Introduction

Wine has always been the most important alcoholic beverage produced by man. Any fruit with enough fermentable sugars content is able to be used for winemaking applying similar technology as utilized with grapes. Therefore these products could be named as “wine of […]” (Brown et al., 1989; Ward, 1991; Varnam & Sutherland, 1997; Duarte et al., 2009). Fruit wines might be considered beneficial for health possibly related to their antioxidant activity (Nuengchamnong & Ingkaninan, 2010).

Antioxidants, including vitamin C and E, polyphenols, carotenoids and terpenoids among others are the most important bioactive compounds present in fruits (Bravo, 1998; Yen et al., 2002; Hensley et al., 2004; Stahl & Sies, 2005).

Antioxidant content in fruits as well as its associated antioxidant activity could be affected by physiological factors such as maturity and also by technological factors like processing and storage conditions (Lindley, 1998; Helyes & Lugasi, 2006).

Citric juices are characterized by important flavonoid and phenilpropanol content as well as ascorbic acid and carotenoids (Rapisarda et al., 1998; Hayat et al., 2010), all of them being responsible for health beneficial properties. Fruit juices pasteurization is a method commonly used for its preservation. Orange juice “partial sterilization” is one of the heat treatment objectives, trying to avoid altering to a lesser extent the nutritional compounds and its organoleptics properties. Besides, it reduces the microbial number so that yeast starter (S. cerevisiae) has less competition to grow and perform the alcoholic fermentation.

Many new compounds are produced and others are transformed during winemaking, mainly influenced by processing conditions, such as temperature, pH and yeast strain used (Torrens, 2000; Del Pozo Bayón, 2011).

Of particular interest is the fruit winemaking at low temperatures (10°C-15°C) in order to produce and preserve the flavors volatile compounds and thus obtain a characteristic aromatic profile. However, these low temperatures may extend the process time (Torija et al., 2002, Beltran et al., 2008). Temperatures over than 20°C may contribute to the loss of some fruit primary aromas and desirable esters generated during fermentation, as well as the production of undesirable superior alcohols (Peynaud, 1981; Molina et al., 2007; Reddy & Reddy, 2011).

Low temperatures improves yeast ethanol tolerance so, it is suppose that must fermented at 10°C-20°C would maintain sensorial properties.

pH is an essential biological stability factor specially for lactic acid bacteria, so it can be considered as a selective parameter over species involved in wine microbiota (Nemeth et al., 2010). On the other hand, technological processes, alimentary and food processing habits considerably affect antioxidant compounds and their bioavailability and bioactivity (Tomás-Barberán, 2003).
Antioxidant activity is a global and reliable estimation of the antioxidant capacity of foods. Besides, it has been described as an interesting parameter to value the dietary activity of the product (Acevedo et al., 2004).

Individual antioxidant compound quantification does not allow an accurate knowledge of the total antioxidant capacity of a mixture, compound or biological fluid because it is determined by synergistic interactions that could occur between them (Benzie & Strain, 1996) as well as each mode of action. Therefore it is necessary to combine methods to correctly assess a sample antioxidant capacity. During the last years many methods have been developed to evaluate antioxidant capacity of foods based on different aspects: ferric ion reducing antioxidant power (FRAP), oxygen radical absorption capacity (ORAC, TRAP), hydroxyl radical scavenging activity (deoxyribose assay), capture of organic molecules radicals (ABTS, DPPH), quantification of products generated by lipidic peroxidation (TBARs, oxidation of LDLs), among others (Frankel & Meyer, 2000; Sánchez Moreno, 2002; Aruoma, 2003).

It was not found any investigation that quantifies bioactive compounds and antioxidant activity in orange wines. Therefore the aim of this study was to determine antioxidant activity (ABTS, DPPH and FRAP methods), ascorbic acid, total phenolics and total carotenoids in those alcoholic fruit substrates in order to evaluate the influence of juice heat treatment, fermentation temperature and pH.

2 Materials and methods

2.1 Orange juice extraction

350 kg of Washington Navel oranges (2012 harvest) were washed under water circulation. The juice was obtained in a FMC extractor, FS BR 1 model (Brasil), and filtered through ASTM#18 sieves. The volume was divided in two fractions: one was reserved as “natural juice” (NJ); the other was pasteurized (JP) to reduce native flora that could compete with inoculated yeasts during alcoholic fermentation, as well as to inactivate the pectinmethylesterase (PME) enzyme that could affects juice stability (Osorio et al., 2008; Maca et al., 2013). Heat treatment was performed at 90°C for 30 s in a Figmay shell and tube heat exchanger (Argentina).

2.2 Analytical methods

Natural and pasteurized juice characterization

Titratable acidity: according to AOAC 9.135 method (Association of Analytical Communities, 1995), expressed as % anhydrous citric acid.

pH: potentiometric measurement, using TOA HM-30V pH meter.

Soluble solids: refractometer method, using Carl Zeiss, Jena, (Germany) refractometer, expressed as °Brix.

Juice yield: expressed as mL juice per 100 g fruit (%).

15 oranges randomly selected were weighted, squeezed in manual cone and cup extractor and filtered through 1 mm sieves.

The volume obtained was measured in a graduated measuring cylinder. Results were expressed in percentage terms according to the Equation 1:

\[
\text{Fruit yield, } \% = \left( \frac{\text{Volume, mL}}{\text{Weight, g}} \right) \times 100
\]  

Direct reducing sugars: according to AOAC 1995, 16th ed., Official Method 923.09 (Association of Analytical Communities, 1995), expressed as g/100 mL juice.

Total reducing sugar: according to Lane-Eynon general volumetric method, AOAC, 1995, 16th ed., Official Method 923.09 (Association of Analytical Communities, 1995). Results were expressed as g/100 mL juice.

Antioxidant compounds determination in juices and wines

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

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 KH₂PO₄ aqueous solution (pH 3 with phosphoric acid), flow rate: 0.7 mL/min; injection volume: 20 μL; peaks were identified at 245 nm (Franke et al., 2004).

Total flavonoids: spectrophotometer method, measured at a wavelength of 285 nm, with methanol as a blank and hesperidin standard solutions. Results were expressed as mg hesperidin/100 mL.

Total Phenolics: determination by the Folin-Ciocalteu method. The assay was performed as follows: X μL of the sample, (850 – X) μL of distilled water, 100 μL of sodium carbonate at 20% p/v and 50 μL of Folin-Ciocalteu reagent were agitated to homogenize. After 30 minutes of reaction at room temperature, absorbance was measured using a UV-visible SHIMADZU spectrophotometer (UV-1603) at 760 nm. The calibration curve was prepared with caffeic acid standard solution 0.1 mg/mL and sodium carbonate (20% p/v). Results were expressed as mM caffeic acid.

2.3 Orange winemaking

The volume of NJ was divided into two fractions: one was reserved as antiseptic. PJ was similarly treated.

The volume obtained was measured in a graduated measuring cylinder. Results were expressed in percentage terms according to the Equation 1:

\[
\text{Fruit yield, } \% = \left( \frac{\text{Volume, mL}}{\text{Weight, g}} \right) \times 100
\]  

Direct reducing sugars: according to AOAC 1995, 16th ed., Official Method 923.09 (Association of Analytical Communities, 1995), expressed as g/100 mL juice.

Total reducing sugar: according to Lane-Eynon general volumetric method, AOAC, 1995, 16th ed., Official Method 923.09 (Association of Analytical Communities, 1995). Results were expressed as g/100 mL juice.

Antioxidant compounds determination in juices and wines

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

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 KH₂PO₄ aqueous solution (pH 3 with phosphoric acid), flow rate: 0.7 mL/min; injection volume: 20 μL; peaks were identified at 245 nm (Franke et al., 2004).

Total flavonoids: spectrophotometer method, measured at a wavelength of 285 nm, with methanol as a blank and hesperidin standard solutions. Results were expressed as mg hesperidin/100 mL.

Total Phenolics: determination by the Folin-Ciocalteu method. The assay was performed as follows: X μL of the sample, (850 – X) μL of distilled water, 100 μL of sodium carbonate at 20% p/v and 50 μL of Folin-Ciocalteu reagent were agitated to homogenize. After 30 minutes of reaction at room temperature, absorbance was measured using a UV-visible SHIMADZU spectrophotometer (UV-1603) at 760 nm. The calibration curve was prepared with caffeic acid standard solution 0.1 mg/mL and sodium carbonate (20% p/v). Results were expressed as mM caffeic acid.

2.3 Orange winemaking

The volume of NJ was divided into two fractions: one was maintained at pH 3.5 (orange juice natural pH) and pH was adjusted to 4 in the other, as it is considered the optimal for S. cerevisiae growth (Fleet & Heard 1993). The pH adjust was made with solid CaCO₃ to facilitate the lees sedimentation at the end of alcoholic fermentation. Commercial sugar was added in sufficient quantity to reach 20 °Bx in order to obtain at least 80 g/L ethanol. In addition, 200 ppm KSO₄ was introduced as antiseptic. PJ was similarly treated.
S. cerevisiae native yeasts, isolated and selected from orange fermented juices (Hours Roque et al., 2005; Ferreyra et al. 2009) were used as pre-culture. It was incubated at 30±1 ºC and yeast viable count was made in Petri plates with orange serum agar. When the count reached close to 5x10⁶ CFU/mL, the pre-culture was used to inoculate NJ and PJ and maintained at 10 ºC y 20 ºC. Yeasts optimal fermentation temperature is considered at 20ºC and was selected because of this reason. Also, as yeasts demonstrated good tolerance at low temperatures, 10 ºC was chosen expecting that more aromatic wines may be obtained in no longer time than in the previous case.

Each substrate was labelled as NJ and PJ, indicating the correspondent pH (3.5 or 4.0) and fermentation temperatures (10ºC or 20ºC).

2.4 Determination of antioxidant activity (AA)

DPPH radical-scavenging assay

The 2, 2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay is based on the scavenging of DPPH radicals (Brand-Williams et al., 1995). It was performed according to the Cavin et al. (1998) protocol: a methanol solution of DPPH⁺ (20 mg/L) was adjusted to a constant absorbance value of 0.32 at 517 nm. Then, 7.5 µL of sample or references compounds were added, and diluted with 250 µL distilled water. Ascorbic acid and hesperidin were used as standard solutions. The decrease in absorbance was measured at 517 nm after incubation at room temperature in the dark for 30 minutes. Free radical scavenging capacity (% discolouration) was determined applying Equation 2:

\[ \% \text{ discolouration} = \frac{X - \bar{X}_c}{\bar{X}_c} \times 100 \]  

(2)

where \( \bar{X}_c \) is the control sample absorbance mean value and \( \bar{X}_o \) the sample absorbance mean value.

According to the ΔA values obtained for the standard compounds concentrations, dilutions of the samples with distilled water were performed in order to produce an absorbance decrease within the expected range. Orange wine samples (NJ) were diluted 1:2 and 1:4 while (PJ) samples did not need dilutions.

ABTS radical-scavenging assay

2, 2-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) radical cation (ABTS⁺) was produced by reacting ABTS (7 mM) and potassium persulphate (2.45 mM) solutions in phosphate buffer solution pH 7.0, prepared 12 hours before use and kept in the dark at room temperature. The concentration of ABTS radical solution was adjusted to an absorbance of 0.700 units at 732 nm. Then, 7.5 µL of standard solutions were determined by adding 900 µL FRAP reagent (X µL sample or standard solution and (100 – X) µL of distilled water. The absorbance was read at 593 nm.

2.5 Statistical analysis

All data are presented as the mean ± SD (standard deviation) of three replicates of each sample. The statistical analysis was performed using Statgraphics Plus 2.0 software. Statistical differences among the groups were obtained by analysis of variance (ANOVA). α<0.05 differences were considered significant. Correlations between parameters were established using Pearson’s coefficient.

3 Results and discussion

Orange juice yield was 48 mL/100 g fruit. Values of the physicochemical parameters analyzed in NJ and in PJ are shown in Table 1.

One of the most important problems in citrus juice quality is the ascorbic acid reduction during heat treatment and storage (Polydera et al., 2003). As shown in Table 1, thermal processing caused a 20.60% vitamin C content decrease and was attributed to it losses due to high temperatures exposure and oxidation. Ravani & Joshi (2011) and Kurozawa et al. (2014).

After pasteurization, flavonoids and carotenoids also reduced their content in fresh juice (15.55% and 38.46%, respectively). Lee & Coates (2003) reported that carotenoids content of Valencia orange pasteurized juices was about 70% lesser than the natural one.

As mentioned above, ascorbic acid, carotenoids, flavonoids and other polyphenols contribute to orange juice antioxidant capacity, so quantification in wines obtained from oranges is also considered very important. It was observed that winemaking was defined as the acid concentration that produces the same antioxidant effect (absorbance decrease) as the sample.

Ferric ion reducing antioxidant power (FRAP)

FRAP reagent consists of acetic acid-sodium acetate buffer (pH 3.6), tripyridyl-triazine (TPTZ) (1 mM) and FeCl₃ (2 mM). This mixture produces TPTZ-Fe³⁺, a brown coloured complex. The standard calibration curve was made using FeSO₄₇H₂O solutions ranging from 100 to 1000 µM. Both samples and the standard solutions were determined by adding 900 µL FRAP reactive, X µL sample or standard solution and (100 – X) µL of distilled water. The absorbance was read at 593 nm.

| Table 1. Mean and standard deviation of physicochemical parameters of natural (NJ) and pasteurized juice (PJ) obtained from Washington Navel oranges. |
|---------------------------------|-----------------|-----------------|
|                                  | NJ              | PJ              |
| pH                              | 3.65±0.00       | 3.84±0.01       |
| Soluble solids (“Brix”)          | 11.00±0.01      | 11.20±0.01      |
| Acidity (% anhydrous citric acid)| 0.94±0.02       | 0.86±0.02       |
| Ascorbic acid (mg/100 mL)        | 76.78±0.01      | 60.96±0.02      |
| Direct reducing sugars (g/100 mL)| 4.32±0.05       | 4.38±0.04       |
| Total reducing sugars (g/100 mL)| 8.71±0.03       | 8.92±0.02       |
| Flavonoids (mg hesperidin/100 mL)| 68.12±0.01      | 57.53±0.01      |
| Carotenoids (mg β-carot./100 mL) | 1.17±0.03       | 0.72±0.04       |
Ascorbic acid decreased between 25-48% in NJ wines and 14-71% for PJ ones during winemaking. When contrasting NJ wines, the highest ascorbic acid content retention (52-75%) was detected in samples fermented at 10°C and pH 3.5 (NJ-3.5-10). The same temperature and pH allowed for the highest ascorbic acid in PJ wines, that is PJ-3.5-10.

Carotenoids showed a great reduction of its initial content in juice. This is probably caused by lees precipitation and subsequent elimination after clarification. Retention was 7-14% and 4-10% in NJ and PJ referring to raw material, respectively.

Hesperidin, the main orange juice glucoside, also decreased its content probably due to an hydrolytic process during fermentation. The aglucon (hesperetin) formed after hesperidin total hydrolysis is highly insoluble, so it may precipitate with lees. ANOVA showed significant differences between vitamin C, β-carotenes and hesperidin in both wines, NJ and PJ, at both fermentation temperatures and pH values with a 95% confidence level. Fermentation temperature is the factor that mostly affected these compounds retention while the other studied compounds exhibited an aleatory behavior, as it can be seen in Table 2.

Phenolic degradation processes begin in the first stages of vinification and continue during storage (Gómez-Plaza & Cano-López, 2011). Ethanol content, temperature, homogenization process and added metabisulphite concentration are the main processing variables that affect phenolic concentration in wines (Garrido et al., 2013).

Researchers have reported identification and quantification of phenolics in orange juices and wines (Kelebek et al., 2008). They found two hydroxybenzoic acids (gallic and protocatechuic), five hydroxycinnamics acids (caffeic, chlorogenic, p-coumaric, ferulic and sinapic) as well as six flavanons (narirutin, naringin, hesperidin, neohesperidin, didimín and apigenin). According to these results, caffeic acid was selected as the standard to express total phenolic content. As can be seen in Table 2, a 3-10% decrease occurs due to temperature effect, in NJ and PJ during alcoholic fermentation.

The aforementioned results indicate that it is necessary to combine at least two methods with different basis in order to evaluate the AA of a sample (Pérez-Jiménez & Saura-Calixto, 2007). In the present study two assays were used: one based on free radical scavenging capacity (DPPH y ABTS) and the other on ferric ion reducing antioxidant power (FRAP). Ascorbic acid and hesperidin were used as standard compounds to plot the calibration curves for DPPH and ABTS assays and to calculate EC$_{50}$ (antioxidant compound concentration necessary to cause a 50% decrease of the initial concentration of the coloured radical in the steady state). Ascorbic acid exhibited the highest antioxidant power. Results of DPPH using both standards are shown in Table 3.

The data obtained using the three aforementioned AA methods (Table 4) showed which radical/ion is the most active, which is therefore providing the antioxidant profile of this new product.

NJ wines showed the AA highest levels, as expected, due to the principal bioactive compound concentration decrease during orange juice heat processing. This difference did not seem to relate to the method used, since NJ wines showed an AA 1- to 2-fold greater than the similar assay performed in PJ wines. Similar results were observed both in free radical scavenging and ferric ion reducing antioxidant power assays.

The correlation analysis (Pearson’s coefficient) between DPPH discolouration percentage and ascorbic acid, flavonoids, carotenoids and total phenolic content indicated more affinity for these compounds.

### Table 2. Mean and standard deviation of antioxidant compounds concentrations present in orange wines produced from natural (NJ) and pasteurized juice (PJ).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Ascorbic acid (mg/100 mL)</th>
<th>Flavonoids (mg hesperidin/ 100 mL)</th>
<th>Total phenolics (mM caffeic acid)</th>
<th>Carotenoids (mg β-Carotene /100 mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NJ-3.5-10</td>
<td>57.60±0.12$^a$</td>
<td>50.20±0.12$^a$</td>
<td>11.9±0.1$^a$</td>
<td>0.009±0.001$^a$</td>
</tr>
<tr>
<td>NJ-3.5-20</td>
<td>55.55±0.13$^a$</td>
<td>49.07±0.20$^a$</td>
<td>10.1±0.3$^a$</td>
<td>0.021±0.003$^b$</td>
</tr>
<tr>
<td>NJ-4.0-10</td>
<td>52.80±0.15$^a$</td>
<td>40.52±0.13$^a$</td>
<td>11.9±0.5$^a$</td>
<td>0.017±0.001$^a$</td>
</tr>
<tr>
<td>NJ-4.0-20</td>
<td>39.60±0.12$^a$</td>
<td>47.87±0.15$^a$</td>
<td>8.6±0.3$^a$</td>
<td>0.017±0.001$^a$</td>
</tr>
<tr>
<td>PJ-3.5-10</td>
<td>52.46±0.15$^a$</td>
<td>36.05±0.16$^a$</td>
<td>7.1±0.2$^a$</td>
<td>0.004±0.002$^a$</td>
</tr>
<tr>
<td>PJ-3.5-20</td>
<td>25.52±0.13$^b$</td>
<td>31.90±0.12$^b$</td>
<td>7.1±0.1$^b$</td>
<td>0.007±0.001$^a$</td>
</tr>
<tr>
<td>PJ-4.0-10</td>
<td>32.92±0.13$^b$</td>
<td>31.08±0.13$^b$</td>
<td>8.0±0.3$^b$</td>
<td>0.003±0.002$^a$</td>
</tr>
<tr>
<td>PJ-4.0-20</td>
<td>29.55±0.16$^b$</td>
<td>32.10±0.14$^b$</td>
<td>6.9±0.2$^b$</td>
<td>0.003±0.001$^b$</td>
</tr>
</tbody>
</table>

Values in the same column followed by different superscript letters are significantly different using Duncan’s multiple range test (α<0.05).

### Table 3. EC$_{50}$ for DPPH$^•$ using ascorbic acid and hesperidin as standards.

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC$_{50}$ (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hesperidin</td>
<td>143.77±0.17</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.203±0.12</td>
</tr>
</tbody>
</table>
between flavonoids (0.8670), less with total phenolics (0.7900) and a weak correlation between carotenoids and ascorbic acid.

The ABTS results showed that Pearson’s coefficient varied from 0.3878 to 0.082 following the sequence: total phenolics, flavonoids, carotenoids and ascorbic acid. These low correlations indicated that samples presented higher activity with DPPH than with ABTS*.

Activity against TPTZ-Fe³⁺ (FRAP assay) exhibited the highest correlation coefficients with flavonoids and total phenolics (0.9026 and 0.8684, respectively), followed by ascorbic acid and total carotenoids (0.7460 and 0.7163, respectively).

These results are in agreement with many studies that reported that even though vitamin C content and AA in fruits are closely correlated (Yoo et al., 2004), this vitamin is not the only responsible for the AA but also other phytochemicals, such as phenolics, thiols, carotenoids or tocopherols (Sun et al., 2002; Guo et al., 2003). It has been pointed out that specifically flavonoids, within phenolics compounds, show AA (García-Alonso et al., 2004).

In addition, AA results expressed by the DPPH method are positively and closely correlated with FRAP assay (0.958), while using ABTS, they did not correlate with the previous methods significantly (0.548 and 0.463, respectively). Therefore, these results suggest that DPPH and FRAP are the recommended methods to asses AA in orange wines.

Ascorbic acid contribution to the AA by DPPH and FRAP assays in NJ wines was 72% and 51%, respectively. Similarly, PJ showed 60% and 56%, respectively. A lower ascorbic acid/AA ratio suggests that other components, such as this vitamin, such as phenolic compounds have more important antioxidant properties in orange wines.

4 Conclusion

The pasteurization process reduced ascorbic acid, carotenoids and flavonoids contents in orange wines. This fact correlates with the variations observed in NJ and PJ wines antioxidant activity.

Results showed that orange wines have greater AA against DPPH radical than ABTS radical. Consequently, a profile of their free radical scavenging activity may be obtained. Orange wines also reduced TPTZ-Fe³⁺ complex (FRAP assay) indicating its reducing capacity. The analyzed orange wines characteristics are attributed both to ascorbic acid as well as to phenolic and carotenoid contents.

The pasteurization process and the alcoholic fermentation temperature resulted as the most important influence both on the bioactive compound content and AA in the orange wines evaluated.

It is important to point out that the occurrence of bioactive compounds in food does not indicate that the food must exhibit AA, because positive and/or negative interactions can occur. The previously mentioned parameters quantified the ability of a sample to capture free radicals in a water solution. The methods performed intend a global characterization of the product, regardless of its individual composition.

Acknowledgements

This research was partially funded by grants received from Secretaría de Ciencia y Técnica, Universidad Nacional de Entre Ríos (Argentina).

References


Orange wines antioxidant activity


