



Ciência e Tecnologia de Alimentos

ISSN: 0101-2061

revista@sbcta.org.br

Sociedade Brasileira de Ciência e
Tecnologia de Alimentos
Brasil

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Ciência e Tecnologia de Alimentos, vol. 37, núm. 3, julio-septiembre, 2017, pp. 449-455
Sociedade Brasileira de Ciência e Tecnologia de Alimentos
Campinas, Brasil

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Bioactive compounds and antioxidant activity analysis during orange vinegar production

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Abstract

Citrus fruits are significant sources of bioactive compounds, such as ascorbic acid, polyphenols and carotenoids, due to their antioxidant properties important for human nutrition. In addition, since oranges possess high sugar content (8-15%), making vinegars from alcoholic orange substrates, with functional characteristics is a possible development of novel products. The aim of this research work was to analyze changes in ascorbic acid, total phenolics, total carotenoids and antioxidant activity during orange vinegar processing. In order to analyze the influence of acetification and aging in these characteristics, samples were taken in three stages: orange alcoholic substrate for acetification (SNA), young orange vinegar or recently obtained (Vn0) and orange vinegar after six month-aging in bottles (Vn6). Statistically significant differences ($p < 0.05$) were found among bioactive compounds concentrations; antioxidant activity decreased along the process, but total phenolics and carotenoids remained constant during aging period (Vn0-Vn6). The highest reduction was recorded during the acetification stage, possibly due to components oxidation caused by continuous air flow to the system. A higher contribution ($p < 0.05$) to antioxidant activity was associated to ascorbic acid and phenolic compounds concentration.

Keywords: orange vinegar; antioxidant activity; bioactive compounds.

Practical Application: Development of a novel product from raw materials rich in bioactive compounds.

1 Introduction

The province of Entre Ríos in Argentina has been characterized by citrus production for decades. A high proportion (15-25%) of Argentinian orange production, very similar to exports percentage, has been derived to industry due to the difficult profitable marketing, during the last five years.

Since oranges possess high sugar content (8-15%), making vinegars from alcoholic orange substrates as an industrial alternative was evaluated. Furthermore, as these fruits are rich in bioactive compounds, new products with functional characteristics could be developed. However, the bibliography does not provide enough information about vinegars made from citrus fruits.

Citrus fruits are significant sources of bioactive compounds, such as ascorbic acid, polyphenols and carotenoids, due to their antioxidant properties important for human nutrition (Passaro Carvalho & Londoño-Londoño, 2012; Gui-Fang et al., 2013; Lee, 2013). Antioxidant activity (AA) is defined as a substance capacity to inhibit oxidative degradation mainly caused by oxygen reactive species, whose reactivity is higher than molecular oxygen. Since they are associated to occurrence and development of cancer, diabetes, neurodegenerative disorders, atherosclerosis, coronary heart disease and aging, consumers became more conscious about finding alternatives to add beneficial components to the daily intake. Consequently, a wide variety of products such as nutritional supplements, functional

foods and fruits with high bioactive compounds content are promoted in markets (Rice-Evans et al., 1997; Passaro Carvalho & Londoño-Londoño, 2012).

Total phenolics, flavonoids among them, have been proved to possess an elevated contribution to AA, even though vitamin C is considered the most abundant antioxidant compound in citrus fruits (Gorinstein et al., 2006; Escobedo-Avellaneda et al., 2014).

Orange juice phenolic composition involves phenolic acids, mainly ferulic and gallic acid, and flavanones, whose most abundant component is hesperidin (Kelebek et al., 2009). These compounds can oxidize to quinones, either by molecular oxygen or enzymatically, because of polyphenol oxydases (PPO) present in vegetable tissues (Damodaran et al., 2010). However, not all phenolic compounds present the same AA because it depends on their chemical structure (Verzelloni et al., 2007; Escobedo-Avellaneda et al., 2014).

Ascorbic acid is the major component in citrus fruits with interesting antioxidant properties, but its content in juices may vary (20-100 mg/100 mL) depending on variety or species. Despite the important role as enzymatic cofactor and antioxidant, humans cannot synthesize or store ascorbic acid in the body (Bernardi et al., 2014). Therefore, it is of great importance to provide it in the daily intake. Besides the numerous ascorbic acid benefits, loss due to heat, air and light exposure must be taken into account

Received 04 Aug., 2016

Accepted 15 Apr., 2017

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during processing (Passaro Carvalho & Londoño-Londoño, 2012; Bernardi et al., 2014).

Carotenoids constitute an important group of natural pigments which impart yellow, orange and red colors to plants and also develop important functions in photosynthesis. Citric fruits are an excellent source of carotenoids for humans and animals. It was recently suggested that these compounds are not only vitamin A precursors but also play an important role in antioxidant capacity (Liu et al., 2014). However, they tend to rapidly oxidize because of their highly unsaturated nature: presence of oxygen or strongly oxidant reactives are main factors in carotenoid oxidation. Moreover, temperature accelerates degradation and turns them more susceptible to breakdown (Damodaran et al., 2010).

Antioxidant content and therefore their associated AA, may be affected by physiological factors, such as maturity, and technological ones, such as processing conditions and post-harvest storage (Connor et al., 2002; Sdiri et al., 2014).

Although vinegar is considered a shelf-stable acid product, physico-chemical and organoleptic changes in the product may occur caused by evolution, oxidation and aging of some compounds during storage in bottles. The presence of oxygen normally triggers chemical and enzymatic reactions. These changes depend on vinegar type, raw material, elaboration process and storage conditions (Casale et al., 2006).

The broad scientific recognition that increased consumption of foods rich in antioxidants results in clear benefits for population health, has led to consumers' increasing interest in knowing the antioxidant richness of food products. For this reason, the aim of this research was to analyze changes in ascorbic acid, total phenolics, carotenoids and antioxidant activity during orange vinegar processing.

2 Materials and methods

2.1 Raw material

Alcoholic substrate used in acetification was prepared from Valencia Late oranges alcoholic fermentation. *S. cerevisiae* native yeasts, isolated and selected from orange fermented juices (Hours et al., 2005; Ferreyra et al., 2009) were used as pre-culture. Once vinification process was finished (13-14% v/v ethanol), the orange wine was racked and filtered through 1 µm porosity membranes.

2.2 Orange alcoholic substrate conditioning

As ethanol concentration could exert an inhibition effect over acetic acid bacteria at values up to 50 g/L (\cong 6% v/v) especially in discontinuous processes (Ory et al., 2002; Garrido-Vidal et al., 2003), orange wine was diluted to 6% v/v. Since this dilution greatly affects the nutrient concentrations, especially in low aminic nitrogen content wines (Ferreyra, 2006), the following solution with minerals and nitrogen source was added: KH_2PO_4 (0.8 g/L), $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ (0.35 g/L) and $(\text{NH}_4)_2\text{SO}_4$ (1.5 g/L) (Davies, 2015).

2.3 Acetification

The acetification stage was carried out in a laboratory NEW BRUNSWICK CIENTIFIC Co., Bioflo 2000 (New Jersey, USA) bioreactor, in submerged culture. *Acetobacter* sp. isolated in laboratory from regional oranges (Gerard, 2015) was utilized as inoculum for the discontinuous acetification processes. The fermentation equipment consisted of a glass vessel with a maximum working volume of 2 L, equipped with PID agitation and temperature controllers. An air flowmeter controls the air flow to the system and a sparger located on the bottom of the vessel homogeneously diffuses air into the vessel.

Once total acidity stabilized its value (around 60 g acetic acid/L) after microbial growth exponential phase, the acetification process was finished in order to prevent acetic acid consumption by acetic acid bacteria (García-García et al., 2009; Tesfaye et al., 2002).

250 mL glass bottles were filled with orange vinegars recently obtained (Vn0) and stored in laboratory at 20-25 °C, simulating market or cupboard conditions once acquired by consumers.

2.4 Antioxidant components assessment

Main orange antioxidant components, ascorbic acid, total phenols (TP) and total carotenoids were assessed according to analytical methods described below.

Since some researchers reported differences between testing systems for antioxidant activity determination, caused by synergistic interactions among the components, the use of at least two methods is recommended (Sánchez-Moreno, 2002; Sakanaka & Ishihara, 2008). Therefore, ABTS and DPPH radical-scavenging assays were utilized for AA determination.

In order to analyze the influence of acetification and aging on the aforementioned quality characteristics, samples were taken in the following stages: orange alcoholic substrate for acetification (SNA), young orange vinegar or recently obtained (Vn0) and orange vinegar after a six-month aging in bottle (Vn6) (Figure 1).

2.5 Analytical methods

L-ascorbic acid: reverse-phase HPLC method using a Hewlett Packard 1100 and a UV detector. Stationary phase: Hewlett Packard, Hypersil BDS C 18 3 µm, 100 × 4.0 mm; guard column: Hewlett Packard, ODS-Hypersil C 18, 5 µm, 20 × 2.1 mm; mobile phase: 1 mM KH_2PO_4 aqueous solution (pH 3 phosphoric acid), flow rate: 0.7 mL/min; temperature: 25 °C; injection volume: 20 µL; peaks were identified at 214 nm. Results were expressed as g ascorbic acid per L of sample (SNA, vinegars).

Total phenolics: method based on the Folin-Ciocalteu reactive reduction was used (Saura-Calixto, 1998). The assay was performed as follows: X µL sample, (850-X) µL distilled water, 100 µL sodium carbonate at 20% p/v and 50 µL Folin-Ciocalteu reagent were agitated to homogenize. After 30 minutes of reaction in darkness, at room temperature (25 ± 1 °C), absorbance was measured at 760 nm using an UV-visible SHIMADZU spectrophotometer, UV-1603 (Japan). Measures were read against an ultra-pure water, sodium carbonate 20% p/v and Folin-Ciocalteu reagent blank

(Zapata, 2014). Gallic acid was used as a standard, therefore results were expressed as mg gallic acid equivalents (GAE) per 100 mL of sample (SNA, vinegars).

Total carotenoids: reverse-phase HPLC method, using a Hewlett Packard 1100 and a UV detector. Stationary phase: Hewlett Packard, Hypersil AA-ODS, 5 μ m, 2.1 \times 200 mm; guard column: Hewlett Packard, ODS-Hypersil, 5 μ m, 20 \times 2.1 mm; mobile phase: methanol; flow rate: 1 mL/min; injection volume: 20 μ L; peaks were identified at 473 nm (Yaping et al., 2002).

Antioxidant activity (AA): determination by ABTS and DPPH methods. L-ascorbic acid was used as reference antioxidant compound and results were expressed as mg ascorbic acid equivalents (AEAC) per 100 mL of sample (SNA, vinegars).

ABTS radical-scavenging assay: 2,2'-azino-bis-(3ethylbenzthiazoline-6-sulphonic acid) radical cation (ABTS^{•+}) was produced by reacting ABTS and potassium persulphate (2.45 mM) solutions and kept in the dark 12 hours before use.

The concentration of ABTS radical solution was adjusted to an absorbance of 0.700 units at 734 nm with the buffer-solution (Re et al., 1999). AA was determined by mixing a volume of sample with other of ABTS^{•+} diluted solution, stirred at 1600 rpm during 30 s. and incubated at room temperature (25 ± 1 °C) in the dark for 20 minutes. Absorbance was measured at 734 nm using an UV-1603 SHIMADZU spectrophotometer (Japan). To prepare the control, a volume of ultra-pure water was utilized instead of sample. A calibration plot was made with five known concentration points of ascorbic acid standard solution, treated as previously explained.

DPPH radical-scavenging assay: 2, 2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay is based on the DPPH[•] radicals absorbance reduction at 517 nm caused by antioxidant action (Brand-Williams et al., 1995). This radical, violet colored, turns colorless or pale yellow when neutralized by antioxidants components.

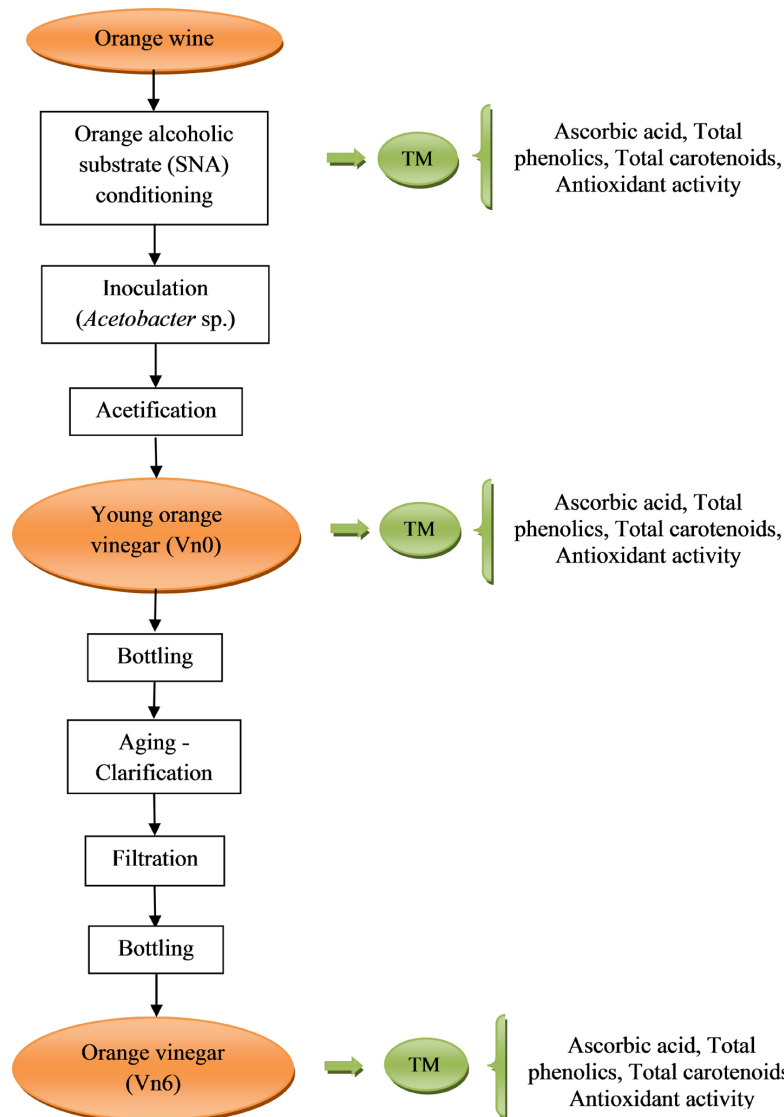


Figure 1. Orange vinegar production flow chart. Sampling scheme (TM) for bioactive compound determination.

Firstly, a DPPH[•] mother solution (200 mg/L) was prepared and stored at 4 ± 1 °C in a brown colored glass bottle wrapped in aluminum paper, until use. This solution was diluted 1/10 with methanol HPLC quality for analytical determinations (Brand-Williams et al., 1995). AA measures were carried out by mixing a volume of the sample with another of DPPH diluted solution. After 30 s. stirring at 1600 rpm, it was incubated at room temperature (25 ± 1 °C) in the dark for 30 minutes. The decrease in absorbance was measured at 517 nm in a UV-visible SHIMADZU spectrophotometer, UV-1603 model (Japan). To prepare the control, a volume of ultra-pure water was utilized instead of sample. Absorbance difference, among blank and sample, was calculated after 30 minutes of radical and sample antioxidant reaction (Zapata, 2014). A calibration plot was prepared with four known concentration points of ascorbic acid standard solution, treated as previously explained.

2.6 Statistical analysis

All data are presented as the mean \pm standard deviation (SD) of three replicates of each sample. Analysis of variance (ANOVA) by Fisher's least significant difference method was used for statistical analysis, with a *p*-value < 0.05 considered statistically significant. In order to assess the relationship between antioxidant compounds and AA, correlation coefficients (*r*) were also calculated. When *p*-value < 0.05, both variables were considered significantly correlated.

Statistical analysis was performed using Statgraphics Centurion XV Corporate software.

3 Results and discussion

3.1 Antioxidant compounds assessment

In general, antioxidant compounds and their associated AA decreased along orange vinegar processing (Table 1).

Ascorbic acid

Ascorbic acid significantly decreased along orange vinegar processing. The highest reduction (49.2%) occurred during SNA acetification period probably due to continuous air flow to the system, as vitamin C is easily degraded in contact with oxygen (Passaro Carvalho & Londoño-Londoño, 2012; Bernardi et al., 2014).

During aging, a 29% decrease was recorded between Vn0 and Vn6. No reference to behaviors in matrices like orange vinegar during this stage was found. However, that reduction

could be attributed to ascorbic acid auto oxidation that occurs, even slowly, in totally anaerobic conditions (Bruchmann, 1980). Also, chemical reactions in which this compound takes part such as browning, are influenced by storage time and temperature (Rodríguez et al., 1991).

Total phenolics (TP)

Total phenolic content decreased about 54.3% during acetification. Andlauer et al. (2000) also recorded reductions of 40%, 13% and 8% in cider, white and red wines respectively, attributed to strong degradation or phenolic transformation during this stage. Different TP proportions recorded in each case were related to individual TP composition, such as polymeric phenols that are oxidized more slowly than monomeric ones.

Vn0 showed no significant changes in TP concentration during aging period. The literature shows studies on high phenolic extraction is achieved when aging is performed in oak barrels or by adding oak chips (Palacios et al., 2002; Morales et al., 2004; Tesfaye et al., 2004; Durán Guerrero et al., 2011; Chen et al., 2013; Cerezo et al., 2014). But there are very few studies on maturation or aging in bottles. Llabé Pino (2008) reported a reduction in hydroxybenzoic and hydroxycinnamic acids and their derivatives in Chardonnay, Cabernet Sauvignon, Moscatel and apple vinegars, after a year storage in glass bottles at 15 °C. In addition to degradation, they observed reactions of new components formation like browning, especially during aging. For this reason, it would be important to have phenolic individual profile to correctly analyze TP changes in each stage.

Total carotenoids

As expected, total carotenoid content showed a 46% decrease during biooxidative period caused by their high susceptibility to the air contact (Damodaran et al., 2010) and particularly during this process, characterized by a constant air supply to the system. However, no concentration changes were observed during the aging stage (Vn0-Vn6).

Total carotenoid concentration in SNA (0.024 ± 0.002 mg/100 mL) was significantly lower when compared to fruit content reported in previous studies (1.170 mg/100 mL in Ferreyra, 2006). This important reduction might be caused by solid phase (lees) deposit during first winemaking stages and eliminated after racking.

Table 1. Mean and standard deviations of antioxidant compounds in different stages along orange vinegar processing.

	Orange alcoholic substrate for acetification (SNA)	Young vinegar (Vn0)	Vinegar after 6-month aging (Vn6)
Ascorbic acid (g/L)	0.124 ± 0.005^a	0.063 ± 0.006^b	0.045 ± 0.002^c
Total phenolics (mg GAE/100 mL)	123.63 ± 9.17^a	56.53 ± 0.99^b	54.14 ± 2.81^b
Total carotenoids (mg/100 mL)	0.024 ± 0.002^a	0.013 ± 0.002^b	0.015 ± 0.002^b
ABTS (mg AEAC/100 mL)	85.48 ± 2.27^a	70.63 ± 0.94^b	50.61 ± 0.88^c
DPPH (mg AEAC/100 mL)	53.24 ± 4.51^a	46.75 ± 2.37^b	24.10 ± 1.61^c

Different letters in each row indicate statistically significant differences (*p* < 0.05) among components means in each stage.

Table 2. Correlation coefficients (*r*) among antioxidant components and antioxidant activity (ABTS and DPPH methods) in orange vinegars (*p* < 0.05).

	ABTS	DPPH
Ascorbic acid	0.92	0.81
Total phenolics	0.82	0.67
Total carotenoids	No correlation	No correlation
ABTS	---	0.97

Antioxidant activity (AA)

Antioxidant activity decreased 40.8% and 54.7% during the whole process studied, according to ABTS and DPPH respectively.

During acetification stages, AA showed a 17.4% and 12.2% decrease when using ABTS and DPPH methods, respectively. A similar value, 16.8%, was reported for a red wine acetification process, using the latter methodology. Researchers attributed this reduction to changes in chemical composition caused by biooxidative process (Cerezo et al., 2010).

During aging period, AA was 28.4% lower when measured according to ABTS, while the reduction was highest using DPPH (48.5%). Probably, these trends are closely related to the reduction in ascorbic acid recorded during this period.

Many research works have reported lower AA in vinegars than in red wines, indicating wine acetification could reduce phenolic content with high AA, and/or lead to the formation of new ones with less AA than those originally present in the alcoholic substrate (Pellegrini et al., 2003; Alonso et al., 2004; Dávalos et al., 2005).

A significant correlation was observed among ascorbic acid and total phenolic concentrations, and antioxidant activity. However, no correlation was observed between AA and total carotenoids (Table 2) indicating these components did not contribute to orange vinegar AA.

These results agree with other studies that indicated ascorbic acid exhibited one of the highest contributions to AA (Miller & Rice-Evans, 1997; Gardner et al., 2000; Arena et al., 2001), while total carotenoid did not show a significant contribution (Gardner et al., 2000).

4 Conclusions

Orange vinegar bioactive compounds as well as their associated antioxidant activity decreased along the whole process studied, but total phenolics and carotenoids remained constant during aging period. The highest reduction was recorded during acetification stage, possibly due to component oxidation caused by continuous air flow to the system.

A higher contribution to antioxidant activity was associated to ascorbic acid and phenolic compounds concentration.

Acknowledgements

This work was funded by Secretaría de Ciencia y Técnica, Universidad Nacional de Entre Ríos (Argentina). The authors would like to thank Lic. Alicia Noceti for language assistance.

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