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Effect of Thermal Processing on the Profile of Bioactive Compounds and Antioxidant Capacity of Fermented Orange Juice

Blanca Escudero-López,[1] Isabel Cerrillo,[1,2] Ángel Gil-Izquierdo,[3] Dámaso Hornero-Méndez,[4] Griselda Herrero-Martín,[1] Genoveva Berná,[1,5] Sonia Medina,[3] Federico Ferreres,[3] Franz Martín,[1,5] and María-Soledad Fernández-Pachón[1,2]

[1]*Area de Nutrición y Bromatología, Departamento Biología Molecular e Ingeniería Bioquímica, Universidad Pablo de Olavide, Carretera de Utrera Km 1, 41013, Sevilla, Spain*

[2]*Facultad de Ciencias de la Salud, Investigador Asociado de la Universidad Autónoma de Chile, Av. Pedro de Valdivia 641, Santiago de Chile, Chile*

[3]*Departamento de Ciencia y Tecnología de Alimentos, CEBAS-CSIC, Campus de Espinardo 25, 30100 Murcia, Spain*

[4]*Departamento de Fitoquímica de los alimentos, Instituto de la Grasa-CSIC, Av. Padre García Tejero 4, 41012, Sevilla, Spain*

[5]*CIBER de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Universidad Pablo de Olavide, Carretera de Utrera Km 1, 41013, Sevilla, Spain*

Correspondence should be addressed to Blanca Escudero López; besclop@upo.es

Abstract

Previously, we reported that alcoholic fermentation enhanced flavanones and carotenoids content of orange juice. The aim of this work was to evaluate the influence of pasteurization on the qualitative and quantitative profile of bioactive compounds and the antioxidant capacity of fermented orange juice. Ascorbic acid (203 mg/L), total flavanones (647 mg/L), total carotenoids (7.07 mg/L) and provitamin A (90.06 RAEs/L) values of pasteurized orange beverage were lower than those of fermented juice. Total phenolic remained unchanged (585 mg/L) and was similar to that of original juice. The flavanones naringenin-7-*O*-glucoside, naringenin-7-*O*-rutinoside, hesperetin-7-*O*-rutinoside, hesperetin-7-*O*-glucoside and isosakuranetin-7-*O*-rutinoside, and the carotenoids karpoxanthin and isomer, neochrome, lutein, ζ-Carotene, zeaxanthin, mutatoxanthin epimers, β-cryptoxanthin and auroxanthin epimers were the major compounds. Pasteurization produced a decrease in antioxidant capacity of fermented juice. However, TEAC (5.45 mM) and ORAC (6353 μM) values of orange beverage were similar to those of original orange juice. The novel orange beverage could be a valuable source of bioactive compounds with antioxidant capacity and exert potential beneficial effects.

Keywords: Orange juice, alcoholic fermentation, thermal pasteurization, bioactive compounds, antioxidant capacity, food composition.

Introduction

In recent years clinical and epidemiological studies have established that the intake of a diet rich in fruits and vegetables decreases the occurrence of diseases related to oxidative stress such as inflammation, cardiovascular diseases, cancer and aging-related disorders (Fernández-Pachón et al., 2008). Citrus fruits, and especially orange juice, are known to be rich sources of bioactive compounds such as ascorbic acid, flavonoid and carotenoid, with demonstrated antioxidant capacity (Stella et al., 2011; Tounsi et al., 2011; Stinco et al., 2012). The health promoting benefits related with the consumption of orange juice have prompted research into the adequate production conditions and technological treatments to maintain or enhance their biological functions and subsequent application in food industry (Klimczak et al., 2007; Agcam et al., 2014). In recent years, fermentation processes have been carried out in fruits (apple (Ajila et al., 2011), mulberry (Kwak et al., 2012), pomenagrate (Mena et al., 2014)), vegetables (onion (Yang et al., 2012)) and legumes (soy germ (Sheih et al., 2014)) resulting in products which provide a higher concentration of bioactive compounds content than the respective substrate. Another advantage presented by some fermented products is its moderate alcohol content. Numerous studies have demonstrated that moderate alcoholic consumption produces positive effects on lipid profile, coagulation system and atherosclerotic process (Wang et al., 2008). So, some fermented products have exerted beneficial health properties (Yoo et al., 2010).

Our previous study evaluated for the first time the influence of 15-days controlled alcoholic fermentation on bioactive compounds profile and the antioxidant capacity of orange juice. This process enhanced total flavanones and carotenoids content and antioxidant capacity of orange juice and induced an alcohol content of 0.85% v/v. The fermentation process of orange juice could be finalized on days 9-11 because in this

time interval fermented orange juice presented the maximum values of total flavanones and antioxidant capacity and a significant increase of total carotenoids (Escudero-López et al., 2013). Otherwise, it is necessary to inactivate microorganisms in order to preserve and make the fermented orange juice available to the consumer. Despite numerous processes like pulsed electric fields, high pressures or microwaves, thermal pasteurization is still regarded as the most suitable preservation process for being inexpensive, efficient and environmentally friendly (Silva & Gibbs, 2008). However, alteration of nutrients and phytochemicals may occur during thermal treatment (Sánchez-Moreno et al., 2005).

The aim of the present study is to evaluate the influence of thermal pasteurization on the content of individual bioactive compounds and the antioxidant capacity of the orange juice fermented during 10 days and to obtain, therefore, a complete characterization of qualitative and quantitative profile in the resulting orange beverage.

Furthermore, we consider that the bioactivity characterization of fermented-pasteurized orange juice is important for the potential applications of this beverage as functional food because its healthy effect was tested in a previous study. The results showed that the consumption of orange beverage reduced cardiovascular risk factors in healthy mice, greater extent than orange juice, due to possible synergistic effects between its bioactive compounds and moderate alcohol content (Escudero-López et al., 2015).

Methods

Chemicals and Reagents

Naringenin-7-*O*-glucoside, naringenin-7-*O*-rutinoside, hesperetin-7-*O*-rutinoside, isosakuranetin-7-*O*-rutinoside, naringenin, homoeriodictyol, hesperetin, isosakuranetin, butylated hydroxytoluene (BHT), 2,2´-Azino-bis(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), L-ascorbic acid, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), iron (II) sulphateheptahydrate, 1,1-diphenyl-2-pricrylhydrazyl (DPPH), gallic acid monohydrate, iron chloride hexahydrate, potassium persulfate, sodium carbonate, sodium di-hydrogen phosphate (anhydrous) and potassium phosphate dibasic were obtained from Sigma–Aldrich Quimica (Alcobendas, Spain). Hesperetin-7-*O*-glucoside was obtained from Faces Biochemical Co., Ltd. (Wuhan, China). Folin-Ciocalteau reagent and methanol were provided from Merck (Mollet del Vallés, Spain). Dimethyl sulfoxide (DMSO) and formic acid were purchased from Panreac (Seville, Spain). HPLC-grade acetone was supplied by BDH Prolabo (Barcelona, Spain). Diethyl ether was purchased from Scharlab (Barcelona, Spain). Acetonitrile was obtained from J.T. Baker (Phillipsburg, New Jersey). HPLC-grade deionised water was produced with a Milli-Q 50 system (Millipore Iberica S.A., Madrid, Spain).

Orange Beverage Production and Sampling

The company Grupo Hespérides Biotech S.L. carried out the orange beverage production. A commercial orange juice made from *Citrus sinensis* L. var. *Navel late* (Huelva, Spain) was used. The criteria for the selection of this orange juice were the compositional homogeneity, microbiological stability and organoleptic quality. These aspects were necessary for adequate development of the fermentation process and consumer acceptance of final product. Table 1 gives the quality parameters of orange juice used: pH, titratable acidity (TA), total glucids (reducing and non-reducing sugar), total soluble solids (TSS) (ºBrix), and alcohol (OIV, 2014).

The fermentation was carried out in 3 parallel pvc tanks (5 L) at 20 °C for 10 days in repose. The yeast specie *Pichia kluyveri* was isolated from the natural microbiota

present in the orange fruit and used for the inoculation of the fermentation. The selected yeast strain only ferments reducing sugars, resulting in a final product with low alcohol content. The thermal pasteurization was achieved in a laboratory pilot scale pasteurizer. Fermented orange juice was placed in a feeding tank and impulsed by a pump (Bio-Rad Econo Pump, Madrid, Spain) to the heat exchanger immersed in a water bath equipped with a temperature control system (FALC BS 70, Treviglio, Italy). The thermal process was carried out at 85 ºC for 30 s. After treatment, the product was cooled to 10 ºC in an ice-water-bath. The quality parameters of fermented and pasteurized orange juice were measured (Table 1).

Samples for the current study were collected at the beginning (orange juice, OJ), at the end of fermentation process (fermented orange juice, F-OJ) and after thermal pasteurization (fermented and pasteurized orange juice, FP-OJ), and immediately stored at -20 ºC until analysis. The supernatant of samples obtained by centrifugation at 3000g (4 ºC, 10 min) was utilized in the evaluation of total phenolic and antioxidant capacity.

Analysis of Ascorbic Acid Content

The assessment of ascorbic acid content was performed with the 2,6-dichlorophenolindophenol method according to Official Methods of Analysis of AOAC International (AOAC, 2005) which has been widely applied to orange juices (Meléndez-Martínez et al., 2007a; Stinco et al., 2012). Equal volumes (5 mL) of sample and aqueous MPA (3% w/v) were mixed and centrifuged at 4000g for 10 min. Five mililiters of supernatant was used for the titration reaction using 2,6-dichlorophenolindophenol. The titration reaction was based on the reduction of the sodium salt of the dye by ascorbic acid resulting in the formation of a colourless derivative and dehydroascorbic acid. The endpoint of titration was indicated by the

appearance of a persistent pink colour. 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 was determined using the Folin-Ciocalteu colorimetric method (Singleton & Rossi, 1965), with some modifications. A previously shaken sample (1.5 mL) was diluted with distilled water (1:5). An aliquot of the diluted sample (20 µL) was added to 1.58 mL water and 100 µL 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 Helios Epsilon UV-vis spectrophotometer (Thermo Scientific, Madison, USA).

Qualitative and Quantitative Analysis of Flavanones

Samples were analyzed by UHPLC-MS/MS, and the content of the individual flavanones was achieved using external standards. Briefly, a volume (10 mL) of each sample was centrifuged at 4500g 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), was filtered through a 0.45 µm PVDF Millex (Millipore) 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 x 2.1 mm, 1.7 µm; Waters, Milford, 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 (MRM) in negative mode was carried out for the characterization and quantification of the flavanones. Standard concentrations ranged from 0.312 to 10 µM and the correlation coefficients ($r^2 > 0.99$) were obtained for each of the compound. 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 ºC; nebulizer pressure: 40 psi; capillary voltage: 4000 V; nozzle voltage: 1000 V. MassHunter Software version B 04.00 (Agilent Technologies) was used for MS control and data gathering, and MassHunter Software version B 03.01 (Agilent Technologies) for data processing, peak integration and linear regression.

Qualitative and Quantitative Analysis of Carotenoids Pigments

The extraction of carotenoids was performed in agreement with the method of Mínguez-Mosquera and Hornero-Méndez (1993) with some modifications, as described by Escudero-López et al. (2013). Briefly, an aliquot (10 mL) of juice sample was centrifuged at 10000g and 4 ºC for 10 min, and the pellet containing the carotenoids was extracted with acetone (3 mL). The extract was dried under nitrogen stream and subsequently dissolved in 3 mL of diethyl ether. 0.5 mL of 20% (w/v) KOH-MeOH was added for saponification during 20 min with periodic agitation. After neutralization with water the upper phase (organic) was collected by centrifugation at 5000g and 4 ºC for 5 min, dried under a nitrogen stream, dissolved in 0.5 mL acetone (containing 0.1% BHT)

and stored at -30 ºC until analysis, which it was carried out in the same day of the preparation of the extracts. All operations were made under dimmed light to prevent isomerization and photodegradation of carotenoids.

The identification and quantification of the carotenoid pigments were carried out as detailed by Escudero-López et al. (2013). Carotenoids were analyzed by HPLC using 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. The column was maintained at 25 ºC. 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). Pigments were quantified in the saponified extracts using calibration curves (6-8 concentration levels) prepared with standard stock solutions for each carotenoid in the concentration range 5-100 µg/mL. The quantification of *cis* isomers was carried out by using the calibration curve of the corresponding all-*trans* counterpart.

LC-MS was performed by coupling a chromatographic system with a Micromass ZMD4000 mass spectrometer equipped with a single quadrupole analyzer (Micromass Ltd, Manchester, United Kingdom) and atmospheric pressure chemical ionization (APCI) probe. The system was controlled with MassLynx 3.2 software (Micromass Ltd). MS conditions were: positive ion mode ($APCI^+$); source temperature: 150 ºC; probe temperature: 400 ºC; corona voltage: 3.7 kV; high voltage lens: 0.5 kV; cone voltage: 30V. 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 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 (such as β-cryptoxanthin).

Oxygen Radical Absorbance Capacity (ORAC) Assay

ORAC assay was performed according to Ou et al. (2001) 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 sodium fluorescein (2.934 mg/L) and incubated for 15 min at 37 ºC. Subsequently, 50 µL of AAPH (221.25 mM) were added and the fluorescence (λ_{ex}: 460 nm; λ_{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, Winooski, USA).

Ferric Reducing Antioxidant Power (FRAP) Assay

The ferric reducing hability was carried out according to Delgado-Andrade et al. (2005). 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 $FeCl_3 \bullet 6H_20$ 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 ºC. The results were obtained from a calibration curve using different concentrations of $FeSO_4 \bullet 7H_2O$ (0.2-1.5 mmol/L). Measurements were recorded on a SynergyTM HT-multimode microplate reader (Biotek Instruments).

Trolox Equivalent Antioxidant Capacity (TEAC) Assay

TEAC assay was realized following the procedure described by Delgado-Andrade et al. (2005). 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 $ABTS^{\bullet+}$ 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 were added to 280 µL of diluted $ABTS^{\bullet+}$ solution. The change at the maximum absorption (730 nm) was evaluated up to 30 min at 30 ºC. 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

DPPH assay was carried out according to previous study (Delgado-Andrade et al., 2005) 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 were added. The solution was incubated in the dark at 30 ºC for 60 min and DPPH absorption was measured at 520 nm. The ability of scavenging the DPPH radical was calculated with the following equation: % Inhibition = $[(A_0-A_1)/A_0]$ x 100, where A_0 is the absorbance of the control 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 and subsequently thermal pasteurization processes were performed in triplicate, and all analyses were in triplicate. The analysis of variance (one-way

ANOVA; Duncan test) was applied. A probability value of $p < 0.05$ was adopted as the criteria for significant differences. These analyses were carried out by SPSS 15.0 Software (SPSS Inc., Chicago, USA).

Results and Discussion

Analysis of Bioactive Compounds of Fermented and Pasteurized Orange Juice

The quantitative and qualitative profile of bioactive compounds of selected OJ in the current study was in agreement with other authors (Gama & Sylos, 2005; Vallejo et al., 2010; Tounsi et al., 2011). Fig. 1 reports ascorbic acid content of OJ, F-OJ and FP-OJ. Ascorbic acid content of OJ kept stable during fermentation process according to our previous study (Escudero-López et al., 2013). However, this content significantly decreased after the pasteurization process ($p < 0.05$), resulting in a 42% loss in relation to F-OJ. The fermented and pasteurized orange juice contained 203 mg/L of ascorbic acid. The influence of pasteurization process on the bioactive compounds content of a fermented orange juice was evaluated for the first time. Thus, the results achieved in this study were related to other studies based on findings from pasteurization of non-fermented orange juices and fermented juices of other fruits. Accordingly, the degree of decrease of ascorbic acid obtained in FP-OJ was intermediate in relation to thermal treatments of other orange juices and lower than other fermented products. In this way, Min et al. (2003) obtained a 19% degradation in ascorbic acid of orange juice after thermal process (90 ºC/90 s), and Gil-Izquierdo et al. (2002) observed a loss of 58% (95 ºC/30 s). Klopotek et al. (2005) reported a loss of 66% in fermented strawberry juice caused by pasteurization process (85 ºC/5 min). Ascorbic acid is a well-known heat-sensitive compound (Sánchez-Moreno et al., 2005) and, therefore, thermal process can greatly affect the rate of its degradation.

In relation to total phenolic content, the fermentation process did not induce any significant change between OJ and F-OJ (Escudero-López et al., 2013). Subsequently, total phenolic content underwent a decline after thermal treatment (9.6% loss) in FP-OJ, but this change was not significant with regard to F-OJ (Fig. 1). Total phenolic content of FP-OJ reached a value of 585 mg/L. Gil-Izquierdo et al. (2002) also described that pasteurization (75 ºC/30 s and 95 ºC/30 s) did not impact on the total phenolic content of orange juice. In the same way, Oliveira et al. (2012) showed that the total phenolic content of peach was unaltered with the pasteurization treatment (90 ºC/5 min). Pasteurization did not promote significant effect on the level of total phenolic compounds that appear to be relatively resistant to the treatment temperatures assayed.

Flavanones are the major phenolic compounds of orange juice (Khan et al., 2014) and numerous studies have shown that citrus flavanones exhibit a wide range of biological activities and may exert beneficial effects against cardiovascular diseases, osteoporosis, and cancer (Espín et al., 2007).Thus, the qualitative and quantitative changes in the flavanone pattern of orange juice due to the fermentation and pasteurization processes were subsequently evaluated. Table 2 shows the chromatographic data and the content of the individual flavanones of OJ, F-OJ and FP-OJ, and also specifies the content in both fractions soluble and pellet. The total flavanones, expressed as the sum of individual flavanone concentrations, are presented in Fig. 2A. After fermentation, the flavanone profile of F-OJ did not vary and total flavanones significantly increased ($p < 0.05$) with respect to OJ (Escudero-López et al., 2013). Due to the pasteurization process, the content of individual flavanones was significantly lower in FP-OJ with respect to F-OJ (Table 2). The decrease percentages of the four major compounds were 26.6% (naringenin-7-*O*-rutinoside), 15.6% (hesperetin-7-*O*-rutinoside), 52% (isosakuranetin-7-*O*-rutinoside), and 51% (hesperetin-7-*O*-glucoside). A total of five

flavanones were quantified in the fermented and pasteurized orange juice: naringenin-7-*O*-glucoside (0.5 mg/L), naringenin-7-*O*-rutinoside (342.1 mg/L), hesperetin-7-*O*-rutinoside (270.0 mg/L), hesperetin-7-*O*-glucoside (8.1 mg/L), and isosakuranetin-7-*O*-rutinoside (25.8 mg/L). Naringenin and hesperetin, previously quantified in OJ and F-OJ, were not detected in FP-OJ. Total flavanones also decreased significantly ($p < 0.05$) from F-OJ to FP-OJ (24.6% loss) (Fig. 2A). However, Gil-Izquierdo et al. (2002) and Sánchez-Moreno et al. (2005) did not obtain decrease in these individual and total flavanones after pasteurization of non-fermented orange juice (95 ºC/30 s). Although pasteurization process caused a decline in total flavanone content, FP-OJ retained an amount (646.6 mg/L) within the range of pasteurized orange juices without previous fermentation process: 289-598 mg/L (Gil-Izquierdo et al., 2001) and 292-703 mg/L (Vallejo et al., 2010). To compare both fractions, fermentation process led an increase in the four major flavanones of the pellet fraction, but only improved the naringenin-7-*O*-rutinoside content of soluble fraction. Fermentation extracted insoluble flavanones but more susceptible to degradation by heat treating. Consequently pasteurization caused losses of 60.1% (isosakuranetin-7-*O*-rutinoside), 52.4% (hesperetin-7-*O*-glucoside), 31.7% (naringenin-7-*O*-rutinoside) and 20.1 % (hesperetin-7-*O*-rutinoside) in pellet fraction. Gil-Izquierdo et al. (2002) also observed the following decreasing percentages after pulp pasteurization: 28% (naringenin-7-*O*-rutinoside), 19% (hesperetin-7-*O*-rutinoside) and 1% (isosakuranetin-7-*O*-rutinoside). However, pasteurization did only affect the content of naringenin-7-*O*-rutinoside in soluble fraction.

Carotenoid pigment composition was also evaluated. These compounds have multiple key functions in human health and exert beneficial effects against cardiovascular disease, cancer, age-related macular degeneration and osteoporosis (Fernández-García

et al., 2012). Table 3 summarizes the chromatographic data and reports the individual carotenoid content of OJ, F-OJ and FP-OJ. Fig. 2B shows total carotenoid content (expressed as the sum of individual carotenoid concentrations) and provitamin A value (RAEs/L) of the samples. The carotenoid profile was identical in OJ and F-OJ. Total carotenoid content and provitamin A value showed significant increase ($p < 0.05$) over the fermentation (Escudero-López et al., 2013). The pasteurization process did also induce changes in the carotenoid profile between F-OJ and FP-OJ. However, all carotenoid pigments significantly decreased ($p < 0.05$) after thermal treatment. The decreasing percentages of major carotenoids were 51.6% (auroxanthin epimer2), 50.7% (auroxanthin epimer3) and 44.9% (β-cryptoxanthin). A total of twenty-one carotenoids were quantified in the fermented and pasteurized orange juice, and the following pigments accounted for principal content: karpoxanthin and isomer (0.68 mg/L), neochrome (0.33 mg/L), lutein (0.47 mg/L), ζ-Carotene (0.64 mg/L), zeaxanthin (0.69 mg/L), mutatoxanthin epimers (0.73 mg/L), β-cryptoxanthin (0.81 mg/L) and auroxanthin epimers (2.02 mg/L). Total carotenoids also decreased significantly ($p < 0.05$) from F-OJ to FP-OJ (45.8% loss). Similar findings were reported by Fratianni et al. (2010) and Stinco et al. (2012) who described significant losses in total carotenoid content (50 and 23.5%, respectively) after pasteurization (85 ºC/1min and 99 ºC/15 s, respectively) of orange juices. In the same way, provitamin A value decreased significantly ($p < 0.05$) due to thermal treatment (a loss 46.6% between F-OJ and FP-OJ). Cortés et al. (2006) reported similar result in orange juice (loss 36%) after pasteurization at 80 ºC/2 min. Because of their highly unsaturated conformation, it was widely presumed that carotenoids in general undergo isomerization and oxidative degradation with thermal processing (Shi & Maguer, 2000) and this would be the principal cause of extensive losses of carotenoids. Despite the fact that thermal

treatment caused a decrease in total carotenoid content and provitamin A value, FP-OJ maintained levels (7.07 mg/L and 90.1 RAEs/L, respectively) within the range of non-fermented and pasteurized orange juices: 5.70-12 mg/L for total carotenoid content (Lee & Coates, 2003; Stinco et al., 2012) and 20.4-89 RAEs/L for provitamin A value (Klopotek et al., 2005).

To summarize, thermal treatment caused a significant decline in ascorbic acid and individual and total flavanones and carotenoids of fermented orange juice. Total phenolic did not show significant change during this process. However, the new orange beverage (FP-OJ) maintained amounts of flavanones and carotenoids within the range of other pasteurized orange juices without a previous fermentation process (Meléndez-Martínez et al., 2007b; Vallejo et al., 2010; Stinco et al., 2012).

Analysis of Antioxidant Capacity of Fermented and Pasteurized Orange Juice

The antioxidant capacity of samples was evaluated using ORAC, FRAP, TEAC and DPPH assays. These methods, 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. Fig. 3 reports the values of antioxidant capacity of OJ, F-OJ and FP-OJ. The antioxidant capacity of OJ was in agreement with other studies on orange juice (Pellegrini et al., 2003; Klimczak et al., 2007; Seeram et al., 2008). After fermentation, TEAC and FRAP values remained constant. However, ORAC and DPPH values of F-OJ increased significantly ($p < 0.05$) with respect to OJ (Escudero-López et al., 2013). Pasteurization process produced a significant decrease ($p < 0.05$) in all values of antioxidant capacity in FP-OJ with respect to F-OJ: 46.3% (ORAC), 38% (FRAP), 16.5% (TEAC) and 57.7% (DPPH). Other authors also reported a lower antioxidant capacity of juices after thermal treatments. Elez-Martínez et al. (2006) observed a significant decrease in DPPH value (26.1%) after thermal treatment (90 ºC/1 min) of

orange juice. FRAP and TEAC value of strawberries juice decreased (30% and 10 %, respectively) after pasteurization (85 ºC/5 min) (Klopotek et al., 2005). Mena et al. (2013) also obtained losses of 10% and 20% in TEAC and DPPH values, respectively, of pomegranate juice due to pasteurization (65 ºC/30 s). The decrease in antioxidant capacity values could be due to partial loss of ascorbic acid and total flavanones. At quantitative level, the decline of carotenoids could not be relevant. Although pasteurization process declined antioxidant capacity of F-OJ, when comparing the values of FP-OJ (ORAC: 6353 μM, FRAP: 5.46 mM, TEAC: 5.45 mM and DPPH: 28.8% inhibition) with the substrate (OJ) it was observed that TEAC and ORAC were maintained without significant changes. The new orange beverage would preserve the antioxidant capacity derived from bioactive compounds content. When comparing the antioxidant capacity of orange beverage with other similar drinks with widely demonstrated beneficial effects, it was observed that FP-OJ exhibits greater antioxidant capacity than white wine and beer, and a similar capacity to that of rosé wine (Pellegrini e al., 2003). If related to other fruit juices, the antioxidant capacity of FP-OJ was higher than that of apple, pineapple, tomato, tropical and apricot juices (Pellegrini et al., 2003; Mullen et al., 2007) and other beverages such as black and green teas (Pellegrini et al., 2003; Seeram et al., 2008).

Conclusions

In this study, orange juice was subjected to alcoholic fermentation during 10 days and subsequent pasteurization to obtain a potential low alcoholic orange beverage. The changes in bioactive compounds and antioxidant capacity after thermal treatment were evaluated in order to establish the composition of this novel product which is important for its potential applications as functional food. The orange beverage showed lower flavanone and carotenoid content than orange juice but remained at comparable levels to

other pasteurized orange juices. The antioxidant capacity was preserved (TEAC and FRAP values) in spite of the partial loss of these compounds. Thus, the orange beverage could be considered an excellent source of bioactive compounds with antioxidant capacity. Future intervention studies in humans are necessary to evaluate its bioavailability and biological activity due to both bioactive compounds and moderate alcohol content and to verify its potentially beneficial effects for nutrition and health.

Declaration of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Figures captions:

Figure 1: Ascorbic acid and total phenolic content of OJ (orange juice), F-OJ (fermented orange juice), and FP-OJ (fermented-pasteurized orange juice). Dark gray bars: Ascorbic acid (mg/L); light gray bars: total phenolic content (mg/L). Values are expressed as mean (n=3) ± SD. The bars in the same measurement with different letters show significant difference ($p < 0.05$).

Figure 2: Flavanone and carotenoid content of OJ (orange juice), F-OJ (fermented orange juice), and FP-OJ (fermented-pasteurized orange juice). A) Flavanone content in the soluble and pellet fractions and total flavanone content. Dark gray bars: flavanone content in the soluble fraction (mg/L); light gray bars: flavanone content in the pellet fraction (mg/L); white bars: total flavanone content (mg/L). B) Total carotenoid and provitamin A contents. Dark gray bars: total carotenoid content (mg/L); light gray bars: provitamin A content (RAEs). Provitamin A content values are on the right axis and total carotenoid content values are on the left. Values are expressed as mean (n=3) ± SD. The bars in the same measurement with different letters show significant difference ($p < 0.05$).

Figure 3: Antioxidant capacity of OJ (orange juice), F-OJ (fermented orange juice), and FP-OJ (fermented-pasteurized orange juice). A) Dark gray bars: FRAP values (mmol Fe^{2+}/L); light gray bars: TEAC values (mmol Trolox equivalents/L). B) Dark gray bars: ORAC values (µmol Trolox equivalents/L); light gray bars: DPPH values (%inhibition). DPPH values are on the right axis and ORAC values are on the left. Values are expressed as mean (n=3) ± SD. The bars in the same measurement with different letters show significant difference ($p < 0.05$).

TABLE 1: Quality parameters of orange juice before and after fermentation and pasteurization.

Quality parameters	Orange juice	Fermented and pasteurized orange juice
pH	3.48±0.20	3.45±0.20
TA (g citric acid/L)	8.48±0.02	8.83±0.02
Total glucids (g/L)	78.2±5.64	52.3±4.23
Reducing sugars (g/L)	48.5±3.63	23.9±2.54
Non-reducing sugars (g/L)	29.7±2.01	29.5±2.33
TSS (ºBrix)	11.0±0.50	10.0±0.50
Alcohol (% v/v)	n.d.	0.85±0.01

Values are expressed as mean (n=3) ± SD. TA, titratable acidity; TSS, total soluble solids; n.d., no detected.

TABLE 2: Structure assignments, retention times (Rt), mass spectral data and concentration of flavanones in orange juice (OJ), fermented orange juice (F-OJ) and fermented-pasteurized orange juice (FP-OJ).

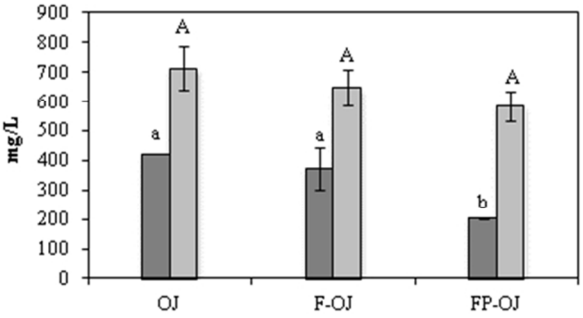
Flavanones	Rt (min)	m/z [M-H]$^-$	MRM transitions (m/z)	Concentration (mg/L)								
				OJ			F-OJ			FP-OJ		
				Soluble	*Pellet*	***Total***	*Soluble*	*Pellet*	***Total***	*Soluble*	*Pellet*	***Total***
Naringenin-7-*O*-rutinoside	4.6	579	[579→271]	136.5±2.2	263.5±27.0	**400.9±24.8**^{A}	162.4±8.8	303.8±2.8	**466.2±11.6**^{B}	135.1±2.2	207.1±11.3	**342.1±13.6**^{C}
Hesperetin-7-*O*-rutinoside	4.8	609	[609→301]	64.9±5.1	239.2±18.3	**304.1±13.2**^{A}	60.9±7.1	259.0±5.4	**319.9±1.6**^{A}	62.3±4.9	208.7±8.2	**270.0±3.4**^{B}
Naringenin-7-*O*-glucoside	5.1	433	[433→271]	0.3±0.0	0.4±0.1	**0.7±0.1**^{A}	0.3±0.0	0.5±0.0	**0.8±0.0**^{A}	0.2±0.0	0.3±0.0	**0.5±0.0**^{B}
Hesperetin-7-*O*-glucoside	5.2	463	[463→301]	0.2±0.1	12.9±2.1	**13.1±2.1**^{AB}	0.1±0.1	16.6±1.6	**16.7±1.6**^{B}	0.2±0.0	7.9±1.6	**8.1±1.6**^{A}
Isosakuranetin-7-*O*-rutinoside	6.3	593	[593→285]	7.3±0.2	36.0±9.9	**43.3±9.8**^{AB}	7.4±2.1	46.4±5.9	**53.8±3.8**^{B}	7.3±0.3	18.5±2.1	**25.8±1.8**^{A}
Naringenin	8.0	271	[271→119]	n.q.	0.2±0.0	**0.2±0.0**^{A}	n.q.	0.1±0.0	**0.1±0.0**^{A}	n.q.	n.q.	**n.q.**
Homoeriodyctiol	8.6	301	[301→151]	n.q.	n.q.	**n.q.**	n.q.	n.q.	**n.q.**	n.q.	n.q.	**n.q.**
Hesperetin	8.7	301	[301→164]	n.q.	0.1±0.0	**0.1±0.0**^{A}	n.q.	0.1±0.0	**0.1±0.0**^{A}	n.q.	n.q.	**n.q.**
Isosakuranetin	10.3	285	[285→164]	n.q.	n.q.	**n.q.**	n.q.	n.q.	**n.q.**	n.q.	n.q.	**n.q.**

Values are expressed as mean (n=3) ± SD. Total values with different superscript capital letters at the same line are significantly different ($p < 0.05$). n.q., no quantified.

TABLE 3: Structure assignments, retention times (Rt), mass spectral data and concentration of carotenoids in orange juice (OJ), fermented orange juice (F-OJ) and fermented-pasteurized orange juice (FP-OJ).

Structure assignment	Rt (min)	m/z $[M+H]^+$	Concentration (mg/L)		
			OJ	F-OJ	FP-OJ
Latochrome	5.38	619	0.17 ± 0.02^{A}	0.19 ± 0.01^{A}	0.12 ± 0.00^{B}
Karpoxanthin	5.86	n.d.	0.37 ± 0.03^{A}	0.48 ± 0.01^{B}	0.30 ± 0.02^{A}
Neochrome	6.05	601	0.40 ± 0.04^{A}	0.54 ± 0.00^{B}	0.33 ± 0.00^{C}
Karpoxanthin isomer	6.31	n.d.	0.36 ± 0.03^{A}	0.50 ± 0.01^{B}	0.30 ± 0.01^{A}
Neochrome isome	6.71	601	0.13 ± 0.00^{A}	0.17 ± 0.00^{B}	0.11 ± 0.00^{C}
Karpoxanthin isomer	6.87	n.d.	0.12 ± 0.01^{A}	0.21 ± 0.00^{B}	0.08 ± 0.00^{C}
Luteoxanthin	8.57	601	n.q.	n.q.	n.q.
Auroxanthin epimer1	8.68	601	0.80 ± 0.02^{A}	0.93 ± 0.04^{B}	0.42 ± 0.02^{C}
Auroxanthin epimer2	8.79	601	1.60 ± 0.03^{A}	1.88 ± 0.04^{B}	0.91 ± 0.02^{C}
Mutatoxanthin epimer1	9.03	585	0.42 ± 0.04^{A}	0.60 ± 0.01^{B}	0.33 ± 0.01^{C}
Auroxanthin epimer3	9.18	601	1.32 ± 0.03^{A}	1.40 ± 0.02^{A}	0.69 ± 0.02^{B}
Mutatoxanthin epimer2	9.31	585	0.51 ± 0.03^{A}	0.70 ± 0.02^{B}	0.40 ± 0.01^{C}
all-trans-Zeaxanthin	9.40	569	0.78 ± 0.08^{A}	1.21 ± 0.15^{B}	0.69 ± 0.09^{A}
all-trans-Lutein	9.61	569	0.39 ± 0.04^{A}	0.56 ± 0.04^{B}	0.33 ± 0.03^{A}
cis-Mutatoxanthin isomer	9.82	585	0.19 ± 0.01^{A}	0.27 ± 0.02^{B}	0.16 ± 0.02^{A}
cis-Mutatoxanthin isomer	9.95	585	0.07 ± 0.00^{A}	0.10 ± 0.01^{B}	0.06 ± 0.01^{A}
9-cis-Lutein	10.23	569	0.03 ± 0.00^{A}	0.05 ± 0.00^{B}	0.03 ± 0.00^{A}
13-cis-Lutein	10.45	569	0.14 ± 0.01^{A}	0.20 ± 0.02^{B}	0.11 ± 0.01^{A}
β-Cryptoxanthin	14.15	553	1.27 ± 0.07^{A}	1.47 ± 0.06^{B}	0.81 ± 0.02^{C}
ζ-Carotene	20.27	541	1.03 ± 0.00^{A}	1.23 ± 0.03^{B}	0.64 ± 0.00^{C}
all-trans-α-Carotene	20.61	537	0.11 ± 0.00^{A}	0.12 ± 0.01^{A}	0.05 ± 0.00^{B}
all-trans-β-Carotene	20.72	537	0.17 ± 0.00^{A}	0.22 ± 0.02^{A}	0.11 ± 0.02^{B}

Values are expressed as mean (n=3) ± SD. Values with different superscript capital letters at the same line are significantly different ($p < 0.05$). n.d., no detected; n.q., no quantified.



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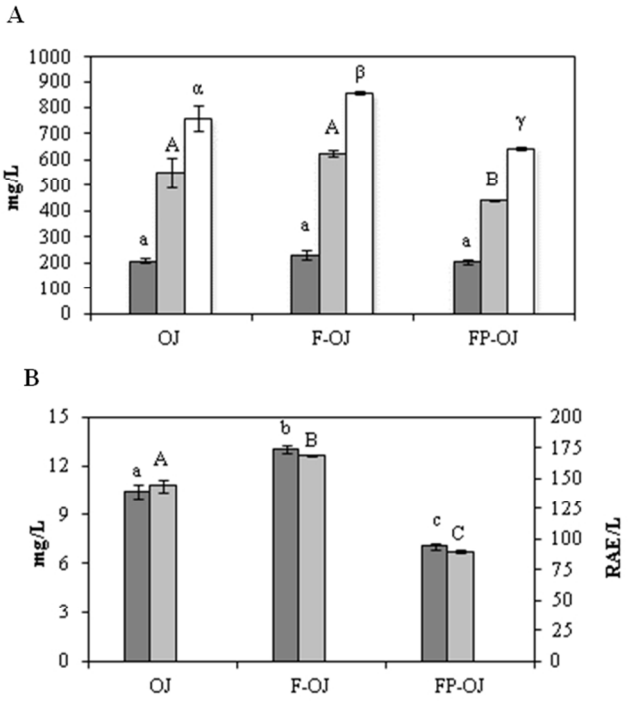

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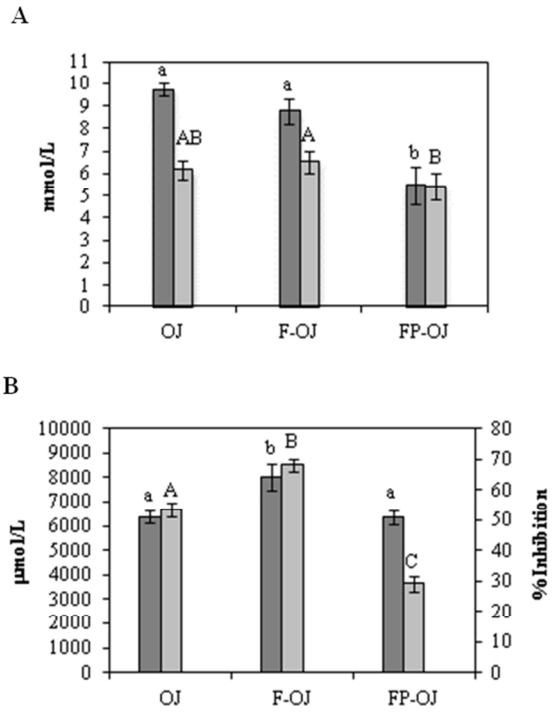

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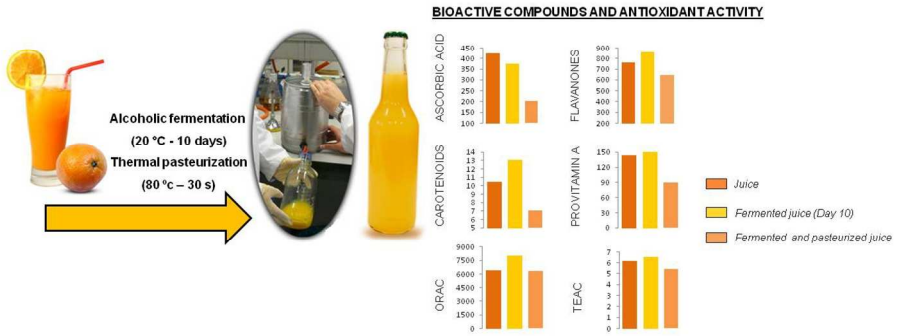

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