric titration using DCIP as titrant for the determination of ascorbic acid in sour orange preparations but

HPLC is a technique increasing in the recent years to analyze ascorbic acid as faster than volumetric titration method (Amiri et al, 2008). DCIP has been a popular reagent for the direct titration of ascorbic acid. This method is based on the reduction of DCIP with ascorbic acid in acidic solution. The dye has often been referred to as 'Tillmans Reagent'. In the official method, the applicability of the method is restricted to only those samples of citrus fruits and multivitamin tablets which do not contain minerals. Materials containing natural or added colors render the end point difficult to judge visibly. Liquid chromatography (LC) has commonly been used for the separation and determination of ascorbic acid, thus resulting in a large number of such methods. Highly sensitive and selective methods have been proposed for the simultaneous determination of ascorbic acid and dehydroascorbic acid in beverages, fruits and biological fluids by HPLC. Despite the fact that so many methods for the determination of ascorbic acid have been developed and more are to come, analytical chemists have not made serious efforts to investigate the functional roles of ascorbic acid.

4. CONCLUSION

As it has been pointed out earlier, sour orange juices suffer a reduction on the ascorbic acid content during storage or industrial processes such as pasteurization. Specifically, oxygen, heat, light, time and temperature of storage affect the ascorbic acid retention in sour orange juices. This work demonstrates that ascorbic acid in sour orange juice concentrates decreased with increasing temperature. Sour orange juice concentrate had the lowest reaction rate at -18 °C when compared to other samples, thus it seems that temperature to be effective in extending shelf life and maintaining ascorbic acid of sour orange juice during storage. Also results in this study show that of material of bottles and presence of an antioxidant additive didn't have significant effect on decreasing ascorbic acid, and dark

glass or polyethylene bottles couldn't inhibit the effect of light in ascorbic acid content. This work demonstrates that the HPLC method is a highly sensitive and selective method for determination of ascorbic acid in sour orange juice. Finally, to prevent the loss of ascorbic acid levels of sour orange juices, they should be kept at refrigerated or frozen condition. Under these conditions sour orange products show a good retention of ascorbic acid.

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