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Characterization of carotenoid and carotenoid esters of astringent persimmon tissues (*Diospyros kaki* Thunb. var. Rojo brillante). Effects of thermal and high pressure non-thermal processing

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Abstract: Carotenoid and carotenoid esters profiles of peel, pulp and whole fruit tissues of astringent persimmon (*Diospyrus kaki* Thunb., var. Rojo Brillante) have been characterized in detail and quantified for the first time. Carotenoids were determined by HPLC-PDA-MS/MS ($APCI^+$), using a reverse phase C_{30} column. A total of 38 carotenoids were identified and quantified, corresponding to 21 free carotenoids (13 xanthophylls and 8 hydrocarbon carotenes) and a total of 17 carotenoid esters. The qualitative profiles are very similar among tissues, differing only in the carotenoids concentration. The most important identified free xanthophylls were (all-*E*)-β-cryptoxanthin, (all-*E*)-antheraxanthin, (all-*E*)-lutein, (all-*E*)-

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zeaxanthin and (all-*E*)-violaxanthin . Hydrocarbon carotenoids found were (all-*E*)-β-carotene, (all-*E*)-α-carotene, (9Z)-β-carotene, (13Z)-β-carotene, (9Z)-α-carotene, and lycopene. The most abundant carotenoid esters were (all-*E*)-lutein-3-O-palmitate, (all-*E*)-zeaxanthin myristate, (all-*E*)-zeaxanthin palmitate and (all-*E*)-cryptoxanthin laurate. Processing by high pressures produced no regular effect on persimmon carotenoids and pasteurization affected negatively the content of all carotenoids from all studied persimmon tissues. This work will contribute to the development of scientific research about the bioaccessibity and bioavailabity of each individual free or esterified persimmon carotenoids in order to a better understanding of the carotenoid compounds impact in human health.

Keywords: Persimmon, *Diospyrus kaki* Thunb., var. Rojo Brillante, astringent**,** carotenoids, carotenoid esters, HPLC-PDA-MS (APCI$^+$), processing effects

1. Introduction

Carotenoids are lipophilic compounds found in fruits, vegetables, flowers, and animals with an important role in nutrition due to vitamin A activity, in addition to antioxidant activity, intercellular communication and immune system activity [1]. Two classes of carotenoids can be distinguished: carotenes, which possess only carbon and hydrogen atoms, and xanthophylls, which also possess oxygenated groups. Approximately, 750 carotenoids have been described in nature and only about 50 of them are identified in the human diet, the most abundant being β-carotene, α-carotene, lutein, zeaxanthin and β-cryptoxanthin [2]. Recent evidence has emerged that consumption of carotenoids may protect from cardiovascular, skin, eye and bone diseases and delay the onset and development of several types of cancer [3].

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The human body is not able to synthesize carotenoids and therefore dietary ingestion is the only source to meet their requirements. Although the existing evidence is insufficient to establish a recommended dietary allowance (RDA) or adequate intake (AI) for provitamin A carotenoids and other carotenoids, several dietary guidelines recommend a RDA = 700-900 retinol activity equivalents (RAE), considering 1 RAE = 6 μg of β-carotene and 12 μg for others provitamin-A carotenoids such as β-cryptoxanthin and α-carotene [4,5]. Since the main food sources of carotenoids are fruits and vegetables, knowledge on carotenoid composition in different edible parts and cultivars will be useful for the selection of nutrient-rich plants for food fortification and proper diet recommendation [6].

Persimmon (*Diospyros kaki* L.) is a widespread fruit that contains high quantities of carotenoids, besides others bioactive compounds including vitamin C, condensed tannins and dietary fiber [7]. The carotenoid composition in fruit and vegetables depends on several factors including variety, ripening stage, de-astringency method or processing [8]. Spanish "Rojo Brillante" variety is an appreciated astringent persimmon cultivar that needs a de-astringency treatment before commercialization to improve its sensorial quality such as exposure to carbon dioxide in high concentrations [9]. Different parts of the persimmon fruit also may contain different types and amounts of carotenoids. For example, the quantity of β-cryptoxanthin, β-carotene, lycopene or lutein is greater in the peel compared to the flesh [10].

Persimmon is a seasonal fruit and only obtainable in fresh form for a short period of time throughout the year (in Europe, from November to January). In order to prolong its availability, persimmon (including whole fruit, flesh and/or peel) could be processed into derivative products which would naturally provide great amounts of health-promoting compounds [11,12]. Nowadays, the development of new fruit-based products in the form of beverages, smoothies or desserts is increasing worldwide since consumers have become more conscious of the importance of healthy and natural food consumption. Persimmon carotenoid

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profile had been reported by several authors [13, 14, 9], but in all of these studies only saponified extracts were characterized and in all cases a C18 column was used by HPLC carotenoid separation, being *trans*-β-cryptoxanthin, *trans*-zeaxanthin, *trans*-lutein, *trans*-β- and α-carotene and lycopene the major carotenoids found in these saponified extracts. Only, Hitaka et al. (2013) [15] reported the characterization of carotenoid fatty acid esters from persimmon peels using the isolation of the main carotenoids and their esters by purification via silica gel column chromatography and the characterization of their structures buy using HR-FAB-MS, ^{1}H- and ^{13}C-NMR. β-carotene, lycopene, β-cryptoxanthin mono myristic acid ester, zeaxanthin di-myristic acid ester, and small amounts of xanthophyll esters of palmitoleic and oleanolic acid were found. No other previously published studies showed the individual profile of carotenoids and carotenoid esters of persimmon fruits.

On the other hand, functional foods have made use of innovations in food technology. To minimize losses of nutritional and organoleptic quality associated with traditional thermal treatment, non-thermal technologies have been applied extensively in the processing of plant foods. In this framework, high hydrostatic pressure (HHP) processing has been identified as a useful tool for extending the shelf-life and quality as well as for preserving the nutritional and functional characteristics mainly of fruit and vegetable products [16]. Recently, the effects of HHP on the retention of potentially health related compounds and antioxidant activity of fruits and vegetables have widely gained attention from researchers [17]. One very important benefit of HHP is that it could produce an improvement on the extraction of bioactive compounds due to induction of many changes on plant food structure during food processing [18, 19, 20, 21]. A previous study of our group [9] reported that the pressurization of persimmon tissues can contribute to an efficient extraction of carotenoids. These studies were carried out in saponified persimmon extracts. From these data, the pressurization of persimmon tissues at 200 MPa was the most interesting HPP condition to continue the

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carotenoid studies in no saponified extracts to analyzed also carotenoid esters with a C30 reverse phase column.

Based on the description above, in this study for the first time an almost complete characterization of carotenoid and carotenoid esters profile of astringent persimmon tissues (peel, pulp, and whole fruit) (*Diospyros kaki* Thunb., var. Rojo Brillante) was made. In addition, the effects of a high pressure treatment and a pasteurization treatment on the composition of individual carotenoid and carotenoid esters of persimmon tissues were evaluated in order to explore the possibility to use them to improve the extraction of these bioactive compounds, carotenoid and carotenoid esters, for the use of persimmon as functional ingredient.

2. Materials and Methods

2.1 Chemicals and reagents

Tetrahydrofuran (THF), methyl tert-butyl ether (MTBE), methanol (MeOH) and diethyl ether, acetone and petroleum ether (30-70°) were purchased from VWR International (Radnor, Pensilvania, USA); anhydrous sodium sulfate, potassium hydroxide (KOH) and sodium chloride (NaCl) from Panreac Química (Barcelona, Spain); butylated hydroxytoluene (BHT) and magnesium carbonate from Acros Organics (New Jersey, USA). Standards for lycopene (L9879, ≥90%, from tomato), lutein (X6250 from marigold) and *trans*-β-apo-8'-carotenal (10810, ≥96%, (UV)) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA); (all-*E*)-β-carotene (HPLC 96%, synth., cryst.), (all-*E*)-α-carotene (HPLC 97%, synth., cryst.), (all-*E*)-β-cryptoxanthin (HPLC 97%, synth., cryst.), (all-*E*)-zeaxanthin (HPLC 97%, synth., cryst.), (all-*E*)-neoxanthin (HPLC 97%, isolated, cryst.) and (all-*E*)-violaxanthin (HPLC 95%, isolated, cryst.) from CaroteNature (Ostermundigen, Switzerland).

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2.2. Persimmon samples

Persimmon fruits (*Diospyros kaki* Thunb., var. Rojo Brillante) were harvested in Ribera del Xúquer (Valencia, Spain) at commercial maturity stage IV, which is based on the external colour according to Salvador et al. (2007) [22]. As corroborated by our previous analysis of "Rojo Brillante" persimmon variety from several areas and local supermarkets, the carotenoid profile remains stable through years (unpublished results). After harvest, fruits were not treated to remove astringency (astringent samples). Selection of those fruits with uniform size and no defects was carried out. Physical and physicochemical characteristics of persimmon fruits (Table 1) were evaluated as described before [9].

Astringent persimmon fruits were washed, drained and hand prepared to obtain three types of tissue: whole fruits, flesh and peel (Control samples). Pieces (20 × 20 mm) of persimmon tissue (approximately 200 g) were vacuum packaged in 200 × 300mm plastic bags (Cryovac®, Sealed Air Corporation, Madrid, Spain), frozen in liquid nitrogen and lyophilized by freeze drying for 5 days at −45°C and 1.3× 10−3 mPa (LyoBeta 15, Azbil Telstar SL, Terrasa, Spain). Each type of freeze-dried persimmon sample was ground by pulverizing (GrindomixGM200, Retsch, Germany) to a fine particle size (<2 mm) before being carefully homogenized and vacuum stored at −36°C in Cryovac® bags until carotenoid analysis.

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Table 1. Physicochemical characteristics of astringent persimmon (*Diospyros kaki* Thunb. var. Rojo brillante)

Persimmon (*Diospyros kaki* Thunb. var. Rojo brillante)	Astringent
Titratable acidity (g citric acid/100 g fw[2])	0.11 ± 0.01
pH	5.45 ± 0.04
Soluble solids (ºBrix at 20 ºC)	15.21 ± 1.15
Total solids (g/100 g fw)	16.28 ± 0.69
Firmness (N)	15.81 ± 1.22
CIELab color parameters	
L^*	58.48 ± 2.37
a^*	7.99 ± 1.65
b^*	23.09 ± 1.39

[1] Values are the mean of three independent determinations ± standard deviation.
[2] fw, fresh weight.

2.3 Technological treatments

2.3.1 High pressure treatment

Samples of different persimmon tissues (80 g) were vacuum packaged in flexible bags (Cryovac®, Sealed Air Corporation, Madrid, Spain), introduced into the pressure unit filled with pressure medium (water) and then treated by high-pressure (200 MPa/25°C/6 min). HPP was performed in a hydrostatic pressure unit with a 2 L capacity, a maximum pressure of 900 MPa, and a potential maximum temperature of 95 °C (Stansted SFP 7100:9/2C, UK). Before pressurization, the pressure chamber was heated/cooled to a desired level by means of a thermostat jacket connected to a water bath. Compression and decompression rates were 2.5 MPa/s. Pressure, time and temperature were controlled by a computer program, being constantly monitored and recorded during the process. After treatment, samples were

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immediately frozen with liquid nitrogen, and lyophilized by freeze drying and stored at −36°C until carotenoid analysis.

2.3.2 Thermal treatment. Pasteurization.

Samples of different persimmon tissues (80 g) were vacuum packaged in flexible bags (Cryovac®, Sealed Air Corporation, Madrid, Spain), placed in an autoclave (Autester-G, Selecta, Barcelona, Spain), heated at 85°C for 15 min, and cooled to room temperature by an ice-water bath. After cooling at room temperature, samples were immediately frozen with liquid nitrogen, and lyophilized by freeze drying and stored at −36°C until carotenoid analysis.

2.6. Carotenoid extraction and saponification

2.6.1. Carotenoid extraction from persimmon tissues

Extraction and saponification procedures were carried out under dim light. Freeze dried samples were turned into a fine powder using a cutter mill. 1 g of freeze-dried sample was combined with 0.5 g of magnesium carbonate and 60 µL of trans-β-apo-8'-carotenal (0.2 mg/mL), as internal standard, and then extracted with 20 ml of tetrahydrofuran (THF) stabilized 0.01 % BHT in a homogenizer (OMNI Macro ES®, OMNI International). The extract was then filtered, and the residue was extracted twice more with 20 mL of THF and filtered. The three filtrates were combined and evaporated to half the volume on a rotatory evaporator at 35 °C, under nitrogen ambient. The concentrated extract was then added to a funnel containing 15 mL of diethyl ether and 25 mL of water saturated with NaCl. Aqueous phase was washed twice with other 15 mL of diethyl ether. The ethereal organic phases were dried with anhydrous sodium sulfate. The dried organic phase (non-saponified extract) was

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completely evaporated by vacuum and controlled temperature (20°C) and nitrogen ambient and then, dissolved to exactly 2 mL with MeOH/MTBE/H_2O (45.5:52.5:2, v/v/v), filtered through a 0.45 µm membrane filter and immediately analyzed by HPLC.

2.6.2. Saponification of carotenoid extracts

In the case of saponified extracts, the dried organic phase was combined with 4 mL of 30% methanolic potassium hydroxide and kept under magnetic agitation for 1.5 hours in nitrogen atmosphere in the dark. The saponified extract was added to a funnel containing 15 mL of diethyl ether and was washed five times with 25 mL of water saturated with NaCl, discarding the aqueous phase each time, to obtain a neutral pH. The extract was then dried with anhydrous sodium sulfate, completely evaporated on a rotatory evaporator with controlled temperature (20°C) and then, re-dissolved to 2 mL with MeOH/MTBE/H_2O (45.5:52.5:2, v/v/v), filtered through a 0.45 µm membrane filter and immediately analyzed by HPLC.

2.7. Carotenoid analysis by HPLC-DAD

The identification and quantification of carotenoids in saponified and non-saponified persimmon extracts from different tissues and samples (control or HPP or P treated samples) were carried out using a 1200 Series Agilent HPLC System (Agilent Technologies, Santa Clara, CA, U.S.A) with a reverse phase C30 column (YMC-Pack YMC C30, 250 x 4.6 mm i.d., S-5 µm, YMC Co., Ltd). The solvents used for separation consisted on a mix of Methanol/MTBE/Water (81:14:4, v/v/v, eluent A) and Methanol/MTBE (10:90, v/v, eluent B) both containing 0.1% of ammonium acetate. The elution gradient was linear, starting at 100% A and ending with 100% B, in 60 minutes. Injection chamber at 4°C in order to preserve carotenoid stabilities. Flow rate was 1 mL/min and the column temperature was 32 °C. Injection volume was 20 µL. Carotenoids were monitored at 450 nm; also chromatograms

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were recorded at 402nm also for carotenoids, 325nm for retinoids, and 294 for tocopherols [23]. Additional UV/Vis spectra were recorded between 300 to 700 nm.

The individual carotenoid identification was carried out according to the following combined information: elution order on C30 and C18 (data no showed in this work) HPLC columns, chromatography with authentic standards, UV-visible spectrum (λ_{max}, spectral fine structure (%III/II), peak cis intensity) and mass spectrum compared with data available in the literature [24, 25, 26, 27, 28, 29]. Prior to quantification by HPLC-DAD, the concentrations of stock solutions of lycopene, lutein, *trans*-β-apo-8'-carotenal, (all-*E*)-β-carotene, (all-*E*)-α-carotene, (all-*E*)-β-cryptoxanthin, (all-*E*)-zeaxanthin, (all-*E*)-neoxanthin and (all-*E*)-violaxanthin were determined spectrophotometrically using their specific absorption coefficients according to Britton (1995) [30] in order to elaborate linear calibration curves. These calibration curves (up to seven concentration levels) were prepared per triplicate with standard stock solutions for each carotenoid in the concentration range 5-100 µg/mL. Calibration curves were constructed by plotting the peak area at 450nm for all carotenoids.

The (all-*E*)-β-carotene calibration was used for quantitation of β-carotene, β-carotene-isomers, while (all-*E*)-violaxanthin, violaxanthin-isomers and (all-*E*)-antheraxanthin were quantitated by violaxanthin calibration. In addition, (all-*E*)-lutein calibration was used for lutein-epoxide quantitation. Other carotenoids such as (all-*E*)-neoxanthin, (all-*E*)-β-cryptoxanthin and lycopene were quantitated by their corresponding standards. Results were expressed micrograms of the corresponding the carotenoid per 100 g of fresh weight. The NAS-NRC conversion factor was used to calculate the vitamin A value [31].

2.8. Liquid chromatography-mass spectrometry (LC-MS/MS (APCI$^+$)

LC/MS analyses were performed with the same HPLC system described above coupled on-line to an Agilent mass spectrometry detector with APCI source model G1947B

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compatible with the LCMS SQ 6120 equipment, according to the procedure described by Breithaupt and Schwack (2000) [24]. Positive ion mass spectra of the column eluate of 13000 Th/s (peak width 0.6 Th, FWHM). Nitrogen was used both as the drying gas at a flow rate of 60 L/min and as nebulizing gas at a pressure of 50 psi. The nebulizer temperature was set at 350°C and a potential of +2779 kV was used on the capillary. Corona was set at 4000 nA both in positive ion mode, and the vaporizer temperature was set at 400°C. Collisium gas was helium and fragmentation amplitude was 0.8-1.2 V. The chromatographic conditions were the same as described for quantitative analyses of carotenoids. The HPLC retention times, UV/Vis spectra, and MS spectral data of carotenoids from whole fruit, peel and pulp of persimmon (*Diospyros kaki* Thunb., var. Rojo Brillante) tissues are showed in Table 2.

2.9. Statistical analysis

The compositional data were expressed as mean and standard deviation (SD). The obtained results were evaluated with variance analysis (ANOVA) and the least significant differences (LSD) were calculated at a $p<0.05$ significance level. The correlation coefficients were determined by Pearson's test at a $p<0.05$ significance level. The statistic software employed was SPSS version 2.7. All the analysis was done at least in triplicate.

3. Results and Discussion

3.1. Characterization of the carotenoid and carotenoid esters profile of Astringent persimmon fruits (Diospyrus kaki Thunb., var. *Rojo Brillante)*

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Carotenoids from lyophilized and rehydrated samples were efficiently extracted with THF. Methanol was not employed in the last extraction step due to the precipitation of tannins which take place due the high amounts of these compounds present in astringent persimmon tissues. Figure 1 shows the chromatograms of HPLC persimmon whole fruit tissue from crude (no saponified) (A) and saponified (B) extracts (Supplementary material: Fig 1S, chromatograms of HPLC persimmon peel fruit tissue and Fig. 2S, chromatograms of HPLC persimmon pulp tissue). The carotenoid profile of non saponified extracts obtained from this astringent variety of persimmon, var. Rojo Brillante, is composed by 38 carotenoids, where all of them were successfully identified and quantified, corresponding to 21 free carotenoids (13 xanthophylls and 8 hydrocarbon carotenes) and a total of 18 carotenoid esters. Saponified extracts showed only 21 identified carotenoids, being 12 xanthophylls and 8 hydrocarbon carotenoids. A residual two peaks corresponding to xanthophyll esters were observed too in the saponified extracts, indicating that the saponification was not completely finished (because this is a reversible reaction).

The qualitative profiles of carotenoids and their carotenoid esters in different astringent persimmon, var. Rojo Brillante, were very similar among different tissues (whole fruit, peel and pulp) (Figure 2 and Figures S1 (persimmon peel) and S2 (persimmon pulp) from Supplementary material). The MS fragmentation pattern of carotenoid esters of persimmon tissues showed the usual fragmentation described for the xanthophyll esters previously reported for other fruits and vegetables as mango and citrus fruits [32], pepper, wolfberry, sea buckthorn, apple, squash [33] and jackfruit [27].

Persimmon fruit carotenoid profile of crude (no saponified) extracts showed the presence of 21 free carotenoids (13 xanthophylls and 8 hydrocarbon carotenes), being the free xanthophylls: (all-*E*)-β-cryptoxanthin, (all-*E*)-antheraxanthin, (all-*E*)-lutein, (all-*E*)-zeaxanthin, (all-*E*)-violaxanthin and small amounts of lutein-5,6-epoxide, 5,6-epoxy-β-

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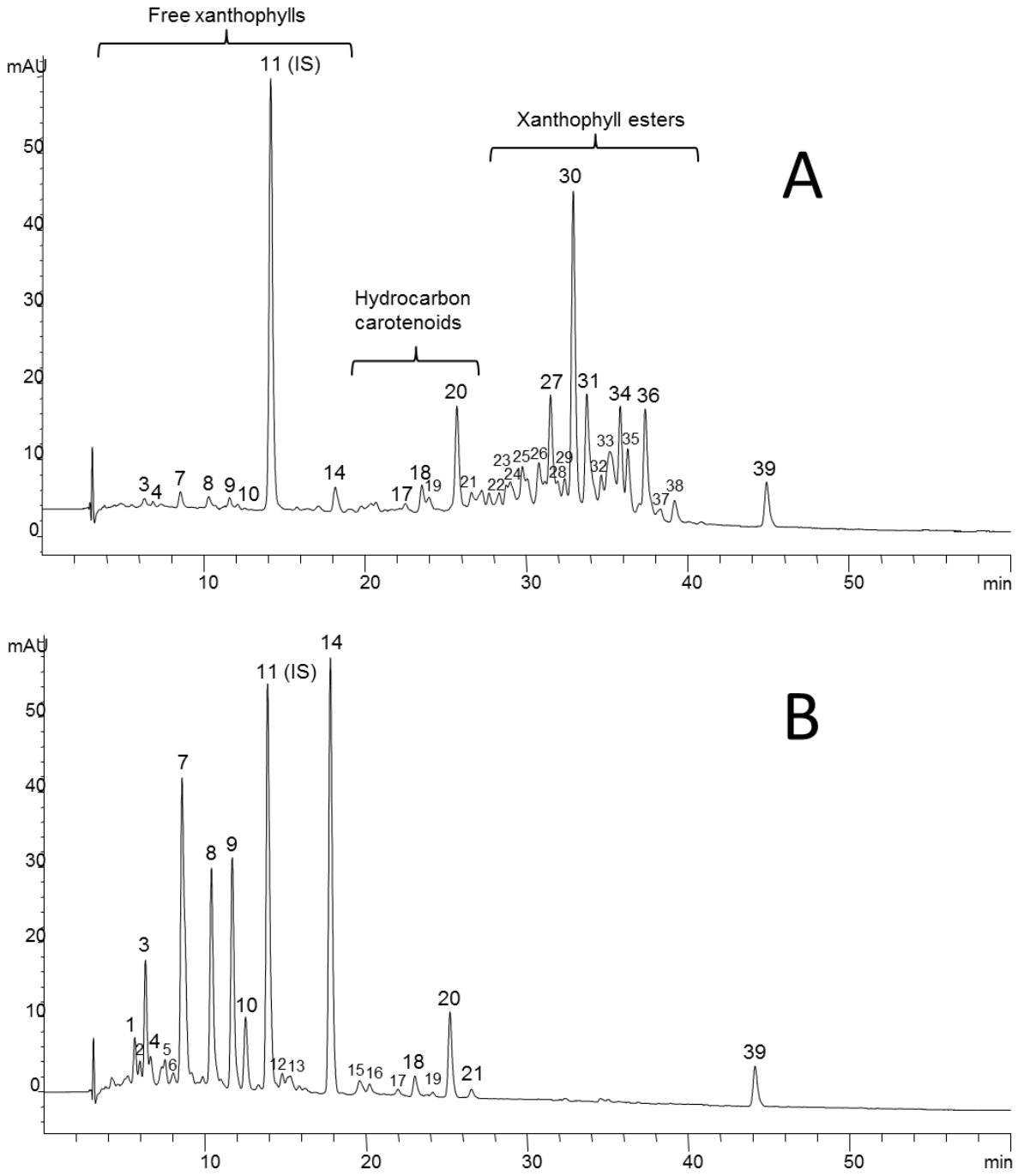

Figure 1: C30 reversed-phase HPLC chromatogram obtained from direct (A) and saponified (B) carotenoid extracts from whole fruit tissue of Spanish astringent persimmon (*Diospyros kaki* Thunb. var. Rojo brillante). UV-vis detection at 450 nm. Peak identities in Table 2. (U) un-identified compounds.

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cryptoxanthin, (all-*E*)-α-cryptoxanthin and (all-*E*)-neoxanthin. Hydrocarbon carotenoids were *trans*-β-carotene, *trans*-α-carotene, (9Z)-β-carotene, (13Z)-β-carotene, (9Z)-α-carotene, and lycopene. In addition, 18 xanthophyll esters were observed in these extracts (Table 2).

The identification is discussed according to the elution order in the C_{30} column and the chromatographic conditions employed in the present study. The use of authentic carotenoid standards as lycopene, (all-*E*)-lutein, (all-*E*)-β-carotene, (all-*E*)-α-carotene, (all-*E*)-β-cryptoxanthin, (all-*E*)-zeaxanthin, (all-*E*)-neoxanthin and (all-*E*)-violaxanthin, and (all-*E*)-β-apo-8'-carotenal as internal standard make easy the identification and quantification of these carotenoids in the extracts.

Also, recently Petry and Mercadante (2016) [32] summarized sources and MS/MS characteristic fragments by APCI (+) for xanthophyll esters. This reported data helped to reinforce the chemical identification of these compounds in the present study about persimmon fruits.

3.1.1. Free xanthophylls in persimmon tissues

Peak **1** (Rt = 5.66 min), Peak **3** (Rt = 6.33 min) and peak **5** (Rt = 7.53 min) were identified as (13Z)-violaxanthin, (all-*E*)-violaxanthin and (9Z)-violaxanthin, respectively. The UV-visible spectrum of peak **3**, with λ_{max} at 414, 438, 468 nm, and the characteristic pronounced fine structure (%III/II = 98) was in agreement with the chromophore consisted of nine conjugated double bonds and at least one β-ring, comparison with the authentic standard and data reported in the literature (Britton, 1995). In contrast, peaks **1** and **5** showed an UV-visible spectra with a less marked fine structures and the appearance of a “cis peak” at 327 nm (327, 414, 436, 468 nm; %III/II = 89 and 326, 416, 440, 468 nm; %III/II = 78, respectively). These three peaks disappeared upon treatment of the extract with diluted HCl (epoxide test).

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Table 2. HPLC retention times, UV/Vis spectra, and MS spectral data of carotenoids from whole fruit, peel and pulp of astringent persimmon (Diospyros kaki Thunb. var. Rojo brillante)

No.	Rt (min)	Compound Identity	HPLC-DAD UV/Vis absorption máxima (nm)	%III/II	% A_B/A_{II}	$[M+H]^+$ m/z	HPLC/APCI(+) MS fragmentation pattern (m/z) fragments ions (m/z)	STD[c]
1	5.66	(13Z)-violaxanthin[a]	326,416,440,468	78	0	601	583 $[M+H-18]^+$, 565 $[M+H-36]^+$, 509 $[M+H-92]^+$, 491 $[M+H-92-18]^+$	n.a.
2	5.98	*(all-E)*-luteoxanthin	406,428,454	nc[f]	0	601	583 $[M+H-18]^+$	n.a.
3	6.33	*(all-E)*-violaxanthin	414,438,468	98	0	601	583 $[M+H-18]^+$, 565 $[M+H-36]^+$, 521 $[M+H-80]^+$	Y
4	6.64	*(all-E)*-neoxanthin	410,434,464	99	0	601	583 $[M+H-18]^+$, 565 $[M+H-36]^+$, 547 $[M+H-54]^+$, 521 $[M+H-80]^+$	Y
5	7.53	(9Z)-violaxanthin	327,414,436,468	89	nc[f]	601	583 $[M+H-18]^+$, 565 $[M+H-36]^+$	n.a.
6	8.04	(9Z)-neoxanthin	327,410,432,462	55	0	601	583 $[M+H-18]^+$, 565 $[M+H-36]^+$, 547 $[M+H-54]^+$, 521 $[M+H-80]^+$	n.a.
7	8.57	*(all-E)*-antheraxanthin	(422),444,472	66	0	585	567 $[M+H-18]^+$, 549 $[M+H-36]^+$, 505 $[M+H-80]^+$	n.a.
8	10.38	*(all-E)*-lutein	(420),444,472	62	0	569	551 $[M+H-18]^+$, 533 $[M+H-36]^+$	Y
9	11.68	*(all-E)*-zeaxanthin	(426),450,470	18	0	569	551 $[M+H-18]^+$, 533 $[M+H-36]^+$	Y
10	12.51	Lutein-5,6- epoxide	(418),440,468	85	nc[f]	585	567 $[M+H-18]^+$, 549 $[M+H-36]^+$, 505 $[M+H-80]^+$	n.a.
11	13.88	*(all-E)*-β-apo-caroten-8´al (internal standard)	462	0	0	417	399 $[M+H-18]^+$, 325 $[M+H-74]^+$	Y
12	14.79	5,6-epoxy- β-cryptoxanthin	(420),445,471	52	nc[f]	569	551 $[M+H-18]^+$, 459 $[M+H-18-92]^+$, 221	n.a.
13	15.30	*(all-E)*-α-cryptoxanthin	(418),442,464	61	nc[f]	553	535 $[M+H-H_2O]^+$, 461 $[M+H-92]^+$	n.a.
14	17.79	*(all-E)*-β-cryptoxanthin	(426),450,476	18	nc[f]	553	535 $[M+H-H_2O]^+$, 461 $[M+H-92]^+$	Y
15	19.27	5,6-epoxy- α-carotene	(418),441,469	10	0	553	535 $[M+H-18]^+$, 495, 205	n.a.
16	19.72	(13Z)-α-carotene	337,(413),438,466	31	0	537	481 $[M+H-56]^+$, 445 $[M+H-92]^+$	n.a.
17	21.78	(13Z)-β-carotene[c]	337,(412),441,468	14	0	537	457 $[M+H-80]^+$, 445 $[M+H-92]^+$, 400 $[M+H-137]^+$, 269 $[M+H-268]^+$, 177 $[M+H-360]^+$, 137 $[M+H-400]^+$	n.a.
18	23,02	*(all-E)*-α-carotene	(422),446,474	66	0	537	457 $[M+H-80]^+$, 413 $[M+H-124]^+$, 177 $[M+H-360]^+$, 137 $[M+H-400]^+$, 123 $[M+H-414]^+$	Y
19	23.66	(9Z)-α-carotene	335,421,440,469	60	0	537	457 $[M+H-80]^+$, 445 $[M+H-92]^+$	n.a.
20	25.07	*(all-E)*-β-carotene[c]	(428),450,476	16	0	537	457 $[M+H-80]^+$, 445 $[M+H-92]^+$, 400 $[M+H-137]^+$, 269 $[M+H-268]^+$, 177 $[M+H-360]^+$, 137 $[M+H-400]^+$	Y

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21	26.50	(9Z)-β-carotene	335,421,440,469	23	0	537	457 $[M+H-80]^+$, 445 $[M+H-92]^+$, 400 $[M+H-137]^+$, 269 $[M+H-268]^+$, 177 $[M+H-360]^+$, 137 $[M+H-400]^+$	n.a.
22	28.31	*(all-E)*-violaxanthin laurate[d]	416,440,466	nc[f]	0	783	765 $[M+H-18]^+$, 747 $[M+H-18-18]^+$, 691 $[M+H-92]^+$, 673 $[M+H-92-18]^+$, 583 $[M+H-12:0]^+$, 565 $[M+H-12:0-18]^+$, 547 $[M+H-12:0-18-18]^+$	n.a.
23	28.75	*(all-E)*-violaxanthin butyrate[d]	416,440,470	nc[f]	nc[f]	671	653 $[M+H-18]^+$, 635 $[M+H-18-18]^+$, 583 $[M+H-4:0]^+$, 579 $[M+H-92]^+$, 565 $[M+H-4:0-18]^+$, 561 $[M+H-92-18]^+$	n.a.
24	29.0	*(all-E)*-neoxanthin dibutyrate[d]	412,436,464	81	0	741	723 $[M+H-18]^+$, 653 $[M+H-4:0]^+$, 649 $[M+H-92]^+$, 635 $[M+H-4:0-18]^+$, 631 $[M+H-92-18]^+$, 565 $[M+H-4:0-4:0]^+$, 547 $[M+H-4:0-4:0-18]^+$	n.a.
25	29.76	*(all-E)*-violaxanthin palmitate[d]	416,440,470	nc[f]	nc[f]	839	821 $[M+H-18]^+$, 803 $[M+H-18-18]^+$, 747 $[M+H-16:0]^+$, 729 $[M+H-92-18]^+$, 583 $[M+H-256]^+$, 565 $[M+H-18-16:0]^+$, 547 $[M+H-16:0-18-18]^+$	n.a.
26	30.79	(9Z) neoxanthin dibutyrate[d]	327,412,436,464	80	16	741	723 $[M+H-18]^+$, 653 $[M+H-4:0]^+$, 649 $[M+H-92]^+$, 635 $[M+H-4:0-18]^+$, 631 $[M+H-92-18]^+$, 565 $[M+H-4:0-4:0]^+$, 547 $[M+H-4:0-4:0-18]^+$	n.a.
27	31.40	(all-*E*)-zeaxanthin palmitate	428,450,476	nc[f]	nc[f]	807	551$[M+H-16:0]^+$, 789 $[M+H-18]^+$, 715 $[M+H-92]^+$, 533 $[M+H-18-16:0]^+$	n.a.
28	32.72	*(all-E)*-antheraxanthin laurate-myristate[d]	419,443,470	33	0	977	959 $[M+H-18]^+$, 777 $[M+H-12:0]^+$, 749 $[M+H-14:0]^+$, 759 $[M+H-12:0-18]^+$, 731 $[M+H-14:0-18]^+$, 549 $[M+H-12:0-14:0]^+$, 531 $[M+H-12:0-14:0-18]^+$	n.a.
29	32.36	*(all-E)*-lutein dimyristate	422,446,474	38	0	nd[g]	761 $[M+H-14:0]^+$, 669 $[M+H-92]^+$, 533 $[M+H-14: 14:0]^+$	n.a.
30	32.89	*(all-E)*-β-cryptoxanthin laurate	424,450,476	25	0	735	643 $[M+H-92]^+$, 535 $[M+H-12:0]^+$, 479 $[M+H-56-12:0]^+$, 443 $[M+H-92-12:0]^+$	n.a.
31	33.72	*(all-E)*-lutein 3-O-palmitate	424,446,474	nc[f]	nc[f]	nd[g]	789 $[M+H-18]^+$, 697 $[M+H-18-92]^+$, 533 $[M+H-18-16:0]^+$	n.a.
32	34.62	*(all-E)*-antheraxanthin 3-O-palmitate[d]	422,444,472	nc[f]	nc[f]	823	805 $[M+H-18]^+$, 787 $[M+H-18-18]^+$, 731 $[M+H-92]^+$, 567 $[M+H-16:0]^+$, 549 $[M+H-16:0-18]^+$, 531 $[M+H-16:0-18-18]^+$	n.a.
33	35.16	*(all-E)*-zeaxanthin myristate	425, 450, 474	nc[f]	0	779	551 $[M+H-14:0]^+$, 761 $[M+H-18]^+$, 687 $[M+H-92]^+$, 533 $[M+H-18-14:0]^+$	n.a.
34	35.81	*(all-E)*-antheraxanthin	420,446,472	31		1033	1015 $[M+H-18]^+$, 941 $[M+H-92]^+$, 805 $[M+H-14:0]^+$, 787	n.a.

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		myristate-palmitate			0		[M+H-14:0-18]$^+$, 771 [M+H-16:0]$^+$, 759 [M+H-16:0-18]$^+$, 549 [M+H-14:0-16:0]$^+$, 531 [M+H-14:0-16:0-18]$^+$	
35	36.28	*(*all-*E)*-β-cryptoxanthin myristate	424,448,476	9	0	763	671 [M+H-92]$^+$, 535 [M+H-14:0]$^+$, 443 [M+H-14:0-92]$^+$	n.a.
36	37.38	*(*all-*E)*-β-cryptoxanthin dipalmitate	424,448,476	10	0	1046	790 [M+H-18-16:0]$^+$, 936 [M+H-92-18]$^+$, 516 [M+H-18-16:0-16:0]$^+$, 954 [M+H-92]$^+$	n.a.
37	38.07	*(*all-*E)*-lutein 3-O-laurate-3´-O-myristate	420,446,474	nc[f]	nc[f]	nd[g]	761 [M+H-12:0]$^+$, 769 [M+H-92]$^+$, 733 [M+H-14:0]$^+$, 533 [M+H-12:0-14:0]$^+$	n.a.
38	39.18	*(*all-*E)*-β-cryptoxanthin palmitate-stearate	426,448,478	nc[f]	nc[f]	1058	801 [M+H-16:0]$^+$, 773 [M+H-18:0]$^+$, 517 [M+H-16:0-18:0]$^+$	n.a.
39	44.16	lycopene	446,472,502	6	0	537	457 [M+H-80]$^+$, 413 [M+H-124]$^+$, 177 [M+H-360]$^+$, 137 [M+H-400]$^+$, 121 [M+H-416]$^+$	Y

n.d.: not detected. n.a.: not available.

[a]: tentatively identified by comparing the D_B/D_{II} value of the putative (9Z)-violaxanthin (0.06) to that reported by Molnár and Szabolcs (1993), i.e. 0.06 (Q=17.50).

[b]:in source fragment [M-H_2O+H]$^+$ in agreement with reference compound.

[c]: tentatively identified by comparing the D_B/D_{II} values if the putative (9Z)-β-carotene (0.06) and (13Z)-β-carotene (0.42) to those reported by Melendez-Martínez et al. (2013), i.e. 0.13 and 0.43, respectively.

[d]: tentative identification according to UV/Vis spectra. The obtained mass spectra were highly ambiguous, hindering its reliable identification.

[e]: ”Y” indicates that the presented analytical data were in agreement with an authentic reference compound.

[f]. %III/II was not calculated because of poor definition of the UV/vis spectrum or because it was not detected.

[g]. [M+H]$^+$ or MS/MS fragments were not detected.

Fatty acid refers to the following mass losses: 88 u = (4:0) butyric acid; 200 u = (12:0) lauric acid; 228 u = (14:0) myristic acid; 256 u = (16:0) palmitic acid; 284 u = (18:0) stearic acid

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Similar process carried out in peak **4** (Rt = 6.64 nm) identified as (all-*E*)-neoxanthin (λ_{max} at 410, 434, 464) and peak **6** (Rt = 8.04 min) identified as (9Z)-neoxanthin (λ_{max} at 327, 410, 432, 462), with the subsequent formation of a new peak, corresponding to a neochrome, generated by acid-catalyzed rearrangement of one 5,6-epoxy group. Neochrome was not detected in crude any persimmon fruit extract, nor peel, pulp or whole fruit. The LC/MS (APCI+) spectra of peaks **3** and **5** showed a protonated molecule $[M+H]^+$ at m/z 601, with was consistent with the molecular formula $C_{40}H_{56}O_4$ (M_w = 601.4257), and with the authentic standard that showed also these $[M+H]^+$ value. Also the presence of various less abundant fragments at 583 $[M+H-H2O]^+$ and 565 $[M+H-2H2O]^+$, derived from neutral losses of water molecules, confirmed the presence of two hydroxyl groups. Similar characteristics showed the carotenoid compound of peak **4**, identified as (all-*E*)-neoxanthin, with three fragments corresponding to the presence in the molecule of three hydroxyl groups, 583 $[M+H-H2O]^+$, 565 $[M+H-2H2O]^+$, and 547 $[M+H-3H2O]^+$. Peak **2** (Rt = 5.98 nm) was tentatively identified as luteoxanthin (406, 428, 454 nm), the %III/II value was not calculated because of poor definition of the UV/vis spectrum. Protonated molecule $[M+H]^+$ at 601 and a fragment 583 $[M+H-H2O]^+$ agree with the chemical structure attributed to this carotenoid. Luteoxanthin is a structural isomer of violaxanthin, with one epoxy group at one side of the molecule and one furanoid group in the other side. Peaks **1**, **2**, **5** and **6** only can be observed in persimmon saponified extracts (Figure 2, B), indicating that they were produced during the saponification step. All these four peaks are cis-isomers of violaxanthin, and neoxanthin.

Peak **7** (Rt = 8.57 min) was identified as (all-*E*)-antheraxanthin. The UV-visible spectrum, with λ_{max} at 422, 444, 472 nm (%III/II = 66). The MS (APCI+) spectrum of antheraxanthin showed a major ion for the protonated molecule $[M+H]^+$ at m/z 585, which was consistent with the molecular formula $C_{40}H_{56}O_3$ (M_w = 584.4229). Also, other fragments were 567 $[M+H-H2O]^+$, 549 $[M+H-2H2O]^+$, 505 $[M+H80]^+$, related to the loss of water molecules and the last with the presence of one 5,6-epoxy group. Peak **8** (Rt = 10.38 min), was identified as (all-*E*)-lutein, showing a UV-visible spectrum, with λ_{max} at 420, 444, 472 nm, by comparison by co-chromatography with a commercial pure standard lutein sample. Also, this compound exhibited a marked spectroscopic fine structure (%III/II = 62), which was consistent with a chromophore

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with nine conjugated double bonds and a potential one β-ring and one ε-ring [30]. Several authors shown in their works that the APCI (+) mass spectrum of lutein can be used for unmistakable determination of the chemical structure of lutein [34]. The main characteristic was the presence of a protonated molecule $[M+H]^+$ at m/z 569 and a most abundant fragment appeared at m/z 551, with was produced by the loss of one water molecule 551 $[M+H-H2O]^+$, followed by a fragment at m/z 533 $[M+H-2H2O]^+$, a loss of two water molecules with very low intensity.

Peak **9** (Rt = 11.68 min) showed a chemical, chromatographic and spectrophotometric properties that was correlated to identify as (all-*E*)-zeaxanthin. UV-visible spectrum with λ_{max} at 426, 450, 470 nm, and a fine structure (%III/II = 18), that could indicate the presence of two β-ring and nine conjugated double bonds [30]. Also, this carotenoid chemical structure was compared by co-chromatography with a commercial pure standard sample of (all-*E*)-zeaxanthin. Protonated molecule $[M+H]^+$ of MS spectrum, showed a value of m/z 569, which is consistent with the molecular formula $C_{40}H_{56}O_2$ (Mw = 582.4229). The most important MS fragments were m/z 551 $[M+H-H2O]^+$ and m/z 533 $[M+H-2H2O]^+$ related to the losses of one or two water molecules, confirming the existence of two hydroxyl groups in the chemical structure of this carotenoid. Peak **10** (Rt = 12.51 min) was identified as lutein-5,6-epoxide, by its spectroscopic characteristics, λ_{max} at 418, 440, 468 nm (%III/II = 85) and MS spectrum, showing a major ion for the protonated molecule $[M+H]^+$ at m/z 585 and ion fragments, 567 $[M+H-H2O]^+$, 549 $[M+H-2H2O]^+$, 505 $[M+H80]^+$. Peak **11** (Rt = 13.88 min) was (all-*E*)-β-apo-8'-carotenal, added to the extracts to check the recovery of carotenoids in the extraction process. Table 2 shows the spectrophotometric characteristics of this carotenoid.

Peak **12** (Rt = 14.79 min) was attributed to 5,6-epoxy-β-cryptoxanthin by spectroscopic characteristics, λ_{max} at 420, 445, 471 nm (%III/II = 52) and MS spectrum showing a major ion for the protonated molecule $[M+H]^+$ at m/z 569 and ion fragments, 551 $[M+H-H2O]^+$, 459 $[M+H-H2O-92]^+$.

The structural assignment of (all-*E*)-α-cryptoxanthin and (all-*E*)-β-cryptoxanthin for peak **13** (Rt = 15.30 min) and **14** (Rt = 17.79 min) respectively was based on the HPLC co-elution with an authentic commercial standard in the case of (all-E-)-β-cryptoxanthin and by published data for (all-*E*)-α-cryptoxanthin [35]. The

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UV-visible spectrum showed λ_{max} at 418, 442, 464 nm (%III/II = 61) for (all-*E*)-α-cryptoxanthin with a mass spectrum of and $[M+H]^+$ at m/z 553. Peak **14,** attributed to (all-*E*)-β-cryptoxanthin showed a λ_{max} at 426, 450, 476 nm (%III/II = 18), that indicated a chromophore consisted of nine double bonds and two β-rings. Mass spectrum was characterized also by a protonated molecule $[M+H]^+$ at m/z 553, corresponding to a molecule $C_{40}H_{56}O$ and a fragment of m/z 535 $[M+H-H2O]^+$ (loss of a water molecule) indicating the presence of an only hydroxyl group, and m/z 461 $[M+H-92]^+$, corresponding to the loss of toluene [35]. Also, injection of (all-*E*)-β-cryptoxanthin standard as well as the chromatogram of saponified extract (Figure 1, B) confirmed this identification.

3.1.2. Hydrocarbon carotenes

Peak **15** (Rt = 19.27 min) was assigned to 5,6-epoxy-α-carotene with λ_{max} at 418, 441, 469 nm (%III/II = 10) and a mass protonated molecule $[M+H]^+$ at m/z 553 and a fragment of m/z 535 $[M+H-H2O]^+$ corresponding to a loss of a water molecule.

Peaks **16** (Rt = 19.72 min) and **17** (Rt = 21.78 min) were assigned to a (13Z) isomers of α-carotene and β-carotene, showing both of them a UV-vis maximum at 337 nm corresponding to a cis structure. Spectroscopic characteristics, λ_{max} at (337), 413, 438, 466 nm (%III/II = 31) and λ_{max} at (337), 412, 441, 468 nm (%III/II = 14) were observed, with mass protonated molecule $[M+H]^+$ at m/z 537 in both compounds. Reverse-phase column facilitate the efficient separation of carotene isomers (Fig. 2). As the correspondent α- and β-carotene, the cis isomers showed similar ions, being the most characteristic but with low abundance, m/z 445 $[M+H-92]^+$ by elimination of neutral molecule of toluene, and m/z 457 $[M+H-80]^+$ by loss of methyl-cyclopentadiene.

Peaks **18** (Rt = 23.02 min) and **20** (Rt = 25.07 min) corresponded to the *trans* isomers of α-carotene and β-carotene respectively. Peak assignment was made by comparison with authentic standards. The UV-visible spectrum of (all-E)-α-carotene showed λ_{max} 422, 446, 474 nm (%III/II = 66) with a mass protonated molecule $[M+H]^+$ at m/z 537. Most of the fragment ions observed in the MS APCI (+) mass spectrum of (all-E-)-α-carotene were the same as those for (all-*E*)-β-carotene (e.g., m/z 137, m/z 413 and m/z 457), peak

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20. However, the most abundant fragment ion in the positive ion tandem mass spectrum of (all-*E*)-α-carotene, corresponding to the α-ionone moiety of m/z 123, was not observed in the APCI(+) mass spectrum of (all-*E*)-β-carotene. Formation of the ion of m/z 123 was facilitated by the position of the double bond in the terminal ring, which helped stabilize the resulting carbocation. Since this ion was not observed in the positive ion APCI tandem mass spectrum of β-carotene, γ-carotene, or lycopene, it may be used to distinguish α-carotene from these isomeric carotenes [35].

Peak **19** (Rt = 23.66 min) and peak **21** (Rt = 26.50 min) were assigned to (9Z) isomers of α-carotene and β-carotenes. The UV-visible spectrum of (9Z)-α-carotene showed λ_{max} 335, 421, 440, 469 nm (%III/II = 60) and (9Z)-β-carotene with λ_{max} 335, 421, 440, 469 nm (%III/II = 23), showing both the appearance of a "cis peak" at 335 nm. As the observed MS fragments of (all-*E*)-β-carotene, the MS spectrum of (9Z)-β-carotene showed similar ions, being the most characteristic but with low abundance, m/z 445 $[M+H-92]^+$ by elimination of neutral molecule of toluene, and m/z 457 $[M+H-80]^+$ by loss of methyl-cyclopentadiene. The observed elution order of carotene isomers followed the reported order of (13Z) isomers, (all-E) isomers and last the (9Z) isomers [36], except for (9Z)-α-carotene (Rt = 26.50 min) which eluted before (all-*E*)-β-carotene (Rt = 25.07 min) in persimmon extracts in the present study.

Finally, peak **39** (Rt = 44.16 min) was assigned to (all-*E*)-lycopene with m/z 537, and a characteristic λ_{max} at 446, 472, 502 nm (%III/II = 6), collaborated by an authentic standard. MS/MS fragments were 457 $[M+H-80]^+$, 413 $[M+H-124]^+$, 177 $[M+H-360]^+$, 137 $[M+H-400]^+$ and 121 $[M+H-416]^+$. No lycopene isomers were found in persimmon extracts in the conditions employed in this study for carotenoid extraction and HPLC analysis.

3.1.3. Xanthophyll esters

In order to identify the nature of each xanthophyll esters from persimmon tissues, a combined information from chromatographic characteristics as elution order, UV/vis spectra (maxima absorption wavelengths (λ_{max}), spectral fine structure (%III/II) and peak cis intensity (%A_B/A_{II}), ad mass spectrum (molecular weight

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and APCI (+) MS fragmentation pattern)) were employed, using the literature references and the available data of these compounds [37].

In the persimmon extracts xanthophyll esters were more retained that hydrocarbon carotenoids (*all*-E)-α- and β-carotene and their isomers (9Z) and (13Z). Mercadante et al. (2017) [37] reported that reliable identification of carotenoid esters based only in the elution order or retention time is not possible and can lead wrong assignments due to co-elution or poor baseline separation. In the present study, no problems were found to assign hydrocarbon carotenoids as showed Fig. 1, regarding elution order of each chemical class of carotenoids (free xanthophylls, hydrocarbon carotenoids, xanthophylls esters and finally, lycopene) in crude (non saponified) and saponified persimmon extracts. Good separation between carotenoids was obtained by reverse-phase C30 column and the chromatographic conditions employed (see Materials & Methods section).

Persimmon carotenoid and carotenoid ester composition was characterized by the presence of xanthophylls in free form and in totally esterified form, in contrast with the carotenoid composition of other fruits as citrus fruits which have the presence of free, partially and totally esterified carotenoids [32]. In persimmon extracts was not necessary to make an additional two-step cleanup procedure to eliminate potential interferences as proposed by Rodrigues et al. (2016) [29].

From time 26.50 min when (9Z)-β-carotene was eluted, the xanthophyll esters began to appear starting by the violaxanthin esters. Violaxanthin esters have an in-source fragmentation pattern previously described by different authors. The detection of a protonated molecule $[M+H]^+$ and losses of water, the FAs (fatty acids) or both together as described different authors [32, 34]. Peak **22** (Rt = 28.31 min), peak **23** (Rt = 28.75 min), and peak **25** (Rt = 29.76 min), were assigned to violaxanthin esters as follow peak **22** (all-*E*)-violaxanthin laurate, peak **23** (all-*E*)-violaxanthin butyrate and peak **25** (all-*E*)-violaxanthin palmitate. The MS/MS spectra showed that these three compounds were xanthophyll monoesters of violaxanthin, with a neutral loss of one and two molecules of water [M+H-18]+ and $[M+H-18-18]^+$, respectively. And also, the corresponding loss of the FA´s, as m/z 583 $[M+H-12:0]^+$ lauric acid, $[M+H-4:0]^+$ butyric acid and $[M+H-16:0]^+$ palmitic acid, and the loss of the corresponding FA´s and water, m/z 565. Loss of a C_7H_8 fragment

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$[M+H-92]^+$ was also observed in the MS/MS spectra of these esters, and $[M+H-92-18]^+$, which are usually found in the xanthophyll esters.

Peak **24** (Rt = 29.00 min), was assigned to (all-*E*)-neoxanthin dibutyrate, and peak **26** (9Z)- neoxanthin dibutyrate (Rt = 30.79 min), both showing a m/z 741. MS/MS fragments were identical in the two isomers of neoxanthin dibutyrate, m/z 565 $[M+H-4:0-4:0]^+$ (loss of two butyric acid fragments), 547 $[M+H-4:0-4:0-18]^+$ (loss of two butyric acid fragments and one water molecule). UV-vis spectrum of (9Z) isomer showed the presence of λ_{max} 327 nm, and % A_B/A_{II} values were 0 and 16, respectively (Table 2). In the present work, an asymmetric neoxanthin was detected in contrast to the reported results for mango and citrus fruits, where no perceptible differences in regioisomeric forms of this carotenoid were observed in the conditions applied for analysis by Petri and Mercadante (2016) [32].

Peak **27** (Rt = 31.40 min) and peak **33** (Rt = 35.16 min) were identified as esters of zeaxanthin, (all-*E*)-zeaxanthin palmitate and (all-*E*)-zeaxanthin myristate, respectively. Peak **27** showed a m/z 807 $[M+H]^+$ and MS/MS fragments 551 $[M+H-16:0]^+$, 789 [M+H-18]+, 715 [M+H-92]+, which were in accordance with the proposed identification. Peak **33**, assigned to (all-E)-zeaxanthin myristate also was possible taking into consideration the a m/z 779 $[M+H]^+$, and the corresponding MS/MS fragments 551 $[M+H-14:0]^+$ (loss of myristic acid), 761 $[M+H-18]^+$ (loss of a neutral water molecule), 687 $[M+H-92]^+$ (loss of C_7H_8, ie.toluene), 533 $[M+H-18-14:0]^+$. No homodiesters or heterodiesters were found in persimmon extracts from different tissues (Figure 1, and Figure 1S and 2S, Supplementary material).

Peaks **28** (Rt = 32.72 min), **32** (Rt = 34.62 min), **34** (Rt = 35.81 min) were identified as antheraxanthin esters. Peak **28** (all-*E*)- antheraxanthin laurate-myristate with m/z 977 $[M+H]^+$, and MS/MS fragments 777 $[M+H-12:0]^+$, 749 $[M+H-14:0]^+$, indicating losses of lauric and myristic acids, respectively. Peak **32** was assigned to (all-E)-antheraxanthin 3-O-palmitate with m/z 823 $[M+H]^+$, and MS/MS fragments 567 $[M+H-16:0]^+$, and losses of water molecules as fragments 549 $[M+H-16:0-18]^+$, 531 $[M+H-16:0-18-18]^+$, or direct loss of one neutral water molecule as m/z 805 $[M+H-18]^+$ or two 787 $[M+H-18-18]^+$. Peak **34** was assigned to (all-*E*)-antheraxanthin myristate-palmitate with m/z 1033 $[M+H]^+$, and MS/MS fragments 805 [M+H-

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14:0]$^+$, 771 [M+H-16:0]$^+$, indicating losses of myristic and palmitic acids, respectively. As the above identified xanthophylls esters, loss of water molecules and loss of C_7H_8 with m/z 941 [M+H-92]$^+$.

Peaks **29** (Rt = 32.36 min), **31** (Rt = 33.74 min), and **37** (Rt = 38.07 min) were identified as lutein esters, (all-*E*)-lutein dimyristate, (all-*E*)-lutein 3-O-palmitate and (all-*E*)-lutein 3-O-laurate-3´-O-myristate. In the case of lutein which has a β- and a ε- rings make easily to unequivocally assign the fatty acid (FA) positions in the molecule in APCI (+)-MS study. Published data for other carotenoid profiles of fruits as mango or citrus fruits reported that in these lutein esters the most abundant in-source fragment ion from neutral loss of the corresponding moiety (hydroxyl group or FA) was in position 3´of lutein [32]. As reported these authors, in persimmon extracts with the conditions employed in the present study, [M+H]$^+$ of all peaks related to lutein was not detected. For their identification the information of MS/MS fragments ions in the compound of peak **29** 761 [M+H-14:0]$^+$ and 533 [M+H-14: 14:0]$^+$ indicated the loss of one or two molecules of myristic acid, and the corresponding UV-vis spectral data λ_{max} 422,446,474 is in accordance with free (all-*E*)-lutein (Table 2). Similar results were obtained regarding peak **31** (Rt = 33.74 min) assigned to (all-*E*)-lutein 3-O-palmitate, with MS/MS ion fragment 533 [M+H-18-16:0]$^+$ (loss of palmitic acid and one water molecule) among others. The third lutein ester found in persimmon tissues was peak **37** assigned to (all-*E*)-lutein 3-O-laurate-3´-O-myristate. The fragmentation pattern of this compound (peak **37**) showed m/z ions 761 [M+H-12:0]$^+$ (loss of lauric acid) , 733 [M+H-14:0]$^+$ (loss of myristic acid), 769 [M+H-92]$^+$ (loss of C_7H_8, ie.toluene) and 533 [M+H-12:0-14:0]$^+$ (loss of lauric and myristic acids).

The last xanthophyll esters identified in persimmon extracts were four β-cryptoxanthin esters, peaks **30** (Rt = 32.89 min), **35** (Rt = 36.28 min), **36** (Rt = 37.38 min) and **38** (Rt = 39.18 min). Peak **30** (Rt = 32.89 min) was assigned to (all-*E*)- β-cryptoxanthin laurate with a m/z 735 [M+H]$^+$, and MS/MS fragment of 535 [M+H-12:0]$^+$ corresponding to the loss of lauric acid (12:0, 200 u) and usual fragments at m/z 643 [M+H-92]$^+$ and 443 [M+H-92-12:0]$^+$. Peak **35** (Rt = 36.28 min) was identified as (all-*E*)-β-cryptoxanthin myristate with m/z 763 [M+H]$^+$ and MS/MS fragments 671 [M+H-92]$^+$ attributed to a loss of C_7H_8 (ie.toluene), 535 [M+H-14:0]$^+$ loss of myristic acid (14:0, 228 u) and 443 [M+H-14:0-92]$^+$ losses of fatty acid and C_7H_8 together (Table 2). Peak **36** (Rt = 37.38 min) was assigned to (all-*E*)-β-cryptoxanthin dipalmitate with m/z

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1046 $[M+H]^+$ and MS/MS fragments 790 $[M+H-18-16:0]^+$ corresponding to a loss of one molecule of water and one molecule of palmitic acid (16:0, 256 u), 516 $[M+H-18-16:0-16:0]^+$ loss of two molecules of palmitic acid (16:0, 256 u) and one molecule of water, 936 $[M+H-92-18]^+$ and 954 $[M+H-92]^+$, fragments usually found in other xanthophyll esters. Finally, peak **38** (Rt = 39.18 min) was identified as (all-*E*)-β-cryptoxanthin palmitate-stearate with a m/z 1058 $[M+H]^+$ and MS/MS fragments 801 $[M+H-16:0]^+$ loss of one molecule of palmitic acid (16:0, 256 u), 773 $[M+H-18:0]^+$ loss of a molecule of stearic acid (18:0, 284 u), 517 $[M+H-16:0-18:0]^+$ loss of the two fatty acids. As reported Mercadante el al. (2017) [37] diesters of dihydroxy and esters of monohydroxy carotenoids, such as β-cryptoxanthin an elimination of fatty acids (FA´s) were not usually accompanied by loss of water. However, in the present study the MS/MS spectrum of peak **36** (all-*E*)-β-cryptoxanthin dipalmitate) showed fragments with the loss of water together with the loss of one or two molecules of fatty acid.

Peaks **1**, **2**, **5**, **6**, **12**, **13**, **15** and **16** were only present in saponified extracts (Figure 1 B, and Figs. 1SB and 2SB) due to different facts. Peaks **1** ((13Z)-violaxanthin), **5** ((9Z)-violaxanthin), **6** ((9Z)-neoxanthin) and **16** ((13Z)-α-carotene) produced by isomerization during the saponification process. Peaks **2** ((all-*E*)-luteoxanthin), **12** (5,6-epoxy- β-criptoxanthin) and **15** (5,6-epoxy- α-carotene) appeared for oxidation and finally, peak **13** ((all-*E*)-α-cryptoxanthin) that was not identified in crude (no saponified) extracts possibly due to its very low concentration.

Previous studies about the carotenoid and carotenoid esters composition of persimmon peel [15] reported the presence of β-carotene, lycopene, β-cryptoxanthin mono-myristic acid ester, zeaxanthin di-myristic acid ester, using silica gel chromatography followed by structural characterization by ^{1}H-NRM and ^{13}C-NMR. Other study described the carotenoids and carotenoid esters in persimmon edible pulp [38], using a reverse phase column C18, identifying β-carotene, lycopene, β-cryptoxanthin myristate, β-cryptoxanthin laurate, zeaxanthin laurate-myristate, zeaxanthin dimyristate, zeaxanthin myristate-palmitate, antheraxanthin dimyristate and antheraxanthin myristate-palmitate and an unidentified ester. In the present study, carotenoid esters found in different tissues of astringent persimmon tissues were accord with these studies, but a higher

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number of carotenoid esters separated by C30 reverse phase column was identified, being 19 all of them in (all-*E*) form and esterified mainly by butyric, lauric, myristic, palmitic and stearic acids.

These differences could be attributed to the fruit variety studied which is an astringent one, the agronomic conditions and the fruit maturity stage (Table 1).

3.2. Carotenoid composition in Spanish astringent persimmon fruits (*Diospyrus kaki* Thunb., var. Rojo Brillante)

The quantitation of carotenoids in the mature Spanish persimmon var. Rojo Brillante tissues are showed in Table 3. Also in this table, the carotenoid and carotenoid esters composition of pressurized and pasteurized persimmon tissues are included. Total carotenoid content (the sum of total free xanthophyls + total hydrocarbon carotenoids + total xanthophyll esters) was greater in peel extracts in all samples, being 11610.1±580.5, 12040.5±602.0 and 12676.7±633.8 µg/100 g fresh weight for control, pasteurized and pressurized peel samples. This higher concentration of carotenoids in the peel comes as no surprise since carotenoids play an important role in attracting animals so they can act as pollinators and seed dispersion vehicles [39]. Total carotenoid content in fruit pulp ranged 2183.3±110.3 (control tissue) to 2058.4±65.7 µg/100 g (pasteurized pulp) fresh weight, while pressurized pulp has 22180.3±155.4 µg/100 g fresh weight. No significant differences were observed between total carotenoids in persimmon peels and pulps non saponified (direct analysis) extracts due to the assayed treatments. Related to whole fruit persimmon samples, the higher total carotenoid content was found in control samples (4164.8±208.2 µg/100 g fresh weight), being significantly different ($p \leq 0.05$) than pasteurized and pressurized whole fruit tissues (3117.6±155.9 and 3237.9±39.5 µg/100 g fresh weight, respectively).

Persimmon peel extracts have 3.8 and 6.0-fold more total free xanthophylls than whole fruit or pulp extracts, respectively (Table 3). The content of individual free xanthophyll in all persimmon tissues (non saponified extracts, direct analysis) was in the following order: (all-*E*)-lutein ≥ (all-*E*)-zeaxanthin ≥ (all-*E*)-β-cryptoxanthin ≥(all-*E*)-antheraxanthin. Other minor abundant free xanthophyll was found in persimmon

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Table 3. Carotenoid content (µg/100g fresh weight) ± standard deviation and retinol activity equivalents (RAE) of direct extracts of astringent persimmon (*Diospyros kaki* Thunb. var. Rojo brillante) submitted to pasteurization (85°C, 15 min) and high-pressure processing (HPP; 200 MPa, 25°C, 6 min).

			Carotenoid content* (µg/100 g fresh weight)								
			Whole Fruit			Pulp			Peel		
	No	Compound	Control	Pasteurization	HPP	Control	Pasteurization	HPP	Control	Pasteurization	HPP
Free xanthophylls	1	(13Z)-violaxanthin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	3	(all-*E*)-violaxanthin	tr.	tr.	tr.	tr.	tr.	tr.	34.8±1.7^{a}	21.1±1.1^{b}	25.4±1.3^{b}
	4	(all-*E*)-neoxanthin	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.
	5	(9Z)-violaxanthin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	6	(9Z)-neoxanthin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	7	(all-*E*)-antheraxanthin	37.1±1.9^{c}	16.7±0.8^{a}	24.0±2.0^{b}	26.8±1.2^{b}	16.1±1.6^{a}	19.2±0.9^{a}	53.4±2.7^{b}	35.6±1.8^{a}	37.5±1.9^{a}
	8	(all-*E*)-lutein	41.5±1.9^{b}	30.2±1.5^{a}	41.7±2.4^{b}	n.d.	n.d.	n.d.	208.0±10.4^{b}	145.6±7.3^{a}	195.3±9.8^{b}
	9	(all-*E*)-zeaxanthin	24.1±1.2^{a}	35.6±1.8^{a}	59.5±7.1^{b}	28.6±3.7^{a}	27.3±2.6^{a}	23.6±4.9^{a}	150.1±7.5^{ab}	127.3±6.4^{a}	168.8±8.4^{b}
	10	Lutein-5,6- epoxide	tr.	tr.	tr.	n.d.	n.d.	n.d.	tr.	tr.	tr.
	12	5,6-epoxy-β-cryptoxanthin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	13	(all-*E*)-α-cryptoxanthin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	14	(all-*E*)-β-cryptoxanthin	35.9±1.8^{a}	34.8±1.7^{a}	41.2±0.2^{b}	30.6±1.5^{ab}	27.1±1.5^{a}	34.9±1.4^{b}	81.3±4.1^{ab}	89.7±4.5^{a}	97.7±4.9^{b}
Hydrocarbon carotenoids[1]	15	5,6-epoxy-α-carotene	24.9±1.2^{b}	18.4±0.9^{a}	25.7±1.8^{b}	12.5±0.9^{ab}	11.3±0.8^{a}	15.9±1.1^{b}	28.4±1.4^{a}	59.9±3.0^{b}	69.4±3.5^{b}
	16	(13Z)-α-carotene	21.3±1.1^{a}	14.5±0.7^{a}	20.9±3.8^{a}	14.8±1.0^{a}	11.7±0.8^{a}	12.8±0.9^{a}	20.8±1.0^{a}	23.6±1.2^{a}	31.3±1.6^{b}
	17	(13Z)-β-carotene	12.2±0.6^{a}	23.5±1.2^{b}	9.0±1.7^{a}	10.4±1.4^{a}	7.7±1.2^{a}	9.7±1.5^{a}	44.9±2.2^{c}	34.7±1.7^{a}	26.8±1.3^{b}
	18	(all-E)-α-carotene	56.9±2.8^{b}	41.7±2.1^{a}	46.7±0.6^{a}	13.5±1.0^{b}	15.5±1.9^{b}	7.6±0.0^{a}	301.7±15.1^{b}	231.9±11.6^{a}	273.5±13.7^{ab}
	19	(9Z)-α-carotene	32.0±1.6^{b}	23.5±1.2^{a}	39.7±1.0^{c}	19.3±2.2^{a}	14.1±3.9^{a}	21.2±0.5^{a}	64.3±3.2^{b}	32.9±1.6^{a}	69.4±3.5^{b}
	20	(all-*E*)-β-carotene	178.6±8.9^{a}	189.6±9.5^{a}	197.1±1.5^{a}	138.1±7.0^{a}	146.1±8.5^{a}	123.1±5.5^{a}	697.6±34.9^{a}	582.6±29.1^{a}	607.1±30.4^{a}
	21	(9Z)-β-carotene	29.2±1.5^{b}	30.1±1.5^{b}	23.0±0.6^{a}	20.8±2.5^{a}	18.0±2.5^{a}	21.5±2.6^{a}	216.1±10.8^{b}	126.4±6.3^{a}	151.8±7.6^{a}
Xantophyll esters	22	(all-*E*)-violaxanthin laurate	37.1±1.9^{b}	28.6±1.4^{a}	23.0±1.3^{a}	18.1±1.8^{a}	17.3±1.2^{a}	25.4±1.5^{b}	104.5±5.2^{b}	72.5±3.6^{a}	107.9±5.4^{b}
	23	(all-*E*)-violaxanthin butyrate	45.8±2.3^{a}	38.1±1.9^{a}	34.2±8.2^{a}	40.4±6.1^{a}	31.7±4.7^{a}	37.7±5.7^{a}	217.1±10.9^{b}	157.3±7.9^{a}	199.0±10.0^{b}
	24	(all-*E*)-neoxanthin dibutyrate	90.0±4.5^{c}	71.7±3.6^{b}	35.5±3.3^{a}	45.3±2.7^{a}	35.8±4.2^{a}	38.5±1.1^{a}	404.9±20.2^{b}	299.5±15.0^{a}	358.1±17.9^{ab}
	25	(all-*E*)-violaxanthin palmitate	120.0±6.0^{c}	95.2±4.8^{b}	68.2±0.9^{a}	55.8±3.3^{a}	63.6±4.2^{a}	64.4±2.4^{a}	685.0±34.3^{b}	431.6±21.6^{a}	479.6±24.0^{a}
	26	(9Z) neoxanthin dibutyrate	133.5±6.7^{b}	105.9±5.3^{a}	92.0±3.6^{a}	33.6±2.0^{a}	20.3±3.7^{a}	27.7±4.2^{a}	404.9±15.2^{a}	362.9±18.1^{a}	370.3±18.5^{a}
	27	(all-*E*)-zeaxanthin palmitate	489.9±12.2^{a}	435.1±21.8^{a}	425.4±11.6^{a}	439.6±11.2^{a}	404.4±24.7^{a}	410.1±20.1^{a}	1343.2±67.2^{a}	1169.8±58.5^{a}	1143.4±57.2^{a}
	28	(all-*E*)-antheraxanthin laurate-myristate	81.8±4.1^{b}	47.6±2.4^{a}	47.9±6.4^{a}	20.9±2.4^{a}	37.3±0.2^{a}	34.4±9.3^{a}	218.3±10.9^{a}	221.4±11.1^{a}	224.2±11.2^{a}
	29	(all-*E*)-lutein dimyristate	141.8±7.1^{c}	103.8±5.2^{b}	80.3±4.1^{a}	81.8±3.8^{b}	52.9±5.0^{a}	80.8±10.2^{b}	318.5±15.9^{a}	264.4±13.2^{a}	271.9±13.6^{a}
	30	(all-*E*)-β-cryptoxanthin laurate	424.3±21.2^{a}	387.8±19.4^{a}	400.0±3.0^{a}	328.5±7.2^{a}	349.9±6.7^{a}	362.9±22.0^{a}	889.6±44.5^{a}	1099.1±55.0^{a}	1070.9±53.5^{a}
	31	(all-*E*)-lutein 3-O-palmitate	662.3±33.1^{b}	405.7±20.3^{a}	433.0±8.9^{a}	340.6±11.5^{b}	244.5±25.3^{a}	331.2±20.8^{b}	1415.8±70.8^{a}	1603.6±80.2^{a}	1665.6±83.3^{a}
	32	(all-*E*)-antheraxanthin 3-O-palmitate	102.5±5.1^{b}	59.5±3.0^{a}	62.9±6.5^{a}	30.7±5.6^{a}	37.9±5.9^{a}	28.0±16.6^{a}	272.9±13.6^{a}	317.8±15.9^{ab}	347.7±17.4^{b}
	33	(all-*E*)-zeaxanthin myristate	505.3±25.3^{b}	352.6±17.6^{a}	413.9±4.6^{a}	244.0±12.0^{a}	276.6±1.4^{a}	267.1±46.6^{a}	1105.3±55.3^{a}	1381.9±69.1^{b}	1477.9±73.9^{b}
	34	(all-*E*)-antheraxanthin myristate-palmitate	279.2±14.0^{b}	190.4±9.5^{a}	217.1±6.8^{a}	65.6±2.8^{a}	56.4±2.3^{a}	49.2±8.9^{a}	686.2±34.3^{a}	991.7±49.6^{b}	1100.8±55.0^{b}
	35	(all-E)-β-cryptoxanthin myristate	95.1±4.8^{c}	53.4±2.7^{a}	80.5±2.6^{b}	n.d.	n.d.	n.d.	178.9±8.9^{a}	242.2±12.1^{b}	267.4±13.4^{b}
	36	(all-*E*)-β-cryptoxanthin dipalmitate	160.1±8.0^{b}	85.8±4.3^{a}	142.0±7.8^{b}	53.9±5.0^{a}	56.2±0.2^{a}	67.6±9.9^{a}	255.9±12.8^{a}	447.1±22.4^{b}	415.2±20.8^{b}
	37	(all-*E*)-lutein 3-O-laurate-3'-O-myristate	95.1±4.8^{b}	47.2±2.4^{a}	45.8±12.3^{a}	19.9±3.0^{a}	32.0±4.8^{a}	24.7±3.7^{a}	151.0±7.5^{a}	169.1±8.5^{a}	178.7±8.9^{a}
	38	(all-*E*)-β-cryptoxanthin palmitate-stearate	31.2±1.6^{b}	19.3±1.0^{a}	30.0±0.7^{b}	11.7±0.5^{a}	9.6±0.9^{a}	13.9±0.1^{a}	49.1±2.5^{a}	82.7±4.1^{b}	90.2±4.5^{b}
Hydrocarbon carotenoids[2]	39	lycopene	176.1±8.8^{b}	131.3±6.6^{b}	77.6±21.5^{a}	37.5±2.5^{a}	37.2±4.2^{a}	27.2±5.7^{a}	1007.7±50.4^{a}	1214.6±60.7^{a}	1158.5±57.9^{a}
		Total free xanthophylls[a]	138.5±6.9^{a}	117.3±5.9^{a}	166.5±24.0^{a}	86.0±4.9^{a}	70.6±2.6^{a}	77.7±7.2^{a}	527.6±26.4^{b}	419.3±21.0^{a}	520.1±26.0^{b}
		Total hydrocarbon carotenoids[a]	531.2±26.6^{a}	458.8±22.9^{a}	439.6±28.4^{a}	266.9±7.4^{a}	261.5±4.3^{a}	239.0±10.4^{a}	2381.6±119.1^{a}	2306.5±115.3^{a}	2387.6±119.4^{a}

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	Total xanthophyll esters[a]	3495.1±174.8[b]	2527.6±126.4[a]	2631.8±12.9[a]	1830.5±51.3[a]	1726.3±67.4[a]	1863.6±137.8[a]	8701.1±435.1[a]	9314.6±465.7[a]	9769.0±488.4[a]
	Total Carotenoids[b]	4164.8±208.2[b]	3117.6±155.9[a]	3237.9±39.5[a]	2183.3±110.5[a]	2058.4±65.7[a]	2180.3±155.4[a]	11610.3±580.5[a]	12040.5±602.0[a]	12676.7±633.8[a]
	Retinol Activity Equivalents[c]	110.2±5.5[a]	96.0±4.8[a]	107.1±2.9[a]	68.6±3.4[a]	69.9±3.0[a]	70.4±4.5[a]	315.6±15.8[a]	316.4±15.8[a]	329.7±16.5 [a]

*Values are the mean of two independent determinations ± standard deviation. Lowercase letters indicate statistically significant differences ($p<0.05$) between treatments for the same tissue.

[1]Hydrocarbon carotenoids part 1.

[2]Hydrocarbon carotenoids part 2.

[a]Represents the algebraic sum of the identified free xanthophylls, hydrocarbon carotenoids and xanthophyll esters, respectively.

[b]Represents the algebraic sum of the identified carotenoids in each sample.

[c]Calculated according to the guidelines of the US Institute of Medicine (2001).

n.d. not detected

tr. traces

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peels was (all-*E*)-violaxanthin, with a content of 34.8±1.7, 21.1±1.1 and 25.4±1.3 µg/100 g fresh weight, for control, pasteurized and pressurized peel extracts.

Total hydrocarbon carotenoids content ranged between 2381.6±119 µg/100 g fresh weight in peel extracts to 266.9±7.4 µg/100 g fresh weight in pulp extracts, being (all-*E*)-β-carotene the most abundant hydrocarbon carotenoid, excluding lycopene, whose content in the different samples is discussed below.

Persimmon fruits are very rich in lycopene (Table 3). As other carotenoids, the persimmon peel is the tissue with the highest content of this carotenoid, control peel samples have 1007.7±50.4 µg/100 g fresh weight, followed by whole fruit samples 176.1±8.8 µg/100 g fresh weight and pulp samples 77.6±21.5 µg/100 g fresh weight. Both treatments, pasteurization and pressurization, did not produced any significant differences ($p \leq 0.05$) in the lycopene content in all tissues, except for whole fruit samples when treatments reduced the lycopene content a 25% in pasteurized and 56% pressurized samples, probably due to a degradation reactions by heat (pasteurization) and by a high enzymatic actions produced by high pressures [16].

In addition, persimmon fruits showed high amounts of xanthophyll esters. Table 3 shows the content of individual content of each xanthophyll ester in peel, pulp and whole fruit extracts and the effects of pasteurization and pressurization treatments on in each individual compound. Again, peel persimmon tissue has the higher amount of total xanthophyll esters (8701.1 ± 431.1 µg/100 g fresh weight) followed by whole fruit (3495.1 ±174.8 µg/100 g fresh weight) and pulp tissues (1830.5 ± 51.3 µg/100 g fresh weight). Processing by high pressures or pasteurization only affects the total xanthophyll esters in whole fruit tissue, where a 26% loss was found. The most abundant xanthophyll esters in Spanish persimmon var. Rojo Brillante whole fruits extracts are in following order: (all-*E*)-lutein-3-O-palmitate (662.3 ± 33.1 µg/100 g fresh weight) ≥ (all-*E*)-zeaxanthin myristate (505.3 ± 25.3 µg/100 g fresh weight) ≥ (all-*E*)-zeaxanthin palmitate (489.9 ± 12.2 µg/100 g fresh weight) ≥ (all-*E*)-criptoxanthin laurate (424.3 ± 21.2 µg/100 g fresh weight). In the case of persimmon pulp the most abundant xanthophyll ester is (all-*E*)-zeaxanthin palmitate (439.6 ± 11.2 µg/100 g fresh weight) followed by (all-*E*)-lutein-3-O-palmitate (340.6 ± 11.5 µg/100 g fresh weight) and (all-*E*)-criptoxanthin laurate (328.5 ± 7.2 µg/100 g fresh weight). In contrast, persimmon peel

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shows the greatest content of (all-*E*)-lutein-3-O-palmitate (1415.8 ± 70.8 µg/100 g fresh weight) and lower amounts of (all-*E*)-zeaxanthin palmitate (1342.2 ± 67.2 µg/100 g fresh weight), (all-*E*)-zeaxanthin myristate (1105.3 ± 55.3 µg/100 g fresh weight) and (all-*E*)-cryptoxanthin laurate (889.6 ± 44.5 µg/100 g fresh weight).

Philip and Chen (1998) [38] studied the quantitative analyses of major carotenoid fatty acid esters in persimmon from California by liquid chromatography. This paper described that the major carotenoid compounds in mature edible persimmon tissue were cryptoxanthin myristate (1404 µg/100 g fresh weight), zeaxanthin dimyristate (576 µg/100 g fresh weight), β-carotene (266 µg/100 g fresh weight), cryptoxanthin (144 µg/100 g fresh weight), zeaxanthin myristate-palmitate (132 µg/100 g fresh weight) and antheraxanthin dimyristate (114 µg/100 g fresh weight). In comparison Spanish astringent persimmon var. Rojo Brillante pulp (edible tissue) showed greater amounts in carotenoids esters that those reported by Philip and Chen (1998) [38], but lower content of β-carotene and lycopene.

Most recent papers about persimmon composition or persimmon derived products, showed only the content of carotenoids from saponified extracts. Total carotenoid content in persimmon flours from vars. Rojo Brillante and Triumph analyzed by spectrophotometric method [40] ranged between 1600 to 1920 µg/g fresh weight), which is a greater content that in fresh fruit due to the dehydration process. Giordani et al. (2011) [11] also reported the carotenoid content from saponified extracts of different astringent and no astringent varieties *Diospyrus kaki* Tumb. In this study, β-criptoxanthin (193 µg/100 g fresh weight), β,β-carotene (all-*E*) (113 µg/100 g fresh weight) and β,ε-carotene (30 µg/100 g fresh weight) were reported.

Processing by high pressures produced no regular effect on the individual carotenoid and carotenoid esters (xanthophyll esters), but attending to the sum of all of them, high pressures only affect the total xanthophyll esters when the whole fruit samples were analyzed. However, pasteurization affects negatively the content of all carotenoid and carotenoid esters in all persimmon tissues.

If the carotenoids of persimmon tissues were analyzed from saponified extracts (Table 4), no significant differences ($p \leq 0.05$) can be observed between control and treated samples for both total free xanthopylls

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Table 4. Carotenoid content (µg/100g fresh weight) ± standard deviation and retinol activity equivalents (RAE) as determined after saponification of extracts of astringent persimmon (*Diospyros kaki* Thunb. var. Rojo brillante) submitted to pasteurization (85°C, 15 min) and high-pressure processing (HPP; 200 MPa, 25°C, 6 min).

			Carotenoid content* (µg/100 g fresh weight)								
			Whole Fruit			Pulp			Peel		
	No	Compound	Control	Pasteurization	HPP	Control	Pasteurization	HPP	Control	Pasteurization	HPP
Free xanthophylls	1	(13Z)-violaxanthin	96.2±4.8^{b}	79.7±3.2^{a}	69.2±3.0^{a}	54.0±2.7^{a}	62.9±3.1^{a}	62.4±3.1^{a}	205.8±8.1^{a}	211.3±14.8^{a}	203.9±16.3^{a}
	3	(all-*E*)-violaxanthin	251.9±12.6^{b}	202.8±8.1^{a}	227.1±13.8^{ab}	107.9±5.4^{a}	98.8±4.9^{a}	115.3±0.2^{a}	908.5±36.3^{a}	846.0±59.2^{a}	763.4±61.1^{a}
	4	(all-*E*)-neoxanthin	58.4±2.9^{b}	49.3±2.0^{a}	51.6±0.8^{ab}	13.2±0.7^{a}	44.8±2.2^{b}	17.8±1.6^{a}	319.7±5.5^{b}	299.1±20.9^{ab}	238.6±19.1^{a}
	5	(9Z)-violaxanthin	54.2±2.7^{b}	33.4±1.3^{a}	55.7±2.8^{b}	28.4±1.4^{a}	39.3±2.0^{b}	36.0±2.3^{b}	193.5±4.0^{a}	192.7±13.5^{a}	184.9±14.8^{a}
	6	(9Z)-neoxanthin	20.5±1.0^{a}	19.8±0.8^{a}	24.5±6.8^{a}	17.6±0.9^{a}	41.7±2.1^{b}	37.9±4.7^{b}	123.0±2.3^{a}	128.9±9.0^{a}	122.6±9.8^{a}
	7	(all-*E*)-antheraxanthin	628.4±31.4^{a}	536.4±21.5^{a}	594.2±7.4^{a}	376.8±18.8^{a}	401.0±20.0^{a}	534.3±21.4^{b}	1098.6±74.6^{ab}	974.8±68.2^{a}	1327.7±106.2^{b}
	8	(all-*E*)-lutein	758.1±37.9^{b}	569.8±22.8^{a}	675.7±14.3^{b}	375.3±17.0^{b}	124.7±6.2^{a}	139.0±2.4^{a}	3544.3±177.2^{a}	3350.1±234.5^{a}	3586.4±286.9^{a}
	9	(all-*E*)-zeaxanthin	776.8±38.8^{b}	604.6±24.2^{a}	783.7±26.1^{b}	375.9±19.1^{a}	300.9±15.0^{b}	300.0±18.0^{b}	2145.0±33.0^{a}	1912.3±134.0^{a}	1955.7±156.5^{a}
	10	Lutein-5,6- epoxide	268.2±13.4^{b}	219.8±8.8^{a}	252.0±0.1^{a}	168.9±8.4^{b}	114.0±5.7^{a}	173.2±8.3^{b}	589.4±6.4^{a}	558.3±39.1^{a}	553.7±44.3^{a}
	12	5,6-epoxy-β-cryptoxanthin	18.4±0.9^{a}	14.1±0.6^{a}	15.3±3.1^{a}	7.4±0.4^{a}	5.9±0.3^{a}	10.0±0.8^{b}	92.0±4.1^{a}	83.2±5.8^{a}	94.8±7.6^{a}
	13	(all-*E*)-α-cryptoxanthin	30.4±1.5^{a}	27.5±1.1^{a}	34.3±2.6^{a}	7.9±0.4^{a}	3.5±0.2^{a}	21.3±8.9^{a}	43.3±6.1^{a}	48.4±3.4^{a}	43.7±3.5^{a}
	14	(all-*E*)-β-cryptoxanthin	557.4±27.9^{a}	534.2±21.2^{a}	606.3±33.6^{a}	402.5±20.1^{b}	299.8±15.0^{a}	386.9±11.6^{b}	1229.8±36.9^{a}	1345.5±94.2^{a}	1135.6±90.8^{a}
Hydrocarbon carotenoids	15	5,6-epoxy-α-carotene	32.0±1.6^{a}	31.4±1.3^{a}	26.4±6.8^{a}	18.5±0.9^{b}	13.7±0.7^{a}	11.7±0.4^{a}	193.7±17.4^{a}	173.6±12.2^{a}	166.2±13.3^{a}
	16	(13Z)-α-carotene	30.9±1.5^{a}	25.1±1.0^{a}	25.5±4.6^{a}	15.4±0.8^{a}	13.7±0.8^{a}	21.5±0.6^{b}	127.2±6.4^{a}	130.6±9.1^{a}	109.6±8.8^{a}
	17	(13Z)-β-carotene	14.1±0.7^{a}	20.1±0.8^{b}	12.1±1.4^{a}	14.1±0.7^{b}	8.4±0.4^{a}	8.9±0.3^{a}	103.7±5.2^{b}	49.7±3.5^{a}	53.2±4.3^{a}
	18	(all-*E*)-α-carotene	38.6±1.9^{a}	38.7±1.5^{a}	43.9±0.6^{a}	19.3±1.0^{b}	13.7±0.7^{a}	10.8±1.4^{a}	255.3±27.6^{a}	221.9±15.5^{a}	218.0±17.4^{a}
	19	(9Z)-α-carotene	18.8±0.9^{b}	13.6±0.5^{a}	15.4±0.3^{a}	10.0±0.5^{b}	7.3±0.4^{a}	9.8±0.7^{b}	31.1±6.8^{a}	30.2±2.1^{a}	32.1±2.6^{a}
	20	(all-*E*)-β-carotene	161.3±8.1^{a}	169.1±6.8^{a}	167.4±18.3^{a}	97.3±4.9^{b}	76.1±3.8^{a}	104.6±4.0^{b}	509.9±35.2^{a}	490.5±34.3^{a}	471.4±37.7^{a}
	21	(9Z)-β-carotene	25.0±1.3^{b}	16.2±0.6^{a}	14.8±3.9^{a}	14.1±0.7^{b}	16.7±0.8^{b}	10.7±0.8^{a}	57.9±11.0^{a}	52.4±3.7^{a}	60.0±4.8^{a}
	39	lycopene	145.8±7.3^{b}	107.8±4.6^{a}	92.8±6.0^{a}	30.6±1.5^{b}	25.7±1.3^{a}	25.2±0.5^{a}	834.4±12.0^{a}	784.9±54.9^{a}	860.2±60.3^{a}
		Total free xanthophylls[1]	3518.8±175.9^{a}	2891.2±115.6^{a}	3389.6±138.2^{ab}	1935.5±96.8^{b}	1537.1±76.9^{a}	1834.1±91.7^{ab}	10493.2±20.5^{a}	9952.6±696.7^{a}	10211.1±816.9^{a}
		Total hydrocarbon carotenoids[1]	466.5±23.3^{a}	421.9±16.9^{a}	398.3±2.5^{a}	219.3±11.0^{a}	175.4±8.8^{a}	203.2±39.4^{a}	2113.3±84.5^{a}	1933.9±135.4^{a}	1970.8±157.7^{a}
		Total Carotenoids[2]	3985.3±199.3^{b}	3313.1±132.5^{a}	3787.9±135.6^{ab}	2154.8±107.7^{b}	1712.5±85.6^{a}	2037.3±81.5^{ab}	12606.5±504.3^{a}	11886.5±832.1^{a}	12181.9±974.6^{a}
		Retinol Activity Equivalents[3]	92.4±4.6^{a}	90.1±3.6^{a}	95.03±0.58^{a}	60.4±3.0^{b}	46.177±2.3^{a}	57.3±2.8^{b}	268.63±27.0^{a}	261.3±18.3^{a}	239.5±19.2^{a}

*Values are the mean of two independent determinations ± standard deviation. Lowercase letters indicate statistically significant differences ($p<0.05$) between treatments for the same tissue.

[1]Represents the algebraic sum of the identified free xanthophylls and hydrocarbon carotenoids, respectively.

[2]Represents the algebraic sum of the identified carotenoids in each sample.

[3]Calculated according to the guidelines of the US Institute of Medicine (2001).

n.d. not detected; tr. Traces

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and total hydrocarbon carotenoids. But a slight decrease in total carotenoids can be observed in persimmon pulp saponified extracts (~20%).

Supplementary material shows the sum of persimmon carotenoids (free and esterified) organized by carotenoid species and by percentage of contribution of each species to total carotenoids and this data when persimmon was submitted to high pressure and pasteurization (in Table S1). Persimmon var. Rojo Brillante peel contents the higher amounts of carotenoids and again, zeaxanthin carotenoids (22.4%) are the most abundant, followed by lutein (18.0%), β-cryptoxanthin (12.5%) and antheraxanthin (10.6%) carotenoids. Hydrocarbon carotenoids content only reach 8.7% for lycopene and 8.3% for β-carotene.

When these data were studied in saponified persimmon extracts from pulp tissue (Table S2), lutein carotenoids were the most abundant (25.2 %), followed by β-cryptoxanthin (19.0 %), antheraxanthin (17.5 %) and zeaxanthin (17.4 %) ones.

Figure S3, in Supplementary material, shows the graphic distribution of the carotenoid proportion (%) of free xanthophylls, hydrocarbon carotenoids and xanthophyll esters (A) in direct extracts (crude) and (B) in saponified extracts; and distribution of carotenoid species (C) in direct extracts (crude) and (D) in saponified extracts of astringent persimmon (*Diospyros Kaki*, L.) cv. Rojo Brillante submitted to pasteurization (85°C, 15 min) and high-pressure processing (HPP; 200 MPa, 25°C, 6 min). This figure facilitates the immediate information of the composition in carotenoid and carotenoid esters in astringent persimmon tissues var. Rojo Brillante studied in the present work.

4. Conclusions

Spanish astringent persimmon fruits (*Diospyrus kaki* Tumb. var. Rojo Brillante) are an interesting source of carotenoid and carotenoid esters. For the first time, 38 carotenoids were identified in different persimmon tissues, being 21 free carotenoids (13 xanthophylls and 8 hydrocarbon carotenes) and a total of 18 carotenoid esters. The qualitative profiles of carotenoids and their carotenoid esters in different tissues (whole fruit, peel and pulp) were very similar, differing only in their individual concentration. The most important identified free xanthophylls were (all-*E*)-β-cryptoxanthin, (all-*E*)-antheraxanthin, (all-*E*)-lutein,

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(all-*E*)-zeaxanthin, (all-*E*)-violaxanthin and small amounts of lutein-5,6-epoxide and (all-*E*)-neoxanthin. Hydrocarbon carotenoids found were (all-*E*)-β-carotene, (all-*E*)-α-carotene, (9Z)-β-carotene, (13Z)-β-carotene, (9Z)-α-carotene, and lycopene. In addition, the most abundant xanthophyll esters were (all-*E*)-lutein-3-O-palmitate, (all-*E*)-zeaxanthin myristate, (all-*E*)-zeaxanthin palmitate and (all-*E*)-cryptoxanthin laurate.

Processing by high pressures produced no regular effect on the individual carotenoid and carotenoid esters (xanthophyll esters), but attending to the sum of all of them, high pressures only affect the total xanthophyll esters when the whole fruit samples were analyzed. However, pasteurization affects negatively the content of all carotenoid and carotenoid esters in all persimmon tissues.

Additional studies must be carried out to investigate the bioaccessibility of persimmon carotenoid and carotenoids esters to better know their potential use as source of functional ingredients.

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Figure captions

Table 1. Physico-chemical characteristics of Spanish astringent persimmon (Diospyros kaki Thunb. var. Rojo brillante)

Table 2. HPLC retention times, UV/Vis spectra and MS spectral data of carotenoids from whole fruit, peel and pulp of astringent persimmon (*Diospyros kaki* Thunb. var. Rojo brillante)

Table 3. Content of carotenoids (µg/100g fresh weight) and retinol activity equivalents (RAE) of direct extracts of astringent persimmon (*Diospyros kaki* Thunb. var. Rojo brillante) submitted to pasteurization (85°C, 15 min) and high-pressure processing (HPP; 200 MPa, 25°C, 6 min)

Table 4. Content of carotenoids (µg/100g fresh weight) and retinol activity equivalents (RAE) as determined after saponification of extracts of astringent persimmon (*Diospyros kaki* Thunb. var. Rojo brillante) submitted to pasteurization (85°C, 15 min) and high-pressure processing (HPP; 200 MPa, 25°C, 6 min)

Figure 1: C30 reversed-phase HPLC chromatogram obtained from direct (A) and saponified (B) carotenoid extracts from whole fruit tissue of Spanish astringent persimmon (*Diospyros kaki* Thunb. var. Rojo brillante). UV-vis detection at 450 nm. Peak identities in Table 2. (U) un-identified compounds.

Supplementary material

Table 1S. Sum of carotenoids (free and esterified) organized by carotenoid species and percentage of contribution of each species to total carotenoids in direct extracts of astringent persimmon (*Diospyros kaki* Thunb. var. Rojo brillante) submitted to pasteurization (85°C, 15 min) and high-pressure processing (HPP; 200 MPa, 25°C, 6 min)

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Table 2S. Sum of carotenoids (free and esterified) organized by carotenoid species and percentage of contribution of each species to total carotenoids in saponified extracts of astringent persimmon (*Diospyros kaki* Thunb. var. Rojo brillante) submitted to pasteurization (85°C, 15 min) and high-pressure processing (HPP; 200 MPa, 25°C, 6 min)

Figure 1S. C30 reversed-phase HPLC chromatogram obtained from direct (A) and saponified (B) carotenoid extracts from peel fruit tissue of Spanish astringent persimmon (*Diospyros kaki* Thunb. var. Rojo brillante) UV-vis detection at 450 nm. Peak identities in Table 2. (U) un-identified compound.

Figure 2S. C30 reversed-phase HPLC chromatogram obtained from direct (A) and saponified (B) carotenoid extracts from pulp fruit tissue of Spanish astringent persimmon (*Diospyros kaki* Thunb. var. Rojo brillante) , UV-vis detection at 450 nm. Peak identities in Table 2. (U) un-identified compound.

Figure 3S. Carotenoid proportion (%) of free xanthophylls, hydrocarbon carotenoids and xanthophyll esters (A) in direct extracts (crude) and (B) in saponified extracts; and of carotenoid species (C) in direct extracts (crude) and (D) in saponified extracts of astringent persimmon (*Diospyros kaki* Thunb. var. Rojo brillante) submitted to pasteurization (85°C, 15 min) and high-pressure processing (HPP; 200 MPa, 25°C, 6 min).