JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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# Fermented Orange Juice: Source of Higher Carotenoid and Flavanone Contents

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**ABSTRACT:** The intake of bioactive compounds and moderate alcohol decreases the risk of cardiovascular diseases. These effects could be joined in a beverage created by a controlled alcoholic fermentation of orange juice. The influence of controlled alcoholic fermentation on the bioactive compound profile of orange juice has not been previously evaluated, and this is the purpose of the present study. Total and individual flavanones and carotenoids significantly increased throughout the fermentation. The reason for this was an enhanced extraction of these compounds from the pulp. Besides, the potential bioavailability of flavanones increased due to a higher content of hesperetin-7-*O*-glucoside (2-fold higher at the end of the fermentation process). Ascorbic acid did not undergo a significant change, and only total phenolics decreased. Antioxidant capacity was also evaluated. TEAC and FRAP values remained constant throughout the process. However, ORAC and DPPH values significantly increased. Correlation analysis concluded that the increase in ORAC and DPPH values could be due to enhancement of flavanones.

**KEYWORDS:** *orange juice, alcoholic fermentation, ascorbic acid, phenolics, flavonoids, flavanones, carotenoids, UHPLC-MS-MS, ORAC, TEAC, DPPH, FRAP*

## INTRODUCTION

Reactive oxygen species (ROS), naturally formed during normal metabolism, can cause oxidative damage in biological structures such as proteins, lipids, or DNA. Human metabolism exhibits an antioxidant defense system involving enzymes and proteins to neutralize the ROS and prevent these effects.[1] However, these defenses can be insufficient under certain circumstances, and oxidative stress may occur. It is established that an oxidation process is involved in the initial development steps of cardiovascular diseases and cancer, two of the most important causes of death in developed countries. It is accepted that the intake of fruits and vegetables, which contain antioxidant compounds such as polyphenols, carotenoids, and ascorbic acid, intensifies and enhances the antioxidant defense system.[2] Numerous epidemiological and intervention studies have established an inverse correlation between the intake of fruits and vegetables and the occurrence of cardiovascular diseases, cancer, and aging-related disorders.[3] Citrus fruits are known to be rich sources of bioactive compounds with antioxidant capacity, and orange juice is recognized by its ascorbic acid, carotenoid, and flavonoid contents.[4] The antioxidant capacity of different varieties of orange and their juices has been characterized.[5] Orange juice presents a greater antioxidant capacity than other similar foods, such as apple juice, pineapple juice, tropical juice, apricot juice, grape juice, or lemon iced tea.[6]

On the other hand, a large number of studies indicate the direct relationship between moderate alcohol consumption and a decrease in the risk of coronary heart diseases.[7]

The healthy effect of dietary bioactive compounds with antioxidant capacity and moderate alcohol consumption, widely shown in wine or beer,[8] could also be joined in a novel developed beverage of low alcoholic graduation prepared from orange juice by controlled alcoholic fermentation.

The main objective of this study was to analyze the evolution of the composition of bioactive compounds and the antioxidant capacity of orange juice during alcoholic fermentation.

At a technological level, this study presents a novelty. Although the influence of freezing, storage, or heat treatment on the antioxidant content as well as on the antioxidant capacity of the orange juice has been widely studied,[9] the influence of a controlled alcoholic fermentation has not been previously evaluated: this is the purpose of the present study. Furthermore, the potential beverage of fermented orange juice would suppose a new manner of orange commercialization and the possibility of providing the consumer with a new potentially healthful beverage that widens the supply of other similar drinks with higher alcohol content, such as wine, beer, or cider.

Received: March 19, 2013
Revised: August 19, 2013
Accepted: August 20, 2013
Published: August 20, 2013

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## ■ MATERIALS AND METHODS

**Samples.** The company Grupo Hespérides Biotech S.L. carried out the controlled alcoholic fermentation of commercial pasteurized juice made from *Citrus sinensis* L. var. Navel late (Huelva, Spain) (patent WO2012/066176A120120524). The homogeneous composition and microbiological stability of orange juice were the qualities used to select the product used in the fermentation process. In addition, its organoleptic properties were more adequate than those of other commercial orange juices with regard to the aromatic quality of the final product (data not shown). The fermentation was carried out in two parallel PVC tanks (5 L) at 20 °C for 15 days in repose. The yeast strain *Saccharomycetaceae* var. *Pichia kluyveri* was isolated from the natural microbiota present in the orange fruit and used for the inoculation of the fermentation. Table 1 gives the quality parameters in both orange juice and fermented product: pH, tritatable acidity (TA), total carbohydrates (reducing and nonreducing sugar), total soluble solids (TSS) (°Brix), percent pulp, and alcohol.[10] The selected yeast strain ferments only reducing sugars, resulting in a final product with low alcohol content (0.87% v/v). The increase of alcohol content was progressive throughout the fermentation. Before sample collection, fermentation liquid was agitated and mixed by the use of magnetic agitators to promote sample homogenization. Sample (25 mL) was collected every 2 days (day 0 (original orange juice), day 1, day 3, day 5, day 7, day 9, day 11, day 13, and day 15) and immediately stored at −20 °C until analysis. The supernatant of samples obtained by centrifugation at 3000*g* (4 °C, 10 min) was utilized in the evaluation of total phenolic, total flavonoids, and antioxidant capacity.

**Table 1. Quality Parameters of Orange Juice before and after Fermentation**[a]

| | orange juice | fermented orange juice |
|---|---|---|
| pH | 3.48 ± 0.20 | 3.43 ± 0.20 |
| [b]TA (g citric acid/L) | 8.48 ± 0.02 | 8.85 ± 0.02 |
| total carbohydrates (g/L) | 78.2 ± 5.64 | 53.7 ± 4.65 |
| reducing sugars (g/L) | 48.5 ± 3.63 | 24.7 ± 2.13 |
| nonreducing sugars (g/L) | 29.7 ± 2.01 | 29.0 ± 2.51 |
| TSS[c] (°Brix) | 11.0 ± 0.50 | 10.0 ± 0.50 |
| % pulp | 12.0 ± 2.00 | 8.00 ± 0.50 |
| alcohol (% v/v) | 0 | 0.87 ± 0.01 |

[a]Values are expressed as the mean ± SD. [b]TA, titratable acidity. [c]TSS, total soluble solids.

**Chemicals and Reagents.** All reagents were of the highest commercial available purity. Naringenin-7-*O*-glucoside, naringenin-7-*O*-rutinoside, hesperetin-7-*O*-rutinoside, isosakuranetin-7-*O*-rutinoside, naringenin, homoeriodictyol, hesperetin, isosakuranetin, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in the crystallized diammonium salt form, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), fluorescein sodium salt, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), metaphosphoric acid (MPA) [40−44% $(HPO_3)N$, 50−60% $(NaPO_3)N$], L-ascorbic acid, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), iron(II) sulfate heptahydrate, 1,1-diphenyl-2-pricrylhydrazyl (DPPH), catechin, sodium dihydrogen phosphate (anhydrous), gallic acid monohydrate, iron chloride hexahydrate, potassium persulfate, aluminum chloride, sodium nitrite, sodium hydroxide, sodium acetate, and sodium carbonate were obtained from Sigma-Aldrich Quimica (Alcobendas, Spain). Hesperetin-7-*O*-glucoside was obtained from Faces Biochemical Co., Ltd. (Wuhan, China). Folin−Ciocalteu reagent, acetic acid, and methanol were provided from Merck (Mollet del Vallés, Spain). Sodium salt 2,6-dichlorophenolindophenol, dimethyl sulfoxide (DMSO), and formic acid were purchased from Panreac (Seville, Spain). HPLC grade methanol and acetone were supplied by BDH Prolabo (VWR International Eurolab, S.L., Barcelona, Spain). Diethyl ether was purchased from Scharlau (Scharlab, S.L., Barcelona, Spain). Acetonitrile was obtained from J. T. Baker (Phillipsburg, NJ, USA). HPLC grade deionized water was produced with a Milli-Q 50 system (Millipore Iberica S.A., Madrid, Spain).

**Analysis of Ascorbic Acid Content.** The assessment of ascorbic acid content of samples was realized with a titrimetric method according to previous studies.[11−13] Equal volumes (5 mL) of sample and aqueous MPA (3% w/v) were mixed and centrifuged for 10 min at 4000*g*. Five milliliters of supernatant was used for the titration reaction using 2,6-dichlorophenolindophenol. The titration reaction is based on the reduction of the sodium salt of the dye by ascorbic acid, resulting in the formation of a colorless derivative and dehydroascorbic acid. The end point of titration is indicated by the appearance of a persistent pink color. The results were obtained from a standard curve using different concentrations of ascorbic acid (0−600 mg/L).

**Analysis of Total Phenolic Content.** The total phenolic content of samples was determined using the Folin−Ciocalteu colorimetric method,[14] with some modifications. A previously shaken and centrifuged sample of fermented orange juice (1.5 mL) was diluted with distilled water (1:5). An aliquot of the diluted sample (20 μL) was added to 1.58 mL of water and 100 μL of Folin−Ciocalteu reagent. After 5 min, 5 mL of $Na_2CO_3$ solution (20% w/v) was added, and the mixture was stored in the dark for 2 h at room temperature. The absorbance was measured spectrophotometrically at 765 nm. The results were obtained from a standard curve using different concentrations of gallic acid (0−500 mg/L). Measurements were recorded on a UV−vis spectrophotometer Helios Epsilon (Thermo Scientific, Madison, WI, USA).

**Qualitative and Quantitative Analysis of Flavonoids.** *Total Flavonoids.* The content of total flavonoids was measured according to the method of Tounsi et al.[5] Briefly, 30 μL of diluted sample (1:2) in methanol (80%) was mixed with 9 μL of $NaNO_2$ (5%). After 6 min, 18 μL of $AlCl_3$ (10%) was added, and 5 min later 60 μL of NaOH (1 M) was added to the mixture. Finally, the mixture was adjusted to 300 μL with distilled water. The absorbance was read at 510 nm. The results were obtained from a standard curve using different concentrations of catechin (0.1−500 mg/L). Measurements were recorded on a SynergyTM HT-multimode microplate reader (Biotek Instruments, Winooski, VT, USA).

*Qualitative and Quantitative Analysis of Flavanones.* Samples were analyzed by UHPLC-MS/MS (Agilent Technologies, Waldbronn, Germany), and the content of the individual flavanones was achieved using external standards. Briefly, a volume (10 mL) of each sample was centrifuged at 4500*g* for 10 min, and both the supernatant and pellet were separated. An aliquot of the supernatant (1 mL) was filtered through a 0.45 μm PVDF Millex filter (Millipore, Tokyo, Japan) and directly injected (10 μL) in the equipment. The pellet was then extracted with DMSO (500 μL) and was filtered through a 0.45 μm PVDF Millex filter and directly injected (10 μL) in the equipment. Samples were analyzed and quantified by a UHPLC-MS/MS (UHPLC-1290 series and a 6460QqQ-MS/MS; Agilent Technologies, Waldbronn, Germany). Separation of the analytes was achieved on an ACQUITY BEH C18 column (150 mm × 2.1 mm, 1.7 μm; Waters, Milford, MA, USA) using a mobile phase that consisted of water/formic acid (99.9:0.1, v/v) (A) and acetonitrile/formic acid (99.9:0.1, v/v) (B) with the following gradient program: 10% B → 30% B at 0−3.5 min; 30% B → 35% B at 3.5−8.0 min; 35% B → 60% B at 8.0−8.01 min; 60% B at 8.01−10.0 min; 60% B → 100% B at 10.0−10.01 min; 100% B at 10.01−12.0 min; and 100% B → 10% B at 12.0−12.01 min. The flow rate was 0.32 mL/min, and the injection volume was 10 μL. Multiple reaction monitoring mode in negative mode was carried out for the characterization and quantification of the flavanones. Figure 1 shows the chromatograms of the flavanones (2.5 μM, each compound) and the MRM transitions for quantification, and Table 2 the chromatographic data and the array of compounds. Standard concentrations ranged from 0.312 to 10 μM, a correlation coefficient ($r^2$ > 0.99) was obtained for each of the compounds, and the limit of detection and limit of quantification are shown in Table 2. Nitrogen was used as the collision gas for the fragmentation by collision-induced dissociation of the compounds at the collision cell of the triple-quadrupole mass spectrometer. Other parameters of the mass spectrometer were set as follows: drying gas flow, 9 L/min; sheath gas flow, 12 L/min; sheath gas temperature, 350 °C; nebulizer pressure, 40 psi; capillary voltage, 4000 V; and nozzle voltage, 1000 V. MassHunter software version B 04.00 was used for MS control and data gathering, and MassHunter

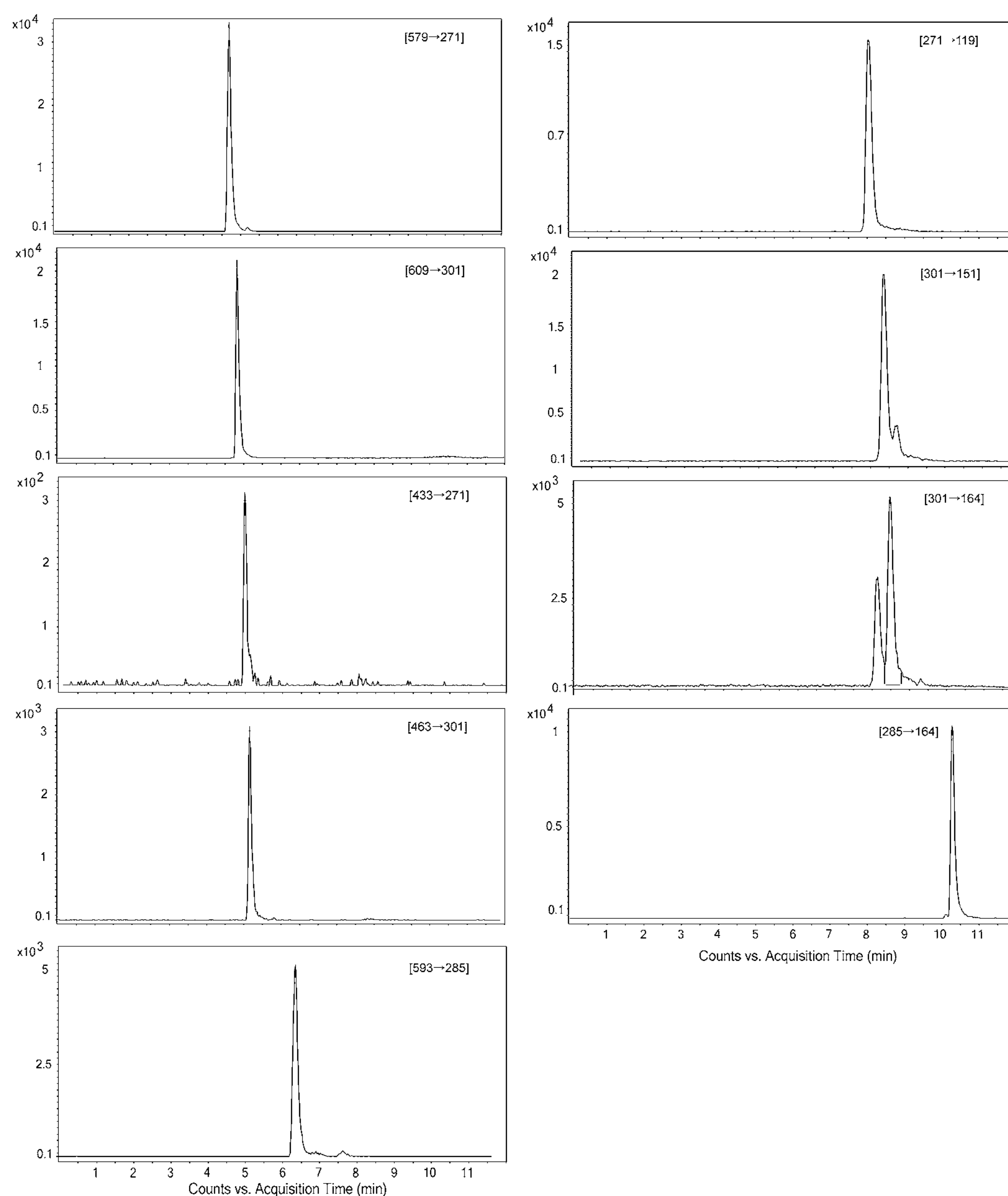


**Figure 1.** Representative UHPLC-MS/MS chromatograms of the flavanones (2.5 $\mu$M, each compound) and the MRM transitions for quantification. See Table 2 for flavanone identification.

**Table 2. Structure Assignments, Retention Times ($t_R$), Mass Spectral Data, LOD, and LOQ of Flavanones Detected in the Negative Mode in Orange Juice and Fermented Orange Juice**

| peak | structure assignment | $t_R$ (min) | $[M - H]^-$ ($m/z$) | MRM transitions ($m/z$) | LOD (ng/mL) | LOQ (ng/mL) |
|---|---|---|---|---|---|---|
| 1 | naringenin-7-*O*-rutinoside | 4.6 | 579 | [579 → 271] | 0.638 | 0.290 |
| 2 | hesperetin-7-*O*-rutinoside | 4.8 | 609 | [609 → 301] | 1.403 | 0.671 |
| 3 | naringenin-7-*O*-glucoside | 5.1 | 433 | [433 → 271] | 0.998 | 0.477 |
| 4 | hesperetin-7-*O*-glucoside | 5.2 | 463 | [463 → 301] | 4.408 | 2.180 |
| 5 | isosakuranetin-7-*O*-rutinoside | 6.3 | 593 | [593 → 285] | 2.791 | 1.366 |
| 6 | naringenin | 8.0 | 271 | [271 → 119] | 0.625 | 0.299 |
| 7 | homoeriodictyol | 8.6 | 301 | [301 → 151] | 0.694 | 0.332 |
| 8 | hesperetin | 8.7 | 301 | [301 → 164] | 1.419 | 0.694 |
| 9 | isosakuranetin | 10.3 | 285 | [285 → 164] | 0.657 | 0.314 |

software version B 03.01 was used for data processing, peak integration, and linear regression.

**Qualitative and Quantitative Analysis of Carotenoids.** *Total Carotenoids.* Extraction of carotenoids from samples was performed in agreement with the method of Mínguez-Mosquera and Hornero-Méndez,[15] with some modifications. Briefly, an aliquot (10 mL) of sample was centrifuged at 10000*g* and 4 °C for 10 min to collect the solids in suspension. The pellet containing the carotenoids was extracted


dx.doi.org/10.1021/jf401240p | J. Agric. Food Chem. 2013, 61, 8773−8782

with acetone (3 mL) and centrifuged at 5000*g* and 4 °C for 5 min. The extract was dried under nitrogen stream. A saponification step was included. The dried extract was dissolved in 3 mL of diethyl ether, and 0.5 mL of 20% (w/v) KOH−MeOH was added, allowing reaction during 20 min with periodic agitation. Three milliliters of distilled water was added, and the sample was centrifuged at 5000*g* and 4 °C for 5 min to separate the organic (upper) and aqueous (lower) phases. The supernatant was collected, dried under nitrogen stream, dissolved in 0.5 mL of acetone, and centrifuged at 12000*g* and 4 °C for 5 min. The absorbance of a known volume of carotenoid solution was read spectrophotometrically at 450 nm. Total carotenoid content was calculated using the extinction coefficient of β-carotene, $E^{1\%} = 2590$, and expressed as milligrams per liter. Measurements were recorded on a UV−vis HP8452A diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA, USA).

*Qualitative and Quantitative Analysis of Carotenoid Pigments.* Routine procedures were used to identify the carotenoid pigments: separation and isolation of pigments by TLC and cochromatography with pure standards, analysis of the UV−visible and mass spectra, comparison with values in the literature,[16] and chemical test for 5,6-epoxide groups. Samples of β-carotene, antheraxanthin, β-cryptoxanthin, zeaxanthin, lutein, violaxanthin, and neoxanthin were isolated and purified from natural sources (*Capsicum annuum* and *Mentha arvensis*).[15] 5,8-Epoxycarotenoids, such as mutatoxanthin, auroxanthin, luteoxanthin, and neochrome were prepared from the corresponding 5,6-epoxide parent pigments by controlled acidic treatment with diluted HCl. Latochrome and karpoxanthin were only tentatively identified due to the lack of pure standards. The tentative identification of *cis* isomers of lutein and mutatoxanthin was based on the presence and relative intensity ($\%A_B/A_{II}$) of the *cis* peak at about 330−340 nm in the UV−visible spectrum; a reduced fine structure and small hypsochromic shift in $\lambda_{max}$ with respect to the *all-E* counterpart; and the chromatographic behavior in the C18 HPLC column (slightly greater retention of *Z* vs *all-E* isomer). Carotenoids were analyzed by HPLC using a method previously developed[15] with slight modifications. Briefly, the method uses a C18 reversed-phase column (Mediterranea SEA18, 20 × 0.46 cm i.d., 3 μm particle size; Teknokroma S.C.L., Barcelona, Spain) and a binary gradient elution system of acetone−deionized water at a flow of 1.0 mL/min. The mobile phase started at 75% acetone and rose linearly to 95% within 10 min and continued isocratically for 7 min, then changing to 100% within 3 min and maintaining this composition for 3 min. Injection volume was 10 μL, and detection was carried out simultaneously at 402, 424, and 450 nm to detect and quantify the diverse family of citrus carotenoids. The column and sample compartment were maintained at 25 and 15 °C, respectively. HPLC analyses were performed with a Waters 2695E Alliance quaternary pump equipped with a Waters 2998 diode array detector and were controlled with Empower2 data acquisition software (Waters Corp., Milford, MA, USA). Pigments were quantified in the saponified extracts by using calibration curves (six to eight concentration levels) prepared with standard stock solutions for each carotenoid in the concentration range of 5−100 μg/mL. LC-MS was performed by coupling a chromatographic system with a Micromass ZMD4000 mass spectrometer equipped with a single-quadrupole analyzer (Micromass Ltd., Manchester, UK) and an atmospheric pressure chemical ionization (APCI) probe. The system was controlled with MassLynx 3.2 software (Micromass Ltd.). MS conditions were as follows: positive ion mode ($APCI^+$); source temperature, 150 °C; probe temperature, 400 °C; corona voltage, 3.7 kV; high-voltage lens, 0.5 kV; and cone voltage, 30 V. Nitrogen was used as the desolvation and cone gas at 300 and 50 L/h, respectively. Mass spectra were acquired within the $m/z$ 300−1200 range. The chromatographic conditions were as described above for carotenoid analysis and quantification.

Provitamin A carotenoid content was expressed as retinol activity equivalents (RAEs) with 1 RAE corresponding to 12 μg of β-carotene or 24 μg of *cis* isomers of β-carotene or any other carotenoid containing an unsubstituted β-ring.

**Antioxidant Capacity Assays.** *Oxygen Radical Absorbance Capacity (ORAC) Assay.* The ORAC assay was performed according to the method of Ou et al.[17] with some minor modifications. All reagents were prepared in phosphate buffer (75 mM, pH 7.4). Briefly, 50 μL of diluted sample (1:300) was added to 100 μL of sodium fluorescein (2.934 mg/L) and incubated for 15 min at 37 °C. Subsequently, 50 μL of AAPH (221.25 mM) was added, and the fluorescence ($\lambda_{ex}$, 460 nm; $\lambda_{em}$, 515 nm) was read every 5 min for 120 min. The area under the curve was calculated. The results were obtained from a standard curve using different concentrations of Trolox (2−38 μM). Measurements were recorded on a SynergyTM HT-multimode microplate reader (Biotek Instruments).

*Ferric Reducing Antioxidant Power (FRAP) Assay.* The ferric reducing ability was estimated according to the procedure of Delgado-Andrade et al.[18] Briefly, 280 μL of FRAP reagent, prewarmed at 37 °C, was mixed with 20 μL of sample diluted (1:20) in distilled water. The FRAP reagent contained 2.5 mL of a 10 mM TPTZ solution in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$, and 25 mL of 0.3 M acetate buffer (pH 3.6). The change at the maximum absorption (595 nm) was evaluated up to 30 min at 37 °C. The results were obtained from a calibration curve using different concentrations of $FeSO_4 \cdot 7H_2O$ (0.2−1.5 mmol/L). Measurements were recorded on a SynergyTM HT-multimode microplate reader (Biotek Instruments).

*TEAC (Trolox Equivalent Antioxidant Capacity) Assay.* The TEAC assay was realized following the procedure described by Delgado-Andrade et al.[18] Briefly, ABTS radical was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12−16 h before use. The ABTS solution obtained was diluted with 5 mM phosphate-buffered saline (pH 7.4) to an absorbance of 0.70 ± 0.02 at 730 nm. Sample was diluted (1:25) in water/methanol (1:1). For the TEAC assay, 20 μL of diluted sample was added to 280 μL of diluted ABTS solution. The change at the maximum absorption (730 nm) was evaluated up to 30 min at 30 °C. The results were obtained from a calibration curve using different concentrations of Trolox (0.06−0.28 mM). Measurements were recorded on a SynergyTM HT-multimode microplate reader (Biotek Instruments).

*DPPH Radical Scavenging Assay.* The DPPH assay was carried out according to previous studies,[18] with minor modifications. For the assay, 40 μL of diluted sample (1:5) in water and 200 μL of methanol were mixed. A 230 mg/L methanolic solution of DPPH was prepared, and 60 μL was added. The solution was incubated in the dark at 30 °C for 60 min, and DPPH absorption was measured at 520 nm. The scavenging ability of the DPPH radical was calculated with the following equation: $\%\ \text{inhibition} = [(A_0 - A_1)/A_0] \times 100$, where $A_0$ is the absorbance of the control (methanol) and $A_1$ is the absorbance in the presence of sample. Measurements were recorded on a SynergyTM HT-multimode microplate reader (Biotek Instruments).

**Statistical Analysis.** Fermentation was performed in duplicate, and all analyses were in triplicate. The analysis of variance (one-way ANOVA; Bonferroni) was applied to establish significant differences between the values obtained during the fermentation. A probability value of $p < 0.05$ was adopted as the criterion for significant differences. Pearson's correlation coefficient ($r$) was used to determine the correlation between the parameters evaluated. A $p$ value of <0.05 was adopted as the criterion for significant correlation. These analyses were carried out by SPSS 15.0 software (SPSS Inc., Chicago, IL, USA).

## RESULTS AND DISCUSSION

**Effect of Alcoholic Fermentation on the Bioactive Compounds of Orange Juice.** The antioxidant content of commercial orange juice before fermentation (day 0) is in agreement with other reported studies in orange juices, although factors such as species cultivars, environmental growing conditions, or maturity stage could vary the composition of the fruits.[19] Thus, ascorbic acid content at day 0 (409 mg/L) was similar to 369 mg/L.[5] The value of total phenolic content obtained in the orange juice was 793 mg/L, and total flavonoids content was 30.8 mg/L, which were comparable to those described by Tounsi et al.[5] (784 and 34.7 mg/L, respectively). In relation to the total carotenoids content, the value observed in

**Table 3. Antioxidant Content and Capacity of Orange Juice during Fermentation[a]**

| days of fermentation | AA[b] (mg/L) | TP[c] (mg/L) | TF[d] (mg/L) | TC[e] (mg/L) | ORAC (μM) | FRAP (mM) | TEAC (mM) | DPPH (% Inh[f]) |
|---|---|---|---|---|---|---|---|---|
| 0 | 409 ± 1.8ab | 793 ± 0.5a | 30.8 ± 8.5a | 5.8 ± 0.3ac | 6044 ± 247a | 10.3 ± 0.4ac | 5.4 ± 0.1a | 58.1 ± 2.6a |
| 1 | 409 ± 1.8ab | 801 ± 7.3ac | 42.3 ± 4.9b | 5.8 ± 0.4abc | 6653 ± 766a | 10.9 ± 0.4ab | 5.5 ± 0.1a | 63.1 ± 2.7ac |
| 3 | 406 ± 0.0a | 786 ± 3.8ac | 40.6 ± 5.3b | 6.0 ± 0.2ab | 6693 ± 791a | 10.8 ± 0.3ab | 5.6 ± 0.1a | 68.0 ± 0.1bc |
| 5 | 406 ± 0.0a | 775 ± 2.7ad | 53.2 ± 2.0c | 5.8 ± 0.1ab | 6517 ± 628a | 10.7 ± 0.4bd | 5.5 ± 0.1a | 70.1 ± 0.8bd |
| 7 | 401 ± 0.0ab | 764 ± 3.2 cd | 61.6 ± 1.4d | 6.1 ± 0.1abc | 7415 ± 599b | 10.3 ± 0.2acd | 5.5 ± 0.1a | 75.6 ± 0.2df |
| 9 | 400 ± 0.9ab | 714 ± 39.5abc | 75.3 ± 0.9e | 6.0 ± 0.3acd | 9355 ± 678c | 9.8 ± 0.2ce | 5.4 ± 0.0a | 82.5 ± 0.7e |
| 11 | 394 ± 5.4ab | 660 ± 1.2b | 95.7 ± 0.6f | 6.5 ± 0.2ce | 8169 ± 652d | 9.9 ± 0.1abe | 5.4 ± 0.0a | 77.4 ± 1.0ef |
| 13 | 391 ± 3.6ab | 667 ± 24.8bc | 92.6 ± 1.2f | 6.5 ± 0.2e | 8229 ± 649d | 10.1 ± 0.1abe | 5.3 ± 0.0a | 67.7 ± 2.0bc |
| 15 | 389 ± 1.8b | 722 ± 12.7c | 67.7 ± 1.0e | 6.3 ± 0.3bde | 7889 ± 382bd | 10.4 ± 0.2abe | 5.4 ± 0.1a | 64.4 ± 2.9bc |

[a]Values are expressed as the mean ± SD. Values with different letters (a−f) in the same column are significantly different at $p < 0.05$. [b]AA, ascorbic acid. [c]TP, total phenolics. [d]TF, total flavonoids. [e]TC, total carotenoids. [f]Inh, inhibition.

orange juice (5.8 mg/L) was an intermediate one compared to that obtained in other studies: 3 and 7.7 mg/L by Gardner et al.[20] and Lee,[21] respectively.

Kelebek et al.[22] determined the antioxidant content of orange juice after alcoholic fermentation, but they used spontaneous yeasts, obtaining orange wine with 12.6% ethanol (v/v). Controlled alcoholic fermentation in orange juice to obtain a product with low alcoholic degree has been used for the first time in this study; also, the influence of this process on the antioxidant content of orange juice was evaluated. Thus, the references to other works taking into account the impact of the fermentation on antioxidant content vary depending on the type of fermentation and the fermentation substrate. Table 3 reports the content of ascorbic acid, total phenolics, total flavonoids, and total carotenoids of orange juice during fermentation. It was observed that vitamin C content slightly decreased throughout the fermentation, but this decline was not significant. Kelebek et al.[22] described vitamin C content in orange juice decreasing significantly during spontaneous alcoholic fermentation. The influence of the fermentation on the ascorbic acid content has been evaluated in other foods, obtaining similar results. In this way, fermentation led to a slight reduction of ascorbic acid content in smoothies,[23] but this decrease was significant in strawberry juice.[24] The alcoholic fermentation induced a nonsignificant decrease in the ascorbic acid content of orange juice. Probably some amount of vitamin underwent degradation because ascorbic acid is very susceptible to chemical and enzymatic oxidation during processing. Time-consuming treatments such as fermentation of the juice reduced the ascorbic acid content because enzymes and oxygen are capable of attacking and inactivating ascorbic acid.[24]

Total phenolics content significantly decreased ($p < 0.05$) from day 0 (793 mg/L) until day 15 (722 mg/L) of the fermentation (Table 3). This decrease in total phenolics content is in agreement with the results reported by Kelebek et al.[22] for orange juice and those described for other foods. Thus, Sun et al.[25] noted that the amount of total phenolics in wine decreased significantly during alcoholic fermentation and the maturation period. The reasons for the decrease of phenolic compounds in orange juice during alcoholic fermentation were probably the precipitation or oxidation during the process, the combination or adsorption of phenolic compounds with solids, proteins, or even yeasts, and polymerization, which induces an important loss of these compounds.[26] In addition, phenolic acids, especially hydroxycinnamic, can be enzymatically degraded during microbial fermentation.[27]

Total flavonoids content significantly increased throughout the fermentation from day 0 (30.8 mg/L) to day 11 (95.7 mg/L) ($p < 0.05$) when the highest value was observed (Table 3). After day 11, total flavonoids decreased until the end of the process at day 15 (67.7 mg/L). Moreover, the difference between days 0 and 15 was also significant ($p < 0.05$), which agreed with other results reported for different foods. The fermentation of grape juice also led to an increase in the content of total flavonoids.[26] It has been recently found that fermentation could change the contents of active components in certain products. The type of microorganism and duration used in fermentation may also affect the concentration and profile of phytochemicals.[28] Therefore, alcoholic fermentation had a positive effect on total flavonoids content of orange juice. The presence of ethanol during fermentation can also contribute to the extraction of flavonoids from the pulp of the orange juice. The final decrease (days 12−15) might be explained by the decomposition of flavonoids due to the long fermentation.

Qualitative change in the flavanone pattern of orange juice during fermentation was subsequently evaluated. Table 4 shows the content of the individual flavanones at day 0 (orange juice), day 11 (peak of total flavonoids), and day 15 (end of fermentation). Flavanones in the soluble and pellet fractions were identified and quantified. The main flavanones in orange juice were the conjugate compounds naringenin-7-*O*-rutinoside (363.7 mg/L), hesperetin-7-*O*-rutinoside (274.9 mg/L), isosakuranetin-7-*O*-rutinoside (47.9 mg/L), and hesperetin-7-*O*-glucoside (11.5 mg/L). Gil-Izquierdo et al.[29] also quantified these as major flavanones in industrial orange juice. Total flavanone of orange juice (698.9 mg/L), expressed as the sum of individual flavanone concentrations, was in the range of previously reported commercial juices (292−703 mg/L).[30] After fermentation, at day 11 (806.2 mg/L) and day 15 (810.7 mg/L), the flavanones significantly increased ($p < 0.05$) with respect to orange juice (698.9 mg/L). Values were very similar on both days. To compare the evolution of both fractions, fermentation did not affect the content of soluble flavanones, except for naringenin-7-*O*-rutinoside and isosakuranetin-7-*O*-rutinoside, which increased between days 0 and 11, but fermentation increased the flavanone content of the cloud fraction. Alcoholic fermentation could extract more flavanones, although less soluble. Other technological treatments such as extraction pressure or industrial squeezing had produced this effect.[31] On the other hand, no hydrolysis of the glycosidic moiety of the flavanones to render the corresponding aglycones was observed. However, special attention must be paid to the partial hydrolysis of the rutinose moiety exerted during the fermentation toward the flavanone-7-*O*-glucosides in the supernatant and pellet

**Table 4. Flavanone Content of Orange Juice and Fermented Orange Juice**[a]

| | orange juice (mg/L) | | | fermented orange juice (mg/L) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | day 0 | | | day 11 | | | day 15 | | |
| flavanone[b] | supernatant | pellet | total | supernatant | pellet | total | supernatant | pellet | total |
| naringenin-7-*O*-glc | 0.2 ± 0.0 | 0.4 ± 0.0 | 0.6 ± 0.0a | 0.2 ± 0.0 | 0.5 ± 0.0 | 0.7 ± 0.0b | 0.2 ± 0.0 | 0.5 ± 0.0 | 0.7 ± 0.0b |
| naringenin-7-*O*-rut | 95.7 ± 4.0 | 268 ± 4.2 | 363.7 ± 8.4a | 113 ± 17.7 | 299 ± 18.5 | 412 ± 0.8b | 108 ± 4.5 | 305 ± 11.5 | 413 ± 7.1b |
| hesperetin-7-*O*-rut | 43.9 ± 5.0 | 231 ± 5.1 | 274.9 ± 10.2a | 43.5 ± 5.4 | 267 ± 3.7 | 310.5 ± 1.7b | 40.3 ± 0.5 | 271 ± 0.5 | 311.3 ± 0.0b |
| hesperetin-7-*O*-glc | 0.4 ± 0.0 | 11.1 ± 0.3 | 11.5 ± 0.7a | 0.2 ± 0.0 | 21.8 ± 2.9 | 22.0 ± 3.0b | 0.1 ± 0.0 | 24.4 ± 0.2 | 24.5 ± 0.1b |
| isosakuranetin-7-*O*-rut | 2.5 ± 0.5 | 45.4 ± 0.7 | 47.9 ± 1.3a | 4.7 ± 0.2 | 56.0 ± 3.3 | 60.7 ± 3.1b | 3.9 ± 1.2 | 57.0 ± 4.1 | 60.9 ± 5.3b |
| naringenin | 0.0 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0a | 0.0 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0a | 0.0 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0a |
| homoeriodyctiol | | | nq[c] | | | nq | | | nq |
| hesperetin | 0.0 ± 0.0 | 0.2 ± 0.0 | 0.2 ± 0.0a | 0.0 ± 0.0 | 0.2 ± 0.0 | 0.2 ± 0.0b | 0.0 ± 0.0 | 0.2 ± 0.0 | 0.2 ± 0.0b |
| isosakuranetin | | | nq | | | nq | | | nq |
| total flavanones | | | 698.9 ± 20.5a | | | 806.2 ± 5.1b | | | 810.7 ± 12.4b |

[a]Values are expressed as the mean ± SD. Total values with different letters (a, b) in the same line are significantly different at $p < 0.05$. [b]Naringenin-7-*O*-glc, naringenin-7-*O*-glucoside; Naringenin-7-*O*-rut, naringenin-7-*O*-rutinoside; Hesperetin-7-*O*-rut, hesperetin-7-*O*-rutinoside; Hesperetin-7-*O*-glc, hesperetin-7-*O*-glucoside; Isosakuranetin-7-*O*-rut, isosakuranetin-7-*O*-rutinoside. [c]nq, not quantified.

because hesperetin-7-*O*-glucoside increased during the fermentation process (overall in the pellet), whereas naringenin-7-*O*-glucoside remained unchanged (Table 4). This point is important because the rutinose moiety represents a metabolic barrier for the absorption rate of the flavanones at the small intestine in humans, the action of the gut microflora being necessary to remove the rhamnoglucoside moiety previous to the absorption by the distal part of the intestine.[32] Several previous papers have described the increase of the hesperetin absorption rate from orange juice when the hesperetin-7-*O*-glucoside is present in a larger extension thanks to a previous enzymatic treatment of the orange juice.[33] The results obtained in our study provide information about a possible and natural partial hydrolysis process of the hesperetin-7-*O*-rutinoside carried out by the yeasts. Besides, the previous studies showed that flavanone rutinosides from the juice soluble fraction are readily available to the body, whereas precipitates of flavanone rutinosides occurring in the juice cloud are not available for absorption.[29,30] However, no further studies have been carried out to observe the absorption rate at the small intestine of hesperetin-7-*O*-glucoside directly provided from the pellet of a citrus product like the fermented orange juice (in a nondependent manner of the gut microbiota action). The transmethylation reaction of the hesperetin developed by microbial transformations was not detected during the fermentation because homoeriodictyol, the isomer of the hesperetin, was not found in the fermented orange juice (Tables 2 and 4).[33] Therefore, the enhancement in the total and individual flavanone contents detected in fermented orange juice after day 11 of fermentation confers to the fermented juice an additional advantage over original juice. Numerous animal experiments and in vitro studies have shown that citrus flavanones exhibit a wide range of biological activities, which indicate that these compounds may exert beneficial effects against cardiovascular diseases, osteoporosis, or cancer.[34] Besides, the higher occurrence of hesperetin-7-*O*-glucoside in the fermented orange juice could suppose a positive effect on the modulation of bone metabolism and on the prevention of osteoporosis thanks to their higher absorption rates according to previous studies.[33,35]

Flavanones comprise the principal group of flavonoids and the major polyphenols in orange juice.[22] However, the obtained values of total flavanones were 10−20-fold higher than values of total flavonoids, and this accounted for only 3.8−14.5% of total phenolics. Although the method used for total flavonoids evaluation has recently been widely used in numerous foods,[36,37] this assay is not suitable for orange juice. The reaction products between aluminum ions and flavanones are low compared to flavanol or flavonol,[38] which occur in small quantities in orange juice.

In relation to total carotenoids, it is observed that the values significantly increased ($p < 0.05$) from day 0 of fermentation (5.8 mg/L) to the end of the process (day 15, 6.3 mg/L) (Table 3). Other authors reported that the carotenoids content did not exhibit any significant change after fermentation of other foods. In this way, Koh et al.[39] did not find significant variation in lycopene content of tomato juice after fermentation. In the present study, alcoholic fermentation led to enhancement of carotenoid pigments in orange juice. The reason for this was probably due to an enhanced extraction of these compounds from the pulp of the orange juice.

The carotenoid pigments profile of orange juice during fermentation was also subsequently evaluated. Figure 2 depicts the chromatogram at 450 nm of the carotenoid profile in the fermented orange juice (at day 15), and Table 5 summarizes the chromatographic data and reports the carotenoid composition at day 0 (orange juice), day 11, and day 15. Twenty-two carotenoids were identified in the orange juice. $\beta$-Cryptoxanthin showed the highest level in orange juice (0.71 mg/L), and one of the auroxanthin isomers (derived from violaxanthin under acidic conditions) presented the second greatest content (0.65 mg/L). These observations are in agreement with previous reports that $\beta$-cryptoxanthin is one of the major carotenoids in orange juice.[40] The total carotenoid content of orange juice, expressed as the sum of individual carotenoid concentrations, was 5.36 mg/L, which has been also found by other authors[41] in similar orange juices (5.7 mg/L). Among 22 identified carotenoids presented in Table 5, 21 of them showed significant changes ($p < 0.05$), increasing with fermentation measured at day 11 as well as at day 15. Despite the increases due to fermentation, the carotenoid pattern in juice did not change with respect to fermented samples. The total carotenoids content also showed a significant increase ($p < 0.05$) over the fermentation in relation to the juice (from 5.36 to 6.41 and 6.64 mg/L, corresponding to days 0, 11, and 15 of fermentation, respectively). Various carotenoids with provitamin A activity ($\beta$-carotene, $\alpha$-carotene, and $\beta$-cryptoxanthin) were present both in the juice and in the

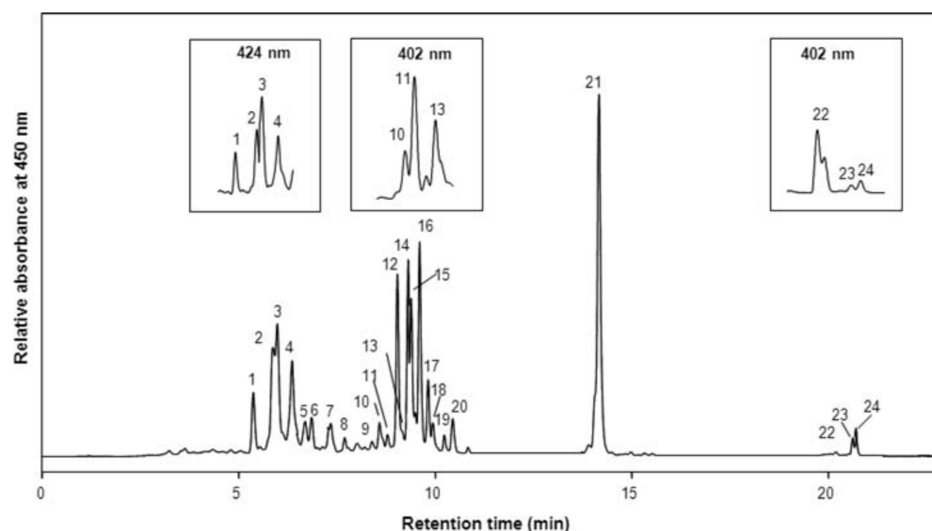


**Figure 2.** C18 reversed-phase HPLC chromatogram obtained for a saponified carotenoid extract prepared from fermented orange juice (at day 15). Peak identification and characterization are given in Table 5.

**Table 5. Structure Assignments, Retention Times ($t_R$), Mass Spectral Data, and Concentration of Carotenoids in Orange Juice and Fermented Orange Juice[a]**

| peak | structure assignment | $t_R$ (min) | $m/z$ [M + H]$^+$ | orange juice(mg/L) day 0 | fermented orange juice (mg/L) day 11 | fermented orange juice (mg/L) day 15 |
|---|---|---|---|---|---|---|
| 1 | latochrome[b] | 5.38 | 619 | 0.15 ± 0.00a | 0.17 ± 0.01b | 0.18 ± 0.00b |
| 2 | karpoxanthin[b] | 5.86 | nd[c] | 0.22 ± 0.01a | 0.28 ± 0.01b | 0.29 ± 0.01b |
| 3 | neochrome | 6.05 | 601 | 0.37 ± 0.01a | 0.44 ± 0.01b | 0.46 ± 0.01b |
| 4 | karpoxanthin isomer[b] | 6.31 | nd | 0.23 ± 0.01a | 0.29 ± 0.01b | 0.29 ± 0.00b |
| 5 | neochrome isomer | 6.71 | 601 | 0.10 ± 0.00a | 0.13 ± 0.00b | 0.13 ± 0.00c |
| 6 | karpoxanthin isomer[b] | 6.87 | nd | 0.10 ± 0.00a | 0.12 ± 0.00b | 0.12 ± 0.00b |
| 7 | not identified | 7.27 | nd | | | |
| 8 | not identified | 7.37 | nd | | | |
| 9 | luteoxanthin | 8.57 | 601 | nq[d] | nq | nq |
| 10 | auroxanthin isomer | 8.68 | 601 | 0.31 ± 0.01a | 0.32 ± 0.01a | 0.36 ± 0.02b |
| 11 | auroxanthin isomer | 8.79 | 601 | 0.65 ± 0.03a | 0.78 ± 0.04b | 0.86 ± 0.03c |
| 12 | mutatoxanthin isomer | 9.03 | 585 | 0.29 ± 0.01a | 0.34 ± 0.01b | 0.36 ± 0.01b |
| 13 | auroxanthin isomer | 9.18 | 601 | 0.43 ± 0.01a | 0.54 ± 0.01b | 0.55 ± 0.02b |
| 14 | mutatoxanthin isomer | 9.31 | 585 | 0.32 ± 0.01a | 0.38 ± 0.02b | 0.40 ± 0.00b |
| 15 | *all-trans*-zeaxanthin | 9.40 | 569 | 0.40 ± 0.02a | 0.47 ± 0.02b | 0.47 ± 0.00b |
| 16 | *all-trans*-lutein | 9.61 | 569 | 0.30 ± 0.01a | 0.35 ± 0.02b | 0.36 ± 0.00b |
| 17 | *cis*-mutatoxanthin isomer[b] | 9.82 | 585 | 0.13 ± 0.00a | 0.15 ± 0.00b | 0.16 ± 0.00b |
| 18 | *cis*-mutatoxanthin isomer[b] | 9.95 | 585 | 0.05 ± 0.00a | 0.06 ± 0.00b | 0.06 ± 0.00b |
| 19 | 9-*cis*-lutein | 10.23 | 569 | 0.02 ± 0.00a | 0.03 ± 0.00b | 0.03 ± 0.00b |
| 20 | 13-*cis*-lutein | 10.45 | 569 | 0.09 ± 0.00a | 0.11 ± 0.01b | 0.11 ± 0.00b |
| 21 | β-cryptoxanthin | 14.15 | 553 | 0.71 ± 0.03a | 0.85 ± 0.04b | 0.85 ± 0.01b |
| 22 | ζ-carotene | 20.27 | 541 | 0.37 ± 0.01a | 0.46 ± 0.02b | 0.46 ± 0.01b |
| 23 | *all-trans*-α-carotene | 20.61 | 537 | 0.04 ± 0.00a | 0.05 ± 0.00b | 0.05 ± 0.00b |
| 24 | *all-trans*-β-carotene | 20.72 | 537 | 0.08 ± 0.00a | 0.09 ± 0.00b | 0.09 ± 0.00b |
| | total carotenoids | | | 5.36 ± 0.21a | 6.41 ± 0.26b | 6.64 ± 0.07c |
| | RAEs[e] | | | 75.3 ± 3.58a | 90.7 ± 3.97b | 90.6 ± 1.53b |

[a]Values are expressed as the mean ± SD. Values with different roman letters (a−c) in the same row are significantly different at $p < 0.05$. [b]Tentative identification. [c]nd, not detected. [d]nq, not quantified. [e]Provitamin A content.

samples collected during the fermentation. Provitamin A values ranged from 75.3 to 90.7 and 90.6 RAEs/L, corresponding to days 0, 11, and 15 of fermentation, respectively. Meléndez-Martínez et al.[42] obtained data in the range of 9.7−94.8 RAEs/L for different types of commercial orange juice. Our results show that provitamin A content was significantly higher ($p < 0.05$) after the fermentation than at the beginning (orange juice). De novo biosynthesis of carotenoids is not possible during fermentation, but some changes in the internal structure of the suspended solids in which the pigments are located may enhance their extractability during the analytical procedure. The increase in the total and individual carotenoid contents detected in


dx.doi.org/10.1021/jf401240p | J. Agric. Food Chem. 2013, 61, 8773−8782

fermented orange juice is an advantage of the original juice. Carotenoids have multiple functions in human health and carotenoid pigments with provitamin A activity have also been assigned an important nutritional role. Most of the beneficial health effects are attributed to the antioxidant capacity of these bioactive compounds.[43]

To summarize, it is observed that fermentation induced a decrease in ascorbic acid and phenolic contents. However, the profile of bioactive compounds, flavanones, and pigments with provitamin A activity was significantly enhanced.

**Effect of Alcoholic Fermentation on the Antioxidant Capacity of Orange Juice.** The antioxidant capacity of the orange juice during fermentation was evaluated using ORAC, FRAP, ABTS, and DPPH assays. These assays, based on different chemical mechanisms, were selected to take into account the wide variety and range of action of antioxidant compounds present in orange juice.

The antioxidant capacity of commercial orange juice before fermentation (day 0) is in agreement with other reported studies on orange juices. Thus, the ORAC value at day 0 was 6044 $\mu$M, which is in accordance with Wang et al.[7] (6820 $\mu$M). The FRAP value obtained in the orange juice (10.3 mM) was similar to that described by Pellegrini et al.[6] (9.4 mM). The TEAC value (5.4 mM) was also comparable to that reported by Kim et al.[44] (4.9 mM). In relation to the DPPH assay, the value observed in orange juice (58.1% inhibition) was slightly greater than other data previously described. Klimczak et al.[9] showed inhibition up to 49.2%.

Table 3 reports the values of antioxidant capacity of orange juice during fermentation. ORAC values significantly increased ($p < 0.05$) from day 5 (6517 $\mu$M) to day 9 (9355 $\mu$M) of the fermentation. Subsequently, this value significantly declined ($p < 0.05$) until day 11 (8169 $\mu$M), remaining constant until the end of the process (day 15, 7889 $\mu$M). Moreover, the total increase between days 0 and 15 was also significant ($p < 0.05$), which agreed with Moore et al.,[45] who evaluated a significant increase of ORAC value of common hard wheat bran after solid-state yeast treatment. Therefore, alcoholic fermentation had a positive effect on the antioxidant capacity of orange juice measured by the ORAC assay.

FRAP values did not experience significant changes between consecutive measurements during fermentation. In addition, there were no significant differences between the beginning (day 0, 10.3 mM) and the end (day 15, 10.4 mM) of the process (Table 3). Studies based on other foods show similar effects on the FRAP value due to varied fermentations. The FRAP value of red wine during alcoholic fermentation was not significantly different.[25] Other authors have reported a significant decrease in FRAP values caused by fermentation. Therefore, the results of the antioxidant capacity of strawberry juice or mash using the FRAP assay showed a significant decrease within the processing fermentation.[24] In the present study, alcoholic fermentation of orange juice did not significantly affect the antioxidant capacity evaluated by FRAP assay.

The TEAC value of orange juice was constant throughout the fermentation. There were no significant differences between any pair of measurements (Table 3). However, other authors have obtained a marked effect of fermentation on the TEAC value, although with different trends. Klopotek et al.[24] indicated a significant decrease of the TEAC value after fermentation of strawberry mash and press juice. On the other hand, a higher antioxidant capacity was shown for yellow onion after fermentation with *Aspergillus kawachii*.[28] In the case of orange juice, alcoholic fermentation did not significantly influence the antioxidant capacity evaluated by the TEAC assay.

With regard to the evolution of the DPPH value during fermentation, a significant increase ($p < 0.05$) was observed from day 0 (58.1%) to day 9 (82.5%). However, from day 9 to day 15 (64.4%) the value decreased also significantly ($p < 0.05$). To compare the beginning (day 0) and the end (day 15) of the fermentation, significant change was observed ($p < 0.05$) (Table 3). Fermentation has a similar effect on the DPPH value of other products. The DPPH value was enhanced significantly after fermentation of onion.[28] Alcoholic fermentation significantly improved the antioxidant capacity of orange juice evaluated by the DPPH assay.

To summarize the antioxidant capacity results, it is observed that fermentation induced a significant increase in ORAC and DPPH values. However, TEAC and FRAP values remained constant through the process.

**Correlation between Antioxidant Content and Antioxidant Capacity of Fermented Orange Juice.** The correlation between antioxidant content (ascorbic acid, total phenolics, total flavanones, and total carotenoids) and antioxidant capacity (ORAC, FRAP, TEAC, and DPPH values) of fermented orange juice was evaluated. Table 6 shows correlation coefficients ($r$). Total flavanones positively and significantly correlated with ORAC and DPPH values ($r = 0.872$ and 0.794, respectively). Therefore, the increased antioxidant capacity of orange juice measured by ORAC and DPPH assays could be due to the enhancement of flavanones total content from fermentation. These results are in concordance with previous data that also described a high correlation between flavonoids content and ORAC and DPPH values.[46] In addition, both assays correlated positively and highly ($r = 0.740$) as shown in Table 6.

Total phenolics positively and highly correlated with FRAP ($r = 0.794$) and TEAC ($r = 0.787$) values and ascorbic acid with the TEAC value ($r = 0.773$). The evolution of these four parameters during fermentation was similar, although the changes observed were significant only for total phenolics. On the basis of these results, the total phenolics content could reasonably explain the antioxidant capacity measured by FRAP and TEAC assays in orange juice, and the ascorbic acid content also could contribute to the TEAC value. These results are in agreement

**Table 6. Correlation Coefficients ($r$) between Antioxidant Content and Capacity of Fermented Orange Juice[a]**

| | AA[b] | TP[c] | TFN[d] | TC[e] | ORAC | FRAP | TEAC | DPPH |
|---|---|---|---|---|---|---|---|---|
| ORAC | −0.707* | −0.794* | 0.872** | 0.614 | 1.000 | | | |
| FRAP | 0.599 | 0.794* | −0.514 | −0.638 | −0.781* | 1.000 | | |
| TEAC | 0.773* | 0.787* | −0.224 | −0.719* | 0.560 | 0.649 | 1.000 | |
| DPPH | −0.263 | −0.487 | 0.794* | 0.302 | 0.740* | −0.605 | −0.025 | 1.000 |

[a]Correlation is significant at the following levels: *, $p < 0.05$; **, $p < 0.01$. [b]AA, ascorbic acid. [c]TP, total phenolics. [d]TFN, total flavanones. [e]TC, total carotenoids obtained with the colorimetric assay.

with those of Thaipong et al.,[47] who concluded that total phenolics and ascorbic acid were well correlated with FRAP and TEAC values.

On the other hand, total carotenoids did not positively correlate with any antioxidant capacity method. Due to the nature of the reaction medium, the TEAC and DPPH assays could evaluate antioxidant capacity of lipophilic compounds as carotenoids; however, there was no correlation between the content of these compounds and TEAC and DPPH values. These results are in agreement with those of Thaipong et al.[47] The content of carotenoids in fermented orange juice could be insufficient to detect their antioxidant capacity with these methods.

In relation to the correlation between the four methods of antioxidant capacity used, a good correlation between only ORAC and DPPH values as above-mentioned was observed. This result is in accordance with other authors who have studied antioxidant capacity in guava fruit extracts.[47] However, poor correlation coefficients were found between the other methods (ORAC−TEAC, ORAC−FRAP, DPPH−TEAC, DPPH−FRAP, and TEAC−FRAP). These results were due to the different nature of the antioxidant compound contained in orange juice, which reacts differently depending on the reaction medium of the method (water- or lipid-soluble), the ability to react with radical species employed, and the nature of the reaction (hydrogen atom transfer/electron transfer). Consequently, comparison of different analytical methods for determining total antioxidant capacity is a key factor to understand the result obtained.

In summary, total flavanones and carotenoids contents of orange juice significantly increased after fermentation. In addition, the relative amount of all analyzed compounds increased significantly. Particularly, hesperetin-7-*O*-glucoside increased in the fermented orange juice, converting this new product in potentially higher bioavailable in flavanones than its corresponding original orange juice, and the pigments with provitamin A activity were also enhanced. Although ascorbic acid and total phenolics decreased, the decrease was significant only for total phenolics. In relation to antioxidant capacity, ORAC and DPPH values underwent a significant enhancement and TEAC and FRAP values remained constant through the process. Therefore, the results suggest that alcoholic fermentation has a positive effect on antioxidant content and activity of orange juice. Correlation analysis indicated that ORAC and DPPH values could be due to the increase of total flavanones.

In this study, we concluded that the fermentation of orange juice could be finalized on days 9−11. In this time interval fermented orange juice presented the maximum ORAC and DPPH values and a significant increase of total flavanones. Thus, the potential beverage produced by alcoholic fermentation of orange juice would have a higher antioxidant content and capacity than the substrate. Subsequent intervention studies are necessary to evaluate the potential health effect of fermented orange juice derived from both bioactive compounds and alcoholic degree.

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### Funding

We are grateful for the support of the Junta de Andalucía through Projects P09-AGR4814M, P08-AGR-03477, and Grupo PAI BIO311 and of national funding agencies through Projects AGL2010-14850/ALI, AGL2011-23690, CSD007-0063 (Consolider-Ingenio 2010 'Fun-C-Food'), and CSIC 201170E041. We are also grateful to the Fundación Séneca - CARM "Group of Excellence in Research" 04486/GERM/06. The Research Project grant of B.E-.L. is supported by the Junta de Andalucía. A.G.-I., F.F., and S.M. are members of the CORNUCOPIA Network 112RT0460, and D.H.-M. of the IBERCAROT Network 112RT0445 financed by CYTED.

### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

We are grateful to Grupo Hespérides Biotech S.L. for providing samples of fermented orange juice.

## ABBREVIATIONS USED

ROS, reactive oxygen species; TA, titratable acidity; TSS, total soluble solids; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; MPA, metaphosphoric acid; TPTZ, 2,4,6-tris(2-pyridyl)-s-triazine; DMSO, dimethyl sulfoxide; DPPH, 1,1-diphenyl-2-pricrylhydrazyl; ORAC, oxygen radical absorbance capacity; FRAP, ferric reducing antioxidant power; TEAC, Trolox equivalent antioxidant capacity; APCI, atmospheric pressure chemical ionization; RAE, retinol activity equivalents

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