JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Impact of High Pressure and Pulsed Electric Fields on Bioactive Compounds and Antioxidant Activity of Orange Juice in Comparison with Traditional Thermal Processing

CONCEPCIÓN SÁNCHEZ-MORENO,[†] LUCÍA PLAZA,[†] PEDRO ELEZ-MARTÍNEZ,[§] BEGOÑA DE ANCOS,[†] OLGA MARTÍN-BELLOSO,[§] AND M. PILAR CANO*,[†]

Department of Plant Foods Science and Technology, Instituto del Frío, CSIC, C/José Antonio Novais 10, Ciudad Universitaria, E-28040 Madrid, Spain, and Department of Food Technology, UTPV-CeRTA, University of Lleida, Avenida Alcalde Rovira Roure 191, E-25198 Lleida, Spain

Bioactive compounds (vitamin C, carotenoids, and flavanones) and DPPH• radical scavenging capacity (RSC) were measured in orange juice (OJ) subjected to different technologies. High pressure (HP) (400 MPa/40 °C/1 min), pulsed electric fields (PEF) (35 kVcm^{-1}/750 μs), low pasteurization (LPT) (70 °C/30 s), high pasteurization (HPT) (90 °C/1 min), HPT plus freezing (HPT+F) (−38 °C/15 min), and freezing (F) were studied. Among the treatments assayed, even though the losses in total vitamin C were <9%, treatments with the higher temperatures tended to show the higher decrease in the content of both forms of vitamin C. HP treatment led to an increased ($P < 0.05$) carotenoid release (53.88%) and vitamin A value (38.74%). PEF treatment did not modify individual or total carotenoids content. Traditional thermal treatments did not exert any effect on total carotenoid content or vitamin A value. With regard to individual carotenoid extraction, HPT and HPT+F led to different releases of carotenoids. With respect to flavanones, HP treatment led to increased ($P < 0.05$) naringenin (20.16%) and hesperetin (39.88%) contents, whereas PEF treatment did not modify flavanone content. In general, pasteurization and freezing process led to a diminished ($P < 0.05$) naringenin content (16.04%), with no modification in hesperetin. HP and PEF treatments did not modify DPPH• RSC. In the case of traditional thermal technologies, HPT treatment showed a decrease ($P < 0.05$) in RSC (6.56%), whereas LPT, HPT+F, and F treatments did not modify RSC. Vitamin C modulated RSC, in terms of antioxidant concentration (EC_{50}) and kinetics ($AE = 1/EC_{50}T_{EC50}$), in the treated and untreated OJ. In summary, HP and PEF technologies were more effective than HPT treatment in preserving bioactive compounds and RSC of freshly squeezed orange juice.

KEYWORDS: Orange juice; high pressure; pulsed electric fields; pasteurization; freezing; carotenoids; flavanones; vitamin C; radical scavenging capacity

INTRODUCTION

Citrus juices are highly consumed in many countries. Orange juice (OJ) accounts for 60% of all Western Europe consumption of fruit juices and juice-based drinks (*1*). In the United States, this juice is the most popular juice per capita, being consumed at ∼5 times the rate of Americans' second choice, apple juice (*2*).

OJ, a rich source of vitamin C (*3*), is one of a number of dietary sources of carotenoids (*4*). OJ carotenoids have diverse biological functions and actions, such as antioxidant activity, provitamin A activity (α- and β-carotene, α- and β-cryptoxanthin), and macula protection (lutein and zeaxanthin) (*5*). In addition, OJ is a major source of antioxidant flavanones (mainly hesperidin and narirutin) in the diet of developed countries (*6*). Recent studies indicate that the intake of OJ improves high-density lipoprotein (HDL) cholesterol plasma levels in subjects with moderate hypercholesteloremia (*7*) and reduces lipid oxidation (8-*epi*$PGF_{2\alpha}$) in smokers (*8*).

Nonpasteurized fresh OJ has a limited shelf life (*9*). To prolong OJ shelf life, the most common method to inactivate microorganisms and enzymes is thermal processing (*10*). However, loss of representative flavor compounds and vitamin C in OJ due to processing temperature (94 °C/30 s) and during storage (4 °C/15 days) has been evidenced (*11*). As other authors have remarked (*12*), little information has been found that describes the behavior of bioactive compounds during the processing of OJ.

Due to consumer demands, the citrus industry has been exploring innovative processing methods with minimal heat

* Author to whom correspondence should be addressed (telephone +34 91 5492300 or +34 91 5445607; fax +34 91 5493627; e-mail pcano@if.csic.es).
[†] Instituto del Frío, CSIC,
[§] University of Lleida.

10.1021/jf048839b CCC: $30.25
Published on Web 04/30/2005

Table 1. Physical and Physicochemical Characteristics of Orange Juices[a]

treatment	pH	soluble solids (°Brix at 20 °C)	total solids (g dw/100 g of fw)	titratable acidity (g of citric acid/100 g of fw)	viscosity (cP)
freshly squeezed	3.59 ± 0.01abc	10.47 ± 0.10a	10.79 ± 0.14a	0.95 ± 0.00cd	27.78 ± 0.70e
HP (400 MPa/40 °C/1 min)	3.63 ± 0.09c	10.97 ± 0.10bc	11.14 ± 0.07b	0.96 ± 0.00d	18.13 ± 0.99b
PEF (35 kV cm^{-1}/750 μs)	3.52 ± 0.01a	10.86 ± 0.04b	10.82 ± 0.08a	0.94 ± 0.00c	18.00 ± 0.35b
LPT (70 °C/30 s)	3.72 ± 0.03d	10.80 ± 0.10b	11.18 ± 0.09b	0.89 ± 0.00b	21.78 ± 0.45d
HPT (90 °C/1 min)	3.60 ± 0.01bc	11.13 ± 0.20cd	10.89 ± 0.23a	0.89 ± 0.00b	17.93 ± 0.23b
HPT (90 °C/1 min) + F (–38 °C/15 min)	3.63 ± 0.05c	11.27 ± 0.10de	11.13 ± 0.07b	0.87 ± 0.01a	16.07 ± 0.19a
F (–38 °C/15 min)	3.54 ± 0.02ab	11.37 ± 0.10e	11.21 ± 010b	0.88 ± 0.01ab	20.80 ± 0.48c

	color				
treatment	*L**	*a**	*b**	*h*	*C*
freshly squeezed	36.82 ± 0.51abc	–1.46 ± 0.15e	36.61 ± 1.12d	87.72 ± 0.32e	36.64 ± 1.11d
HP (400 MPa/40 °C/1 min)	36.74 ± 0.50abc	–3.19 ± 0.26b	34.45 ± 1.12abc	84.71 ± 0.27b	34.59 ± 1.14abc
PEF (35 kV cm^{-1}/750 μs)	36.30 ± 0.18a	–2.17 ± 0.18cd	34.22 ± 1.47ab	86.37 ± 0.13cd	34.29 ± 1.39ab
LPT (70 °C/30 s)	36.98 ± 0.13bc	–3.11 ± 0.32b	36.12 ± 0.68cd	85.08 ± 0.44b	36.25 ± 0.70cd
HPT (90 °C/1 min)	36.50 ± 0.24ab	–3.78 ± 0.18a	35.69 ± 0.97bcd	83.96 ± 0.19a	35.89 ± 0.98bcd
HPT (90 °C/1 min) + F (–38 °C/15 min)	36.72 ± 0.23abc	–2.55 ± 0.07c	35.59 ± 0.53abcd	85.90 ± 0.13c	35.68 ± 0.53bcd
F (–38 °C/15 min)	37.16 ± 0.31c	–1.90 ± 0.13d	33.82 ± 1.13a	86.79 ± 0.27d	33.87 ± 1.13a

[a] Values are means ± standard deviation, $n = 6$. Mean values within a column with different letters are significantly different at $P < 0.05$. dw, dry weight; fw, fresh weight; HP, high pressure; PEF, pulsed electric fields; LPT, low pasteurization; HPT, high pasteurization; F, freezing.

treatment, to increase markets by improving the nutritional and flavor qualities of OJ (*13*). Due to technological development, high-pressure (HP) processing and pulsed electric fields (PEF) processing have received increased attention during the past decade (*14*). The flavor and nutritional quality of OJ is usually unaffected by these technologies, although microorganisms and enzymes may be inactivated (*10*, *11*, *15*−*19*). In contrast, knowledge about the effects of these emerging nonthermal technologies on the fate of antioxidants in fruit juices, including OJ, is scarce, and it is generally limited to vitamin C (*11*, *16*, *20*, *21*). In a previous research, we have studied the impact on several bioactive compounds of different processes that combine HP and thermal treatment to preserve OJ as freshly squeezed during a 10-day period of refrigerated storage (*22*, *23*). In addition, our research group has shown that OJ, preserved by these HP (*24*) and PEF (*25*) emerging technologies, retains the bioavailability of vitamin C and antioxidant protection of freshly squeezed orange juice (FSOJ) in humans.

From the methodological point of view, the widespread use of the 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) radical scavenging model is recommended as fast, easy, and accurate, with regard to measuring the potential radical scavenging capacity (RSC) of plant-derived foods (*26*−*28*). The protocol used by our group takes into account not only the antioxidant concentration but also the reaction time to reach the plateau of the scavenging reaction (*29*). This characteristic could be an advantage over other methods using only the antioxidant concentration (*27*, *30*).

Therefore, the aim of this study was to evaluate the impact of HP and PEF emerging technologies in comparison with traditional thermal technologies on the vitamin C, carotenoid, and flavanone contents of OJ. In addition, their effect on RSC was assayed.

MATERIALS AND METHODS

Chemicals. L-(+)-Ascorbic acid was purchased from Merck KGaA (Darmstadt, Germany). DPPH$^{\bullet}$, DL-dithiothreitol, and hesperetin were obtained from Sigma Chemical Co. (St. Louis, MO). Hoffman-La Roche (Basel, Switzerland) kindly provided β-carotene, β-cryptoxanthin, lutein, and zeaxanthin. Naringenin was purchased from Aldrich Chemical Co. (Milwaukee, WI).

Orange Juice Samples. Orange fruits (*Citrus sinensis* L. var. Navel late) (Valencia, Spain) were purchased from a local supermarket and kept at 4 °C before being processed. The OJ was obtained using a squeezer (Lomi model 4, Madrid, Spain) and then was filtered using a 2 mm steel sieve. Initial characteristics of the FSOJ are shown in **Table 1**. All treatments were carried out with this FSOJ.

Nonthermal Processing Technologies. *High-Pressure (HP) Treatment.* FSOJ samples were vacuum packed in plastic bags (Doypack) and then introduced into the pressure unit filled with pressure medium (water) as described in previous works *(22*, *23*, *31*, *32*). HP treatment at 400 MPa was performed in a hydrostatic pressure unit with 2350 mL capacity, at a maximum pressure of 500 MPa, with a potential maximum temperature of 95 °C (GEC Alsthom ACB 900 HP, type ACIP no. 665, Nantes, France). Temperature and time selected were 40 °C and 1 min, respectively. Pressure was increased and released at 2.5 MPa/s. According to previous results of the group, this combined treatment was selected due to the effect on the stability of carotenoids, vitamin C (*22*), and flavanones (*23*) in OJ during storage (10 days/4 °C). In addition, its effect on enzymatic inactivation (*32*) in OJ was considered.

Pulsed Electric Fields (PEF) Treatment. PEF treatment was carried out in a continuous flow bench scale system (OSU-4F, The Ohio State University, Columbus, OH) using square-wave pulses. The treatment system consisted of a series of eight collinear treatment chambers. Each chamber consisted of two stainless steel electrodes separated by a gap of 0.29 cm. The flow rate of the process was adjusted to 200 mL/min and was controlled by a variable speed pump (model 75210-25, Cole Palmer, Vernon Hills, IL). The product was refrigerated in the space provided between the chambers by means of an ice−water shaking bath. The PEF processing conditions for FSOJ were 35 kV/cm electrical field applied in a bipolar mode, 800 Hz pulse frequency, 4 μs pulse width, and 750 μs total treatment time. Temperature never exceeded 50 °C. According to previous results of the group, these conditions were selected due to the induced inactivation of enzymes (*17*, *33*) and microbial reduction (*18*, *34*).

Traditional Thermal Technologies. *Low Pasteurization (LPT) Treatment.* FSOJ samples were placed in an autoclave (Autester-G, Selecta, Barcelona, Spain) and heated at 70 °C for 30 s.

High-Pasteurization (HPT) Treatment. FSOJ samples were placed in an autoclave (Autester-G, Selecta, Barcelona, Spain) and heated at 90 °C for 1 min.

High-Pasteurization plus Freezing (HPT+F) Treatment. FSOJ samples were placed in an autoclave (Autester-G, Selecta) and heated at 90 °C for 1 min. Then, after being cooled to 30 °C, samples were frozen at −38 °C for 15 min in a pilot freezer equipment (Aga Frigoscandia, Lidingö, Sweden).

Freezing (F). FSOJ samples were frozen at −38 °C for 15 min in pilot freezer equipment (Aga Frigoscandia).

Physical and Physicochemical Assays. *pH, Titratable Acidity, Soluble, and Total Solids.* These parameters were measured according to conventional methods in unprocessed and processed OJ, as described elsewhere (*29*).

Viscosity. Viscosity was measured on ∼35 mL of OJ with a model DV-II viscometer (Brook-field Engineering Laboratories, Inc., Stoughton, MA). Viscosity was expressed as centipoise (cP).

Color. The color of OJ was measured using a tristimulus reflectance colorimeter (HunterLab, model D25 A9, Hunter Associates Laboratory, Inc., Reston, VA) calibrated with a white standard tile ($X = 82.51$; $Y = 84.53$; $Z = 101.23$). Samples were placed in Petri dishes and filled to the top, and color was recorded using the CIE*Lab* uniform color space. L^* (lightness), a^* (green-red tonality), and b^* (blue-yellow tonality) values were recorded. The CIE*Lab* value hue angle h and the chroma or saturation were used to express the OJ color, $h = \tan^{-1}(b^*/a^*)$, $C = (a^{*2} + b^{*2})^{1/2}$.

Determination of Vitamin C. L-Ascorbic acid (L-AA) and total vitamin C [L-AA plus l-dehydroascorbic acid (L-DHAA)] were determined by HPLC as we have described previously (*22*). Total vitamin C was determined by the reduction of L-DHAA to L-AA, using dl-dithiothreitol as reductant reagent, according to a modification of a previous procedure (*35*). Results were expressed as milligrams of total vitamin C per 100 mL of OJ.

Extraction, Separation, Identification, and Quantification of Carotenoids. The extraction was carried out according to a previous method (*22*).

HPLC analysis was based on the method described elsewhere (*36*, *37*). Total carotenoid content was expressed as micrograms of α-cryptoxanthin plus micrograms of β-cryptoxanthin plus micrograms of zeaxanthin plus micrograms of lutein plus micrograms of α-carotene plus micrograms of β-carotene. Results were given as micrograms of the corresponding carotenoid per 100 mL of OJ.

Vitamin A values have been given as retinol activity equivalents (RAE) per 100 mL of OJ, according to the equation RAE = [μg of β-carotene/12] + [μg of other provitamin A carotenoids (β- and α-cryptoxanthin and α-carotene)/24] (*38*).

Extraction, Hydrolysis, Identification, and Quantification of Flavanones. The extraction method employed was based on the procedure described elsewhere (*39*), modified as described in Sánchez-Moreno et al. (*29*). HPLC analysis was based on the method described elsewhere (*29*). Results were given as milligrams of the corresponding flavanone per 100 mL of OJ.

Scavenging Effect on DPPH• Radical. The determination of the RSC in the unprocessed and processed OJ was evaluated with the DPPH• stable radical. The method is described extensively elsewhere (*23*). The parameters EC_{50}, which reflects 50% depletion of initial DPPH• radical, and the time needed to reach the steady state at EC_{50} concentration (T_{EC50}) were calculated. The antiradical efficiency (AE = $1/[EC_{50}T_{EC50}]$), a parameter that combines both factors, was also calculated.

Statistical Analysis. Results were given as mean ± standard deviation of six independent determinations. One-way analysis of variance (ANOVA) was used to compare the means. Differences were considered to be significant at $P < 0.05$. All statistical analyses were performed with Statgraphics Plus 2.1 (Statistical Graphics Corp., Inc., Rockville, MD).

RESULTS AND DISCUSSION

Physical and Physicochemical Characterization. The effects of HP and PEF technologies on the physical and physicochemical properties of OJ were compared with FSOJ and conventionally processed OJ (**Table 1**). Except in the case of LPT-treated OJ, the pH did not change over the course of the treatments. All of the treatments showed significantly higher °Brix than FSOJ. HP and PEF treatments led to similar titratable acidity values in comparison to FSOJ, whereas pasteurization and freezing treatments led to lower titratable acidity. The characterization of the processed OJ by these parameters was in agreement with literature data for OJ (*40*). Viscosity has traditionally been considered an important quality parameter for citrus juices. The consistency and amount of pulp of OJ give viscosity. Loss of stability is due to the action of the endogenous enzyme pectin methyl esterase, which demethoxylates pectin, allowing calcium pectate precipitates to fall out of solution and giving a clarified appearance (*41*). In our work, all of the treated OJ showed significantly lower viscosity than FSOJ. HP, PEF, and thermal treatments are known to inactivate a portion of the total enzyme (*11*, *41*−*44*). Also, it is known that changes associated with freezing can cause aggregation and conformational modifications of enzyme molecules, leading to a reduction of enzymatic activity (*45*). However, the measurement of pectin methyl esterase could clarify this point. Previously, our group has observed 20% of pectin methyl esterase residual activity in PEF-treated OJ (*17*).

Table 2. Vitamin C Content (Milligrams per 100 mL) of Orange Juices[a]

treatment	L-ascorbic acid	total vitamin C
freshly squeezed	40.07 ± 0.98b	44.37 ± 1.09d
HP (400 MPa/40 °C/1 min)	36.91 ± 1.71a	42.79 ± 0.55cd
PEF (35 kV cm^{-1}/750 μs)	37.27 ± 0.92a	41.26 ± 1.01abc
LPT (70 °C/30 s)	38.80 ± 1.44ab	44.15 ± 1.11d
HPT (90 °C/1 min)	36.85 ± 1.44a	40.14 ± 1.21ab
HPT (90 °C/1 min) + F (–38 °C/15 min)	36.76 ± 1.33a	39.67 ± 1.57a
F (–38 °C/15 min)	40.07 ± 0.59b	41.78 ± 0.69bc

[a] Values are means ± standard deviation, $n = 6$. Mean values within a column with different letters are significantly different at $P < 0.05$. HP, high pressure; PEF, pulsed electric fields; LPT, low pasteurization; HPT, high pasteurization; F, freezing.

The lightness parameter, L^*, did not change due to treatment. HP-, PEF-, and F-treated OJ showed significantly lower chroma values, which represent color intensity, than FSOJ. After HP, PEF, and F processing, b^* values decreased significantly ($P < 0.05$), indicating a less yellow color, whereas in the case of pasteurized OJ, no changes were found. With regard to a^* values, after all of the treatments, it shifted in a negative direction, indicating a less red color. The hue angle of treated OJ was significantly lower ($P < 0.05$) than that of FSOJ.

Vitamin C Content. Table 2 shows the L-AA and the total vitamin C contents in the processed and nonprocessed OJ. HP, PEF, HPT, and HPT+F treatments caused a significant decrease in L-AA content (∼7.79%) in comparison with FSOJ. On the other hand, LPT and F treatments did not exert any change. With regard to total vitamin C, PEF, HPT, HPT+F, and F led to decreases (∼8.24%), whereas HP and LPT did not exert any change. Putting these findings together, we observed that among the treatments assayed, even though the losses were <9%, treatments with the higher temperature tended to show the higher decrease in the content of L-AA and L-DHAA. However, one has to bear in mind that the losses in vitamin C in the tested OJ are relatively low. Vitamin C is typically a heat-sensitive nutrient (*11*) and is also vulnerable to enzyme-catalyzed oxidation (specifically, ascorbate oxidase and peroxidase) (*3*). Thus, in our work the treatments applied (nonthermal and thermal) may have eliminated in part some of the enzymes responsible for vitamin C loss. Previous studies by our research group in OJ show that the depletion of L-AA after combined treatment of HP/temperature is dependent mainly on temperature intensity, showing low losses just after treatment (400 MPa/40 °C/1 min) (*22*). Also, other authors find stability of L-AA in OJ pressurized at mild temperatures (*46*, *47*). Several studies have investigated

Table 3. Individual Carotenoid, Total Carotenoid Content (Micrograms per 100 mL), and Vitamin A Value (Retinol Activity Equivalents per 100 mL) of Orange Juices[a]

treatment	β-cryptoxanthin	α-cryptoxanthin (as β-cryptoxanthin)	zeaxanthin	lutein	β-carotene	α-carotene (as β-carotene)	total carotenoids	vitamin A
freshly squeezed	145.28 ± 8.05a	56.17 ± 3.32ab	330.83 ± 15.89ab	323.59 ± 9.62bc	53.67 ± 4.97ab	30.09 ± 2.89a	939.63 ± 35.49ab	14.12 ± 0.98a
HP (400 MPa/40 °C/1 min)	208.06 ± 22.93b	81.94 ± 7.26d	478.13 ± 58.73d	567.69 ± 51.16d	69.90 ± 6.22c	40.25 ± 3.64b	1445.97 ± 135.33c	19.59 ± 1.82b
PEF (35 kV cm^{-1}/750 μs)	142.27 ± 6.78a	49.47 ± 2.93a	284.25 ± 13.65a	304.37 ± 8.16b	44.79 ± 4.15a	26.92 ± 2.58a	851.97 ± 30.29a	12.84 ± 0.83a
LPT (70 °C/30 s)	137.57 ± 12.03a	57.47 ± 6.69ab	374.74 ± 30.92bc	313.95 ± 34.33b	49.27 ± 9.22ab	27.42 ± 3.19a	960.42 ± 87.52ab	13.38 ± 1.57a
HPT (90 °C/1 min)	160.68 ± 19.69a	66.95 ± 8.77c	454.88 ± 66.00d	248.82 ± 33.48a	55.72 ± 6.43b	31.27 ± 3.72a	1018.32 ± 136.62ab	15.43 ± 1.85a
HPT (90 °C/1 min) + F (–38 °C/15 min)	151.73 ± 12.75a	57.16 ± 4.76ab	420.86 ± 32.95cd	222.84 ± 17.39a	56.79 ± 7.53b	30.46 ± 3.94a	939.84 ± 75.93ab	14.71 ± 1.47a
F (–38 °C/15 min)	161.74 ± 18.07a	62.04 ± 7.25bc	375.89 ± 45.71bc	366.88 ± 29.63c	50.28 ± 6.18ab	28.01 ± 3.60a	1044.84 ± 108.60b	14.68 ± 1.71a

[a] Values are means ± standard deviation, $n = 6$. Mean values within a column with different letters are significantly different at $P < 0.05$. HP, high pressure; PEF, pulsed electric field; LPT, low pasteurization; HPT, high pasteurization; F, freezing

the effects of PEF processing on vitamin C content in vegetable foods. Recent studies showed that vitamin C losses in OJ due to its processing by PEF under 5.0% are very small and significantly lower ($P < 0.05$) compared with the thermal pasteurization procedure (90 °C/90 s) (*16*, *48*). In our work, the L-AA losses after PEF treatment were comparable (~6.98%) to those found by these authors for PEF-treated OJ. Freezing is a technique used in the industry to preserve the OJ when products exceed market demand. However, little information has been found regarding the stability of vitamin C during freezing. Some authors have described in OJ an increase in L-DHAA (26%) and a decrease in the L-AA content after freezing. However, this reciprocal effect leads to no effect in the total vitamin C content (*12*). More research could be done to clarify the stabilization of both forms of vitamin C after OJ freezing treatments.

Carotenoid Content and Vitamin A Value. On the basis of their antioxidant and provitamin A activities certain carotenoids were systematically quantified in the treated and untreated OJ (α- and β-carotene, α- and β-cryptoxanthin, lutein, and zeaxanthin) (**Table 3**) to compare nonthermal and traditional thermal processing technologies. Interestingly, HP-treated OJ showed the highest carotenoid content compared with the rest of the tested OJ. Each carotenoid showed a significant increase in comparison with the untreated OJ: α-carotene (33.76%), β-carotene (30.24%), α-cryptoxanthin (45.87%), β-cryptoxanthin (43.21%), zeaxanthin (44.52%), and lutein (75.43%). According to its higher provitamin A carotenoid content, HP-treated OJ showed the higher vitamin A value. These results were in agreement with those reported by our research group showing an increase in extractable carotenoids due to HP treatment in OJ (*23*), persimmon puree (*36*), and tomato puree (*31*). Carotenoids are tightly bound to macromolecules, in particular protein and membrane lipids (*49*), and HP processing is known to affect macromolecular structures, such as proteins and polymer carbohydrates (*50*) and the membranes in vegetable cells (*51*). Thus, in OJ, HP increased the release of carotenoids from the suspended pulp particles, making it more accessible to the extraction. Additionally, it was observed that the higher polarity, the higher the extraction on carotenoids. Accordingly, it is described that the release of lutein into an aqueous environment is higher than that of β-carotene because of its lower lipophilicity compared with β-carotene (*52*). In addition, there are indications that disruption of the matrix affects the bioavailability of various carotenoids differentially (*53*). Thus, it could be speculated that due to the different structural arrangement of oxygen and hydrocarbon carotenoids in the intracellular locations of juice vesicles, HP led to different release of carotenoids. It is now generally accepted that the application of PEF (5 kV/cm) with nanosecond to microsecond duration leads to the permeabilization of plant membranes (*54*). PEF-treated OJ did not modify individual or total carotenoids content. This is the first time that the behavior of carotenoids during OJ PEF processing is described. Further research is needed on the consequences of membrane permeabilization induced by PEF treatment. Traditional thermal treatments did not exert any significant effect on total carotenoid content or vitamin A value in comparison with FSOJ. With regard to individual carotenoid extractions, LPT and F treatments did not exert any change. No loss of β-carotene and lutein is observed during 2 h of freezing of acetone extracts from green vegetables (*55*). Also, it is known that freezing (especially quick-freezing) generally preserves the provitamin A carotenoids (*4*). In the case of HPT-treated OJ, this treatment led to an increase in α-cryptoxanthin (19.19%) and zeaxanthin (37.49%) and to a decrease in lutein (23.10%), whereas no changes were found in the extraction of β-cryptoxanthin or hydrocarbon carotenoids. In a similar way to HPT treatment, HPT+F treatment increased zeaxanthin (27.21%) and decreased lutein (31.13%). The main cause of carotenoid degradation is isomerization and oxidation. It is widely presumed that carotenoids in general undergo isomerization with thermal processing (*56*). Heat treatment may lead to significant losses of lutein and, to a lesser extent, carotenes (*5*). In our work, the temperature of pasteurization (90 °C/1 min) led to a decrease in lutein; thus, this xanthophyll is likely to be converted to epoxy carotenoids as heat proceeds.

Table 4. Flavanone Content (Milligrams per 100 mL) of Orange Juices[a]

treatment	naringenin	hesperetin	total flavanones
freshly squeezed	3.72 ± 0.36b	10.18 ± 0.50ab	13.89 ± 0.78a
HP (400 MPa/40 °C/1 min)	4.47 ± 0.30c	14.24 ± 0.48c	18.70 ± 0.51b
PEF (35 kV cm^{-1}/750 μs)	3.49 ± 0.36ab	10.64 ± 0.50ab	14.13 ± 0.78a
LPT (70 °C/30 s)	3.13 ± 0.29a	10.92 ± 0.59b	14.05 ± 0.84a
HPT (90 °C/1 min)	3.11 ± 0.45a	10.73 ± 1.22ab	13.84 ± 1.56a
HPT (90 °C/1 min) + F (–38 °C/15 min)	3.45 ± 0.37ab	9.46 ± 1.08a	12.91 ± 1.24a
F (–38 °C/15 min)	3.13 ± 0.35a	9.41 ± 0.86a	12.54 ± 0.63a

[a] Values are means ± standard deviation, $n = 6$. Mean values within a column with different letters are significantly different at $P < 0.05$. HP, high pressure; PEF, pulsed electric fields; LPT, low pasteurization; HPT, high pasteurization; F = freezing.

Flavanone Content. Table 4 shows the flavanone content after the hydrolysis of flavanone glycosides [hesperidin (3′,5,7-trihydroxy-4′-methoxyflavanone-7-rutinoside) and narirutin (4′,5,7-trihydroxyflavanone-7-rutinoside)] to their corresponding aglycons, hesperetin and naringenin, respectively, in the processed and unprocessed OJ. These values were in the range of those found in FSOJ by other authors (*57*, *58*). HP-treated OJ gives

Table 5. Radical Scavenging Parameters of Orange Juices[a]

treatment	EC_{50} (mL/g of DPPH•)	T_{EC50} (min)	AE × 10^{-3} [b]
freshly squeezed	194.20 ± 11.35a	1.89 ± 0.11ab	2.74 ± 0.10b
HP (400 MPa/40 °C/1 min)	184.69 ± 13.22a	2.00 ± 0.20b	2.73 ± 0.09b
PEF (35 kV cm^{-1}/750 μs)	197.79 ± 5.26ab	1.84 ± 0.06a	2.71 ± 0.02b
LPT (70 °C/30 s)	183.14 ± 9.90a	1.99 ± 0.15b	2.76 ± 0.09b
HPT (90 °C/1 min)	206.90 ± 10.89b	1.89 ± 0.10ab	2.56 ± 0.04a
HPT (90 °C/1 min) + F (–38 °C/15 min)	193.91 ± 6.32a	1.95 ± 0.14b	2.66 ± 0.13ab
F (–38 °C/15 min)	191.70 ± 9.68a	1.92 ± 0.18b	2.73 ± 0.12b

[a] Values are means ± standard deviation, $n = 6$. Mean values within a column with different letters are significantly different at $P < 0.05$. dw = dry weight. HP, high pressure; PEF, pulsed electric fields; LPT, low pasteurization; HPT, high pasteurization; F = freezing. [b] Expressed as 1/[EC_{50} (mL/g of DPPH•) T_{EC50} (min)].

the higher naringenin (20.16%) and hesperetin (39.88%) contents among the tested OJ. Thus, it may be possible that, as in the case of carotenoid release, there were some structural changes in the cell walls of treated OJ vesicles, which led to a higher extraction of flavanones. Consistently, as we show in the present work, different HP treatments (350 MPa/30 °C/2.5 min and 400 MPa/40 °C/1 min) applied to OJ lead to a relative increase in the case of hesperetin but not in naringenin content (*23*). PEF treatment did not modify the naringenin or the hesperetin content. In general, pasteurization and freezing processes led to diminishing naringenin content (~16.04%). Accordingly, pasteurization of OJ has been shown to lead to narirutin degradation (28.12%) (*12*). On the contrary, neither pasteurization nor freezing treatment modified hesperetin content in OJ. It is described that flavanones tend to precipitate at low pH, from the soluble fraction to the cloud in OJ. This leads to an increase in the proportion of flavanones in the cloud during processing, especially at low temperatures (*59*). Thus, a lower release of naringenin in the pasteurization and freezing processes in OJ during chemical extraction could occur.

DPPH• RSC. We compare RSC of FSOJ with nonthermally and thermally processed OJ (**Table 5**). HP and PEF processes did not modify the RSC of OJ. Despite the increase in flavanone content in HP-treated OJ compared with FSOJ, the RSC of HP-treated OJ was not affected, because the contribution of phenols to the antioxidant activity in OJ is low (*29*, *60*). With regard to traditional thermal treatments, in the case of HPT treatment, a significant decrease in RSC, in terms of antioxidant concentration (EC_{50}), was observed, whereas LPT, HPT+F, and F treatments did not modify RSC. We found a significant correlation between both L-AA and total vitamin C versus AE ($r = 0.6958$, $P = 0.0005$; and $r = 0.7530$, $P = 0.0001$, respectively). Thus, it seems that the RSC of OJ toward DPPH• is mainly modulated by vitamin C both in antioxidant concentration and in kinetic activity. Consistently, antioxidant activity measured according to the $ABTS^{\bullet+}$ radical cation assay in blond and blood OJ is shown to be strictly related to vitamin C content (*60*). Our group has previously shown that vitamin C is the major bioactive contributor to RSC in commercial OJ (*29*). Accordingly, due to the relative high stability of L-AA in OJ during the different processing techniques, shown in this work (losses of ~7.79%), RSC did not give significant differences, with the exception of HPT-treated OJ. Among all OJ tested, a significant inverse correlation was found between the EC_{50} parameter and total carotenoids ($r = -0.5307$, $P = 0.0133$) or total flavanones ($r = -0.4617$, $P = 0.0351$), whereas no correlation was found between these compounds and the AE parameter, indicating their lack of relevant effect in RSC kinetic toward DPPH•. We do not reject the possibility that other phytochemicals present in the OJ may also have contributed to the total RSC.

HP and PEF nonthermal technologies were more effective than HPT in preserving bioactive compounds and the RSC of FSOJ. Vitamin C modulates, in terms of antioxidant concentration and kinetics, DPPH• RSC in treated and untreated OJ.

ABBREVIATIONS USED

AE, antiradical efficiency; *C*, chroma or saturation; *a**, CIE*Lab* green-red tonality parameter; *b**, CIE*Lab* blue-yellow tonality parameter; DPPH•, 2,2-diphenyl-1-picrylhydrazyl; dw, dry weight; F, freezing; fw, fresh weight; FSOJ, freshly squeezed orange juice; HP, high pressure; *h*, hue angle; L-AA, L-ascorbic acid; L-DHAA, L-dehydroascorbic acid; *L**, CIE*Lab* lightness parameter; OJ, orange juice; PEF, pulsed electric fields; RSC, radical scavenging capacity; LPT, low pasteurization; HPT, high pasteurization; HPT+F, high pasteurization plus freezing.

LITERATURE CITED

(1) Fry, J.; Martin, G.; Lees, M. Authentication of orange juice. In *Production and Packaging of Non-carbonated Fruit Juices and Fruit Beverages*; Ashurts, P. E., Ed.; Blackie Academic and Professional: New York, 1995; pp 1−52.

(2) ASFS. Americans choose juice; http://www.asfsa.org (accessed July 2004).

(3) Davey, M. W.; Van Montagu, M.; Inzé, D.; Sanmartin, M.; Kanellis, A.; Smirnoff, N.; Benzie, I. J. J.; Strain, J. J.; Favell, D.; Fletcher, J. Plant L-ascorbic acid: Chemistry, function, metabolism, bioavailability and effects of processing. *J. Sci. Food Agric.* **2000**, *80*, 825−860.

(4) Rodríguez-Amaya, D. B. *Carotenoids and Food Preparation: The Retention of Provitamin A Carotenoids in Prepared, Processed, and Stored Foods*; Office of Health and Nutrition, U.S. Agency for International Development: Washington, DC, 1997.

(5) Van den Berg, H.; Faulks, R.; Granado, F.; Hirschberg, J.; Olmedilla, B.; Sandmann, G.; Southon, S.; Stahl, W. The potential for the improvement of carotenoid levels in foods and the likely systemic effects. *J. Sci. Food Agric.* **2000**, *80*, 880−912.

(6) Gil-Izquierdo, A.; Gil, M. I.; Ferreres, F.; Tomás-Barberán, F. A. In vitro availability of flavonoids and other phenolic in orange juice. *J. Agric. Food Chem.* **2001**, *49*, 1035−1041.

(7) Kurowska, E. M.; Spence, J. D.; Jordan, J.; Wetmore, S.; Freeman, D. J.; Piché, L. A.; Serratore, P. HDL-cholesterol raising effect of orange juice in subjects with hypercholesterolemia. *Am. J. Clin. Nutr.* **2000**, *72*, 1095−1100.

(8) Sánchez-Moreno, C.; Cano, M. P.; De Ancos, B.; Plaza, L.; Olmedilla, B.; Granado, F.; Martín, A. Effect of orange juice intake on vitamin C concentrations and biomarkers of antioxidant status in humans. *Am. J. Clin. Nutr.* **2003**, *78*, 454−460.

(9) Feller, P. J. Shelf life and quality of freshly squeezed, unpasteurized polyethylene-bottled citrus juice. *J. Food Sci.* **1988**, *53*, 1699−1702.

(10) Jia, M.; Zhang, Q. H.; Min, D. B. Pulsed electric field processing effects on flavor compounds and microorganisms of orange juice. *Food Chem.* **1999**, *65*, 445−451.

(11) Yeom, H. W.; Streaker, C. B.; Zhang, Q. H.; Min, D. B. Effects of pulsed electric field on the quality of orange juice and comparison with heat pasteurization. *J. Agric. Food Chem.* **2000**, *48*, 4597−4605.

(12) Gil-Izquierdo, A.; Gil, M. I.; Ferreres, F. Effect of processing techniques at industrial scale on orange juice antioxidant and beneficial health compounds. *J. Agric. Food Chem.* **2002**, *50*, 5107−5114.

(13) Deliza, R.; Rosenthal, A.; Silva, A. L. S. Consumer attitude towards information on non conventional technology. *Trends Food Sci. Technol.* **2003**, *14*, 43−49.

(14) Señorans, F. J.; Ibáñez, E.; Cifuentes, A. New trends in food processing. *Crit. Rev. Food Sci. Nutr.* **2003**, *43*, 507−526.
(15) Polydera, A. C.; Stoforos, N. G.; Taoukis, P. S. The effect of storage on the antioxidant activity of reconstituted orange juice which had been pasteurized by high pressure or heat. *Int. J. Food Sci. Technol.* **2004**, *39*, 783−791.
(16) Hodgins, A. M.; Mittal, G. S.; Griffiths, M. W. Pasteurization of fresh orange juice using low-energy pulsed electrical field. *J. Food Sci.* **2002**, *67*, 2294−2298.
(17) Elez-Martínez, P.; Suárez-Recio, M.; Espachs-Barroso, A.; Barbosa-Cánovas, G. V.; Martín-Belloso, O. High-intensity pulsed electric field inactivation of pectin methyl esterase in orange juice. Presented at the 12th World Congress of Food Science and Technology, 7E-9, Chicago, IL, 2003.
(18) Elez-Martínez, P.; Escolà-Hernández, J.; Soliva-Fortuny, R. C.; Martín-Belloso, O. Inactivation of *Saccharomyces cerevisiae* suspended in orange juice using high-intensity pulsed electric fields. *J. Food Prot.* **2004**, *67*, 2596−2602.
(19) Butz, P.; Serfert, Y.; Fernández-García, A.; Dieterich, S.; Lindauer, R.; Bognar, A.; Tauscher, B. Influence of high-pressure treatment at 25 °C and 80 °C on folates in orange juice and model media. *J. Food Sci.* **2004**, *69*, SNQ117−SNQ121.
(20) Fernández-García, A.; Butz, P.; Bonàr, A.; Tauscher, B. Antioxidative capacity, nutrient content and sensory quality of orange juice and an orange-lemon-carrot juice product after high-pressure treatment and storage in different packaging. *Eur. Food Res. Technol.* **2001**, *213*, 290−296.
(21) Nienaber, U.; Shellhammer, T. H. High-pressure processing of orange juice: Combination treatments and a shelf life study. *J. Food Sci.* **2001**, *66*, 332−336.
(22) Sánchez-Moreno, C.; Plaza, L.; De Ancos, B.; Cano, M. P. Vitamin C, provitamin A carotenoids, and other carotenoids in high-pressurized orange juice during refrigerated storage. *J. Agric. Food Chem.* **2003**, *51*, 647−653.
(23) Sánchez-Moreno, C.; Plaza, L.; De Ancos, B.; Cano, M. P. Effect of high-pressure processing on health-promoting attributes of freshly squeezed orange juice (*Citrus sinensis* L.) during chilled storage. *Eur. Food Res. Technol.* **2003**, *216*, 18−22.
(24) Sánchez-Moreno, C.; Cano, M. P.; De Ancos, B.; Plaza, L.; Olmedilla, B.; Granado, F.; Martín, A. High-pressurized orange juice consumption affects plasma vitamin C, antioxidative status and inflammatory markers in healthy humans. *J. Nutr.* **2003**, *133*, 2204−2209.
(25) Sánchez-Moreno, C.; Cano, M. P.; De Ancos, B.; Plaza, L.; Olmedilla, B.; Granado, F.; Elez-Martínez, P.; Martín-Belloso, O.; Martín, A. Pulsed electric fields-processed orange juice consumption increases plasma vitamin C and decreases F_2-isoprostanes in healthy humans. *J. Nutr. Biochem.* **2004**, *15*, 601−607.
(26) Da Porto, C.; Calligaris, S.; Celotti, E.; Nicoli, M. C. Antiradical properties of commercial cognacs assessed by the DPPH test. *J. Agric. Food Chem.* **2000**, *48*, 4241−4245.
(27) Sánchez-Moreno, C. Methods used to evaluate the free radical scavenging activity in foods and biological systems. *Food Sci. Technol. Int.* **2002**, *8*, 121−137.
(28) Espín, J. C.; Soler-Rivas, C.; Wichers, H. J.; García-Viguera, C. Anthocyanin-based natural colorants: A new source of antiradical activity for foodstuff. *J. Agric. Food Chem.* **2000**, *48*, 1588−1592.
(29) Sánchez-Moreno, C.; Plaza, L.; De Ancos, B.; Cano, M. P. Quantitative bioactive compounds assessment and their relative contribution to the antioxidant capacity of commercial orange juices. *J. Sci. Food Agric.* **2003**, *83*, 430−439.
(30) Martínez-Valverde, I.; Periago, M. J.; Provan, G.; Chesson, A. Phenolic compounds, lycopene and antioxidant activity in commercial varieties of tomato (*Lycopersicon esculentum*). *J. Sci. Food Agric.* **2002**, *82*, 323−330.
(31) Sánchez-Moreno, C.; Plaza, L.; De Ancos, B.; Cano, M. P. Effect of combined treatments of high-pressure and natural additives on carotenoid extractability and antioxidant activity of tomato puree (*Lycopersicum esculentum* Mill.). *Eur. Food Res. Technol.* **2004**, *219*, 151−160.
(32) Cano, M. P.; Hernández, A.; De Ancos, B. High-pressure and temperature effects on enzyme inactivation in strawberry and orange products. *J. Food Sci.* **1997**, *62*, 85−88.
(33) Elez-Martínez, P.; Aguiló-Aguayo, I.; Barbosa-Cánovas, G. V.; Martín-Belloso, O. Inactivation of peroxidase in orange juice processed by high-intensity pulsed electric fields. *Proceedings of a Workshop on Nonthermal Food Preservation*, Wageningen, The Netherlands; IFT: Chicago, IL, 2003; pp 40−42.
(34) Elez-Martínez, P.; Escolà-Hernández, J.; Espachs-Barroso, A.; Barbosa-Cánovas, G.; Martín-Belloso, O. Inactivation of *Lactobacillus brevis* in orange juice by high-intensity pulsed electric fields. Presented at the Institute of Food Technologists' Annual Meeting, 92C-35, Chicago, IL; 2003.
(35) Sánchez-Mata, M. C.; Cámara-Hurtado, M.; Diez-Marques, C.; Torija-Isasa, M. E. Comparison of high-performance liquid chromatography and spectrofluorimetry for vitamin C analysis of green beans (*Phaseolus vulgaris* L.). *Eur. Food Res. Technol.* **2000**, *210*, 220−225.
(36) De Ancos, B.; Gonzalez, E.; Cano, P. Effect of high-pressure treatment on the carotenoid composition and the radical scavenging activity of persimmon fruit purees. *J. Agric. Food Chem.* **2000**, *48*, 3542−3548.
(37) Hart, J. D.; Scott, K. J. Development and evaluation of an HPLC method for the analysis of carotenoids in foods, and the measurement of the carotenoid content of vegetables and fruits commonly consumed in the UK. *Food Chem.* **1995**, *54*, 101−111.
(38) Institute of Medicine. Food and Nutrition Board. *Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium and Zinc*; National Academy Press: Washington, DC, 2001.
(39) Mattila, P.; Astola, J.; Kumpulainen, J. Determination of flavonoids in plant material by HPLC with diode-array and electro-array detection. *J. Agric. Food Chem.* **2000**, *48*, 5834−5841.
(40) Chen, C. S.; Shaw, P. E.; Parish, M. E. Fruit juice processing technology. In *Orange and Tangerine Juices*; Nagy, S., Chen, C. S., Shaw, P. E., Eds.; Agscience: Auburndale, FL, 1993; pp 110−166.
(41) Parish, M. E. Orange juice quality after treatment by thermal pasteurization or isostatic high pressure. *Lebensm. Wiss. Technol.* **1998**, *31*, 439−442.
(42) Yen, G.-C.; Lin, H.-T. Comparison of high-pressure treatment and thermal pasteurization effects on the quality and shelf life of guava puree. *Int. J. Food Sci. Technol.* **1996**, *31*, 205−213.
(43) Giner, J.; Gimeno, V.; Espachs, A.; Elez, P.; Barbosa-Cánovas, G. V.; Martín, O. Inhibition of tomato (*Lycopersicon esculentum* Mill.) pectin methylesterase by pulsed electric fields. *Innovations Food Sci. Emerging Technol.* **2000**, *1*, 57−67.
(44) Giner, J. G., V.; Palomes, M.; Barbosa-Cánovas, G. V.; Martín, O. Lessening polygalacturonase activity in a commercial enzyme preparation by exposure to pulsed electric fields. *Eur. Food Res. Technol.* **2003**, *217*, 43−48.
(45) Calligari, S.; Falcone, P.; Anese, M. Color changes of tomato purees during storage at freezing temperatures. *J. Food Sci.* **2002**, *67*, 2432−2435.
(46) Van den Broeck, I.; Ludikhuyze, L.; Weemaes, C.; Van Loey, A.; Hendrickx, M. E. Kinetics for isobaric−isothermal degradation for L-ascorbic acid. *J. Agric. Food Chem.* **1998**, *46*, 2001−2006.
(47) Donsi, G.; Ferrari, G.; Di Matteo, M. High-pressure stabilization of orange juice: Evaluation of the effects of process conditions. *Ital. J. Food Sci.* **1996**, *8*, 99−106.
(48) Min, S.; Jin, Z. T.; Zhang, Q. H. Commercial scale pulsed electric field processing of tomato juice. *J. Agric. Food Chem.* **2003**, *51*, 3338−3344.
(49) Clinton, S. K. Lycopene: Chemistry, biology, and implications for human health and disease. *Nutr. Rev.* **1998**, *56*, 35−51.

(50) Butz, P.; Tauscher, B. Emerging technologies: Chemical aspects. *Food Res. Int.* **2002**, *35*, 279−284.
(51) Smelt, J. P. P. M. Recent advances in the microbiology of high-pressure processing. *Trends Food Sci. Technol.* **1998**, *9*, 152−158.
(52) Van het Hof, K. H.; West, C. E.; Weststrate, J. A.; Hautvast, J. G. A. J. Dietary factors that affect the bioavailability of carotenoids. *J. Nutr.* **2000**, *130*, 503−506.
(53) Van het Hof, K. H.; Tijburg, L. B. M.; Pietrzik, K.; Weststrate, J. A. Bioavailability of carotenoids and folate from different vegetables. Effect of disruption of the vegetable matrix. *Br. J. Nutr.* **1999**, *82*, 203−212.
(54) Knorr, D.; Angersbach, A.; Eshtiaghi, M. N.; Heinz, V.; Le, D.-U. Processing concepts based on high-intensity electric field pulses. *Trends Food Sci. Technol.* **2001**, *12*, 129−135.
(55) De Sá, M. C.; Rodríguez-Amaya, D. M. Carotenoid composition of cooked green vegetables from restaurants. *Food Chem.* **2003**, *83*, 595−600.
(56) Shi, L.; Le Maguer, M. Lycopene in tomatoes: Chemical and physical properties affected by food processing. *Crit. Rev. Food Sci. Technol.* **2000**, *40*, 1−42.
(57) Puppin, A. M.; Dennis, M. J.; Toledo, M. C. F. Flavanone glycosides in Brazilian orange juices. *Food Chem.* **1998**, *61*, 275−280.
(58) Rapisarda, P.; Tomaino, A.; Lo Cascio, R.; Bonina, F.; De Pasquale, A.; Saija, A. Antioxidant effectiveness as influenced by phenolic content of fresh orange juices. *J. Agric. Food Chem.* **1999**, *47*, 4718−4723.
(59) Tomás-Barberán, F. A.; Clifford, M. N. Flavanones, chalcones and dihydrochalcones—nature, occurrence, and dietary burden. *J. Sci. Food Agric.* **2000**, *80*, 1073−1080.
(60) Arena, E.; Fallico, B.; Macarone, E. Evaluation of antioxidant capacity of blood orange juices as influenced by constituents, concentration process and storage. *Food Chem.* **2001**, *74*, 423−427.

Received for review July 13, 2004. Revised manuscript received January 7, 2005. Accepted January 19, 2005. This research was supported by funding from Comunidad Autónoma de Madrid (Grants 07G/0040/2000-07G/0041/2000 and 07G/0053/2003) and the Ministry of Science and Technology (Grant AGL2002-04059-C02-02). C.S.-M. was supported by a Ramón y Cajal Research Contract from the Ministry of Science and Technology.

JF048839B