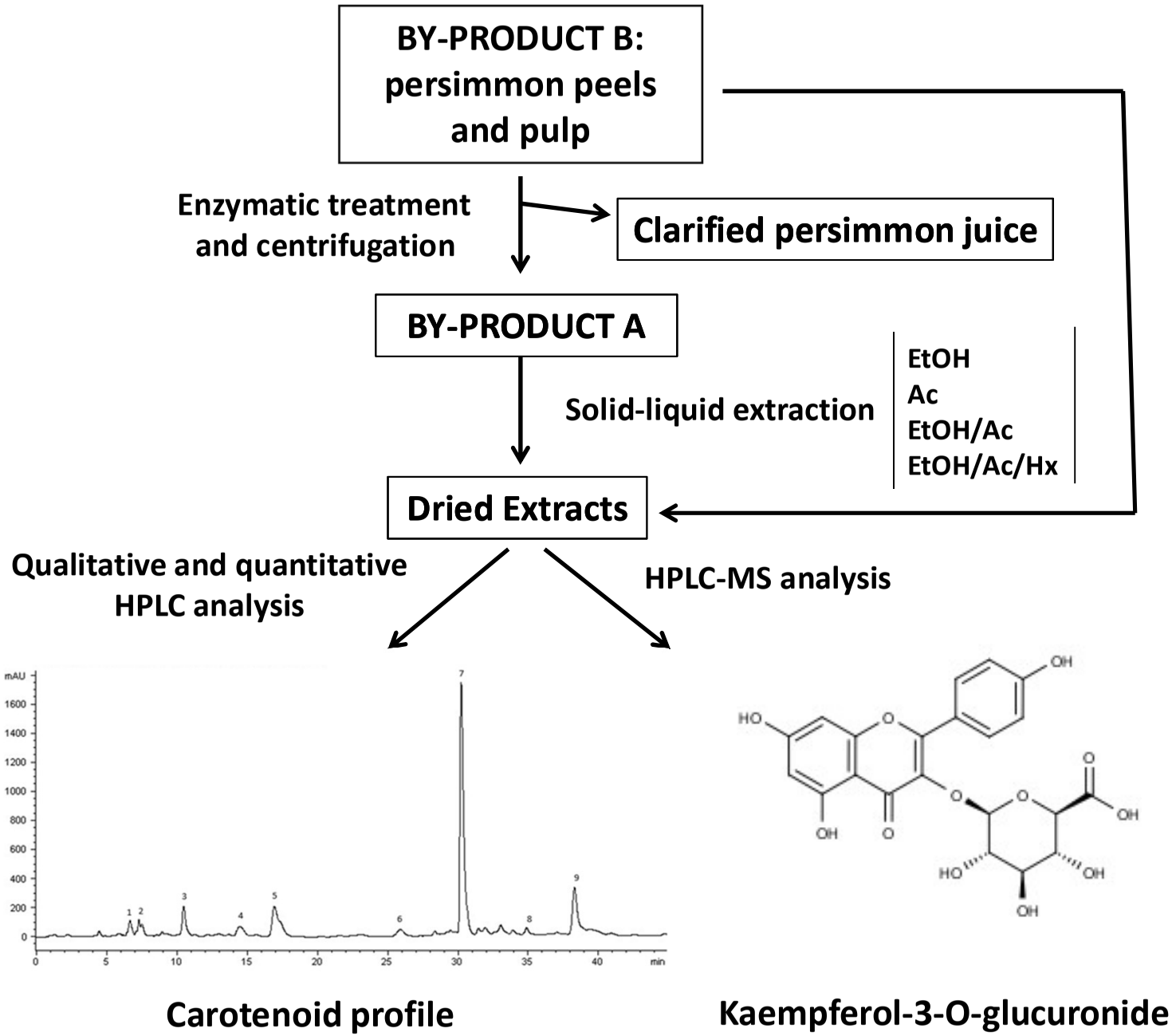
Graphical Abstract



Highlights

Revalorization of by-products derived from industrialization of persimmon juice was approached ► By-product A resulting from an enzymatic treatment was especially suitable for recovery carotenoids ► Acetone extract from by-product A showed the largest amount of these bioactive pigments ► Total carotenoid content contributed greatly to antioxidant activity of this acetone extract ► Twenty two phenolic compounds were also identified by HPLC-MS in this persimmon by-product extract.

Carotenoids from persimmon juice processing

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Abstract

The aim of this study was the use and revalorization of two persimmon by-products A and B generated in the juice production process. The by-product A resulting from an pectinase enzymatic treatment of peels and pulp to optimize juice extraction was especially suitable for recovery of valuable bioactive carotenoids. The extraction solvents and solvent combinations used were: ethanol, acetone, ethanol/acetone (50:50 v/v) and ethanol/acetone/hexane (25:25:50 v/v/v). HPLC analysis detected and identified a total of nine individual carotenoids namely neoxanthin, violaxanthin, zeaxanthin, lutein, antheraxanthin, β-cryptoxanthin 5,6-epoxide, β-cryptoxanthin, α-carotene, and β-carotene. β-cryptoxanthin and β-carotene represented 49.2% and 13.2% of the total carotenoid content in the acetone extract from by-product A. Total carotenoid content contributed greatly to antioxidant activity of acetone extract derived from this by-product. Twenty two phenolic compounds belonging to different chemical classes were also identified by HPLC-MS in this persimmon extract. Pectinase enzymatic treatment of persimmon peels and pulp followed by absolute acetone extraction of carotenoids could be an efficient method to obtain a rich extract in these compounds that could be used as nutraceutical ingredient.

Keywords: *Diospyros kaki*; Persimmon; By-products; Solid-liquid extraction; Carotenoids; Polyphenols

1. Introduction

The persimmon (*Diospyros kaki* Thunb.) is a tree of the Ebenaceae botanical family and is cultivated in subtropical climates (Martínez-Calvo et al., 2012). It originates from China, Japan and Korea, but nowadays it has been extended to other countries such as Brazil, United States of America (USA), Australia and some countries present in the Mediterranean Sea coast such as Israel, Italy or Spain. After China and Korea, Spain is today the third largest world producer (404,131 t) ahead of Japan and Brazil (FAOSTAT, 2017). The western area of Andalucía mainly in the province of Huelva (specifically, in the municipalities of Cartaya, Lepe, Isla Cristina and Villablanca) and Sevilla, and the Valencia Region highlight as the largest producers. According to MAPA estimates, in 2018 production of persimmon in Andalucía was 56,780 t from the variety Sharon or Triumph marketed under the Sharoni brand, while Valencia Region produced 425,075 t of the Rojo Brillante variety marketed under the protected denomination of origin (PDO) 'Kaki Ribera del Xúquer'.

It is remarkable that thousands tons of persimmon fruits (in particular, the Spanish Association of Persimmon currently estimates discarded fruits in the Valencia Region by about 18,000 t) are discarded every year due to a combination of the high quality standards of supermarkets, strict government regulations and the high expectations that consumers have when buying these fruits in terms of size, shape and color (Porter et al., 2018). Surplus fruits, damaged fruits that are unusable for fresh consumption, and those fruits unacceptable to the consumer that result from prolonged storage at low temperatures and/or chemical treatments to eliminate astringency, in the case of varieties with high content of soluble polyphenolic tannins (Arnal and Del Rio, 2003), require the development of new derivative products. In addition, fruits must be processed to facilitate their consumption and for commercial, logistic and economical

75 reasons (Ayala-Zavala et al., 2011). This generates large quantities of by-products
76 including peels, seeds and unused flesh in different steps of processing chain. These by-
77 products are rich in valuable compounds which can be utilized in various industries as
78 novel, economical and natural sources of dietary fiber, antioxidants, pectin, enzymes,
79 organic acids, food additives, essential oils, and others using different methods of
80 extraction, purification and fermentation (Kodagoda and Marapana, 2017; Lapornik et
81 al., 2005). The actions of reusing plant by-products agree with Sustainable
82 Development Goal number 12 (SDG 12) of the 2030 Agenda for Sustainable
83 Development of the United Unions (UN General Assembly, 2015).

84 Persimmon fruits are rich dietary source of bioactive compounds such as vitamin
85 C, dietary fiber, polyphenols and carotenoids (Gorinstein et al., 2001; Pérez-Burillo et
86 al., 2018; Veberic et al., 2010) which may act in concert to provide their antioxidant,
87 anti-inflammatory and other health-related properties useful to protect against non-
88 communicable chronic diseases (Aune et al., 2017; Hosseini et al., 2018). Nowadays,
89 the carotenoids have a great interest in the industry. Extensive research is allocated to
90 the obtention and production of these compounds because of their functional properties.
91 They are used as feed additives in animal nutrition (Jamilah et al., 2009), natural
92 colorants in foods, nutraceuticals and cosmetics (Berman et al., 2015; Jaswir et al.,
93 2011). On the other hand, carotenoids are used for their beneficial effect in the
94 prevention of diseases such as cancers (Bolhassani, 2015), cardiovascular diseases
95 (Csepanyi et al., 2015), and degeneration of optical vision (Harrison, 2019).

96 The aim of this study was to obtain an extract rich in carotenoid pigments, using
97 various solvents with different combinations such as ethanol, acetone, ethanol/acetone
98 (1:1), and ethanol/acetone/hexane (25:25:50). As plant material two by-products derived
99 from the industrial production of persimmon juice were used. Various extracted

carotenoids were identified and quantified by high-performance liquid chromatography (HPLC), using diode array detector (DAD) and analytical standards. Moreover, the polyphenolic profile of diverse extracts derived from different persimmon by-products is presented here for the first time.

2. Material and methods

2.1. Chemicals

The carotenoid standards: Neoxanthin (purity ≥ 97%), violaxanthin (purity ≥ 95%), zeaxanthin (purity ≥ 97%), lutein (purity ≥ 99%), antheraxanthin (purity ≥ 95%), β-cryptoxanthin (purity ≥ 97%), α-carotene (purity ≥ 97%) and β-carotene (purity ≥ 96%) were obtained from CaroteNature (Lupsingen, Switzerland). Methanol (MeOH), acetone (Ac), ethanol (EtOH), *n*-hexane (Hx), diethyl ether, acetic acid, acetonitrile, and potassium hydroxide (KOH, purity = 90%) were from Panreac Química SLU (Castellar del Vallès, Barcelona, Spain). All HPLC organic solvents were of analytical grade. Folin-Ciocalteu reagent, potassium persulfate, sodium carbonate, gallic acid, ABTS [2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)], and AAPH [2,2'-Azobis (2-amidinopropane) dihydrochloride] were purchased from Sigma-Aldrich Corp. (Saint Louis, Missouri, USA). Fluorescein (FL) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Fluka Chemika (Neu-Ulm, Germany). Ultrapure water was obtained from a purified water system Q-Gard® 1 from Merck Millipore (Darmstadt, Germany) with a resistivity of 18.0 MΩ·cm. Gas nitrogen has been obtained from Air Liquide (Madrid, Spain).

2.2. Plant material

124 The two fresh by-products A and B used in this study were purchased from
125 Mitra Sol Technologies (Elche, Spain). Both derive from persimmon fruits of the
126 Sharon or Triumph variety (non-astringent, seedless and hard) and are composed of
127 peels and pulp resulting from different stages of industrial processing of persimmon
128 juice (Fig. 1). By-product A was obtained by pectinase enzymatic treatment of by-
129 product B to optimize juice extraction. So by-product A had homogeneous granular
130 appearance and orange color, while by-product B, which was obtained liquefying the
131 edible parts of persimmon fruits through a Nutrifaster N450 commercial juicer
132 (Nutrifaster Inc., Seattle, Washington DC, USA), showed a more viscous and
133 heterogeneous appearance.
134 Moisture content (%MC) for the two by-products was determined. 10 g samples
135 were dried at 60°C ± 1°C in a drying oven (JP Selecta, Barcelona, Spain) and led to
136 constant weight.
137
138 *2.3. Solid-liquid extraction*
139 The methodology for carotenoids extraction was described by Olives Barba et al.
140 (2006). Initially, fresh by-products were washed with water (25:75 w/v) to eliminate
141 remaining sugars that could interfere with the subsequent drying process of extracts.
142 Then, by-products were divided into 4 portions and each of them were individually
143 washed three times with one of the solvents or solvent combinations assayed for 20 min
144 at 40ºC and 150 rpm, until a colorless liquid was obtained. Extraction solvents and
145 solvent combinations used were: ethanol, acetone, ethanol/acetone (50:50 v/v) and
146 ethanol/acetone/hexane (25:25:50 v/v/v). The different extracts were reduced using a
147 rotary evaporator low pressure (Series R-210, Buchi) at 40ºC in darkness. Residue was
148 saponified with 5 mL of KOH (30%) during 1 h at 56ºC according to Müller (1997).

149 Obtained solution was transferred to a funnel and mixed with 100 mL ethyl ether to
150 separate the organic phase, which was subsequently washed three times with water
151 according to Izuchi et al. (2009). Extracts were dried in a Genevac™ miVac Centrifugal
152 Vacuum Concentrator (SP Scientific) at 40ºC and lyophilized (Telstar Cryodos-80,
153 Terrassa, Barcelona, Spain) for removing the residual water. The final dry weight of
154 extracts were used to calculate extraction yields (%YE). Extracts were stored at -80ºC.
155 The different extracts were obtained in triplicate.
156
157 *2.4. Preparation of standards, calibration curve and HPLC analysis*
158 All standard stock solutions were prepared and kept under nitrogen atmosphere
159 at -20 °C until analysis. Neoxanthin, violaxanthin, zeaxanthin, lutein, antheraxanthin,
160 β-cryptoxanthin, α-carotene and β-carotene were separately dissolved in 1 mL of
161 chloroform. Standards curves were built with five concentrations for each carotenoid
162 (3.125, 6.25, 12.5, 25.0, and 50.0 mg/L). HPLC analysis for all standard solutions was
163 performed and the peak area for each compound at its individual maximum wavelength
164 was plotted against the concentrations. The developed calibration curve was used to
165 determine the concentration of each component in the extract samples analyzed by
166 HPLC. β-cryptoxanthin 5,6-epoxide was expressed as β-cryptoxanthin equivalents.
167 Four extracts were dissolved in acetone at a concentration of 20 mg/mL and
168 were filtered through a 0.45 µm PVDF syringe filter. In each sample, carotenoids
169 composition was analyzed by HPLC-DAD (Rivera and Canela-Garayoa, 2012).
170 Injection volume was 10 µL. YMC Carotenoid HPLC Column, C30, 4.6 x 250 mm, 5
171 µm (Teknokroma Analítica, Sant Cugat del Vallès, Barcelona, Spain) was used.
172 Analysis was performed at 25°C. Mobile phase consisted in a gradient of 60:40 (v/v)
173 methanol/acetone (A), and 60:40 (v/v) acetone/water (B). Flow rate was 0.5 mL/min.

174 The gradient profile of the mobile phase was set as follows: decreasing from 60% B to
175 30% B in 3 min; 30% B for 19 min; decreasing from 30% B to 10% B in 4 min; 10% B
176 for 15.5 min; and increasing from 10% B to 60% B in 3.5 min (total run time 45 min).
177 Chromatograms were recorded at 450 nm wavelength. Carotenoids were identified by
178 comparison of the retention times (RTs) with those of authentic standards, when
179 available, or by matching the observed versus literature retention time under identical
180 chromatographic conditions.
181
182 *2.5. Determination of total phenols*
183 For total phenolic compounds (TPC) determination, 5 mg extract were dissolved
184 in 1 mL ethanol. TPC were determined with Folin-Ciocalteu reagent in a SPECTROstar
185 Omega UV/VIS absorbance microplate reader (BMG LABTECH GmbH, Offenburg,
186 Germany) (González et al., 2015). 10 µL of extract sample dilution, 50 µL Folin-
187 Ciocalteu reagent, 100 µL of aqueous 20% Na_2CO_3 and 100 µL of distilled water were
188 mixed. The mixture was allowed to stand for 30 min at room temperature before
189 measuring absorbance at 750 nm. Gallic acid was used as standard. Results were
190 expressed as gallic acid equivalents (mg GAE/100 g extract).
191
192 *2.6. Antioxidant activity*
193 The antioxidant activity of the extracts was evaluated by both the ABTS radical
194 scavenging and the oxygen radical absorbance capacity (ORAC) antioxidant assay. The
195 radical cation was prepared by the reaction between a 7 mM solution of ABTS in water
196 mixed with a 2.45 mM solution of potassium persulfate (González et al., 2015). The
197 mixture was incubated 24 h in the dark at room temperature. Then, this solution was
198 diluted with ethanol to reach an absorbance of 0.7 ± 0.02 at 734 nm, measured in the

199 microplate reader SPECTROstar Omega. To determine the antioxidant activity of
200 extracts, 200 µL of the $ABTS^{\bullet+}$ dissolution was mixed with 20 µL of extract and after 6
201 min the absorbance was measured at 734 nm, obtaining the value of the decrease in
202 absorbance. This determination was carried out with 5 mg/mL ethanolic dilutions of
203 extracts. Trolox was used as standard and results were expressed as Trolox equivalents
204 (mM TE/100 g extract).

205 To assay the capacity of extracts to scavenge peroxyl radicals a validated ORAC
206 method which uses FL as the fluorescent probe ($ORAC_{FL}$) was utilized (Vegara et al.,
207 2014). The automated ORAC assay was carried out on a FLUOstar Galaxy fluorescence
208 microplate reader (BMG LABTECH GmbH). Several dilutions of Trolox were used to
209 construct the calibration curve. A freshly prepared AAPH water solution was used for
210 each determination. The temperature of the incubator was set at 37ºC and the FL
211 fluorescence was recorded every minute after the addition of AAPH. The ORAC values
212 were calculated by using a regression equation between the Trolox concentration and
213 the net area of the FL decay curve (area under curve, AUC). ORAC values were
214 expressed as Trolox equivalents (mM TE/100 g extract).

215

216 *2.7. Purification of extracted polyphenols*

217 Sep-Pak® plus C18 (WA020515) from Waters Corporation (Milford,
218 Massachusetts, USA) was used. This cartridge contains a silicon matrix with high
219 affinity for hydrophobic substances such as polyphenols. The cartridge was activated
220 circulating 5 mL of methanol through it, followed by 5 mL of diethyl ether, 5 mL of
221 methanol and, finally, 5 mL of miliQ water using a syringe. Once activated, 0.5 mL of
222 extract sample obtained by refluxing with 80% ethanol and 4.5 mL of water was passed
223 through the Sep-Pak® using a syringe. The retained substances were eluted by passing

0.5 mL diethyl ether twice. The ether eluted material was analyzed by HPLC-Mass Spectrometry (MS).

2.8. HPLC-MS analysis

The Agilent 1100 Series HPLC system equipped with a DAD and an auto sampler (Agilent Technologies, Palo Alto, California, USA) was coupled to an Esquire 3000 Plus Ion Trap Mass Spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an Electrospray Ionization (ESI) interface. Injection volume was 20 µL. Poroshell 120, SB-C18, 4.6 x 150 mm, 2.7 µm HPLC column (Agilent Technologies) was used for separation. Analysis was performed at 25°C. Mobile phase consisted of A (0.5% acetic acid) and B (acetonitrile). A linear gradient of elution was programmed as follows: 0-10 min, 0-20% B; 10-15 min, 20-30% B; 15-20 min, 30-50% B; 20-25 min, 50-75% B; 25-30 min, 75-95% B; 30-35 min, 95-100% B; followed by 5 min equilibration of 100% B (total run time 40 min). Flow rate was 0.7 mL/min. The compounds separated were monitored with DAD at 280 nm wavelength.

Mass spectra were recorded at the range of mass-to-charge (*m*/*z*) ratio 50-2000 in negative mode. The capillary voltage was set at 4500 V; capillary temperature was set at 365°C with drying nitrogen gas flow of 9.0 L/min and nebulizing gas pressure of 45 psi.

2.9. Statistical analysis

Experiments were carried out in triplicate and results were expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) was performed with GraphPad Prism® version 6.0 (San Diego, California, USA) and differences between means were

248 estimated by Tukey's HSD (honestly-significant-difference) test. Regression analysis
249 was used to describe the relationship between the variables.
250
251 **3. Results**
252 *3.1. %MC of persimmon by-products*
253 The %MC of the two persimmon by-products varied greatly. Estimated %MC,
254 expressed as g water per 100 g fresh weight (FW), were found to be 69.23 ± 0.08% and
255 94.10 ± 0.73% for by-product A and B, respectively. The high %MC of by-product B
256 suggests a large sugars content that would explain its sticky appearance.
257
258 *3.2. %YE*
259 The %YE for both by-products (A and B) and each one of assayed organic solvents
260 or solvent combinations are shown in Fig. 2A. In general, solid-liquid extraction for by-
261 product B was more effective than for by-product A. Regardless of the solvent used in
262 the extraction process, there was a statistically significant increase in the amount of
263 final dried extract. The major increment was obtained using absolute acetone as organic
264 solvent [10,413.66 ± 72.90 mg/100 g dry weight (DW)], followed by using ethanol and
265 the mixture of both solvents. The lowest %YE was obtained using the solvent
266 combination ethanol/acetone/ hexane (3,537.01 ± 119.90 mg/100 g DW). Statistically
267 significant differences were also observed between the estimated %YEs for by-product
268 A. The highest %YE was obtained with the solvent combination ethanol/acetone
269 (1,550.19 ± 76.29 mg/100 g DW) although it did not differ from that obtained only with
270 ethanol. The lowest %YE was obtained using acetone as solvent (902.82 ± 21.14
271 mg/100 g DW) (Fig. 2A).
272

3.3. Extracted carotenoid quantity

Determination of total extracted carotenoid content (TCC) from the two persimmon by-products A and B was performed by HPLC-DAD, adding up the individual carotenoid concentrations measured in each extract obtained using one of the organic solvents or combinations of them studied. TCCs were higher in by-product A than in by-product B, for all solvents and combination used. Results displayed statistically significant differences among extracts derived from each persimmon by-product (Fig. 2B). The lowest TCC values were recorded for by-product B with values varying from 2,444.54 ± 566.61 mg/100 g ethanol/acetone extract to 111.61 ± 13.39 mg/100 g ethanol/acetone/hexane extract. By-product A provided the largest TCCs, i.e., 33,970.25 ± 1,542.61 mg/100 g acetone extract and 13,953.20 ± 891.87 mg/100 g ethanol extract (Fig. 2B).

3.4. Identification and quantification of extracted carotenoids

Representative HPLC-DAD chromatograms of carotenoids in different extracts from the persimmon by-product A are shown in Figs. 3A-D. Nine carotenoids including neoxanthin, violaxanthin, zeaxanthin, lutein, antheraxanthin, cryptoxanthin 5,6-epoxide, β-cryptoxanthin, α-carotene, and β-carotene were identified by RTs coincident with the respective commercial standards or already reported values under identical chromatographic conditions 6.7, 7.4, 10.6, 14.5, 17.0, 25.9, 30.3, 34.9, and 38.4 min.

Chromatographic profile of main carotenoids found in persimmon by-products was similar for both sources of plant material as expected. All extracts regardless of by-product origin and solvent or solvents combination used clearly showed the nine identified carotenoids. On the contrary, individual concentration of all carotenoids present in both by-products was clearly higher in by-product A than in by-product B

298 (Figs. 4A and 4B). Quantitative analysis of carotenoids was also influenced by the
299 organic solvent or the combination of solvents used for its extraction. The acetone
300 extract derived from by-product A was the richest in carotenoids. The predominant
301 carotenoid was β-cryptoxanthin (16,709.90 ± 518.70 mg/100 g extract) followed by β-
302 carotene (4,479.07 ± 713.71 mg/100 g extract), antheraxanthin (4,250.83 ± 27.90
303 mg/100 g extract), and zeaxanthin (2,265.48 ± 19.37 mg/100 g extract) (Fig. 4A). In
304 comparison with the previous extract, the major carotenoid in ethanol extract was β-
305 cryptoxanthin (6,436.06 ± 286.17 mg/100 g extract) followed by antheraxanthin
306 (2,161.05 ± 160.04 mg/100 g extract).
307 In by-product B, the highest individual carotenoid amounts were measured in the
308 ethanol/acetone extract (Fig. 4B). Carotenoid relation in decreasing order of
309 concentration was β-carotene, β-cryptoxanthin (688.34 ± 45.20 - 645.65 ± 88.35
310 mg/100 g extract), zeaxanthin, and violaxanthin (443.94 ± 262.73 - 411.43 ± 120.09
311 mg/100 g extract).
312
313 *3.5. TPC*
314 TPC determination indicated considerable variations among extracts from by-
315 product A which was selected for its higher concentration in carotenoids since levels
316 oscillated between ~49 and ~101 mg GAE/100 g extract (Table 1). Data in the four
317 extracts were very homogeneous. The lowest value was recorded in the ethanol/acetone
318 extract and the highest in acetone one, with 48.88 ± 3.84 and 101.28 ± 6.53 mg
319 GAE/100 g extract, respectively. No large differences were found among TPC's of
320 different extracts.
321
322 *3.6. Antioxidant activity*

In this study, in vitro antioxidant activity of persimmon extracts was measured by two different analytical methods: ABTS and ORAC. Results are summarized in Table 1. Concerning ABTS, values varied significantly from ~3 to ~139 mM TE/100 g extract. As in TPC, whereas the highest values were found in acetone extract from by-product A, the lowest ones were recorded both in ethanol/acetone and ethanol/acetone/hexane extracts. ORAC results were higher than those already remarked for ABTS and expressed a similar sequence with respect to the biological activity assayed (Table 1). Acetone extract displayed a moderately large antioxidant activity (~455 mM TE/100 g extract) while ethanol, ethanol/acetone and ethanol/acetone/hexane extracts showed values between ~210 and ~302 mM TE/100 g extract.

TPC results were correlated to ABTS (r = 0.9783; R^2 = 95.7141) and ORAC (r = 0.9116; R^2 = 83.1099) at the 95% (P = 0.0217 < 0.05) and 90% (P = 0.0884 < 0.10) confidence level respectively. The low confidence levels of these correlations suggest that TPC contributes minority and only partially to the antioxidant activity of the extracts studied in this work. The initial washing with water of the fresh by-product drastically decreased the extractable amount of these compounds. In contrast, there was a statistically significant relationship between total carotenoid content in the different extracts from by-product A and ORAC values at the 99% (r = 0.9999; R^2 = 99.9836; P = 0.0001 < 0.01) confidence level, suggesting that the carotenoids contribute greatly to the antioxidant activity of the persimmon by-product extracts.

3.7. Identification of purified polyphenols by HPLC-MS

The RTs, mass spectra data and corresponding identified phenolic components in purified samples from both the ethanol and acetone extracts are summarized in

348 Tables 2 and 3, respectively. Although at least 10 compounds were coincident, the
349 phenolic profile of purified sample derived from the ethanol extract, with 14
350 constituents (Table 2), was noticeably different from that of sample corresponding to
351 acetone extract, which showed 22 (Table 3). Peaks 10 and 13, with same molecular
352 formula at m/z 326.7, in the MS spectra of purified sample derived from acetone extract
353 (p-coumaroyl-glucoside, $C_{15}H_{18}O_8$) were detected at 28.1 and 29.9 min, indicating the
354 occurrence of isomeric structures that significantly differ in their elution behavior.
355 Qualitatively flavonoids abound with representatives of different subfamilies (flavanols,
356 flavones, flavanones, and flavan-3-oles) and some polyflavonoid. Most of the identified
357 compounds have been found in fruits of numerous plant species, with the exception of
358 castalagin derivatives I and II, found in oak and chestnut wood, as well as in stem barks
359 of *Anogeissus leiocarpus* Guill. & Perr. and *Terminalia avicennioides* Guill. & Perr. or
360 leaves of *Syzygium samarangense* (Blume) Merril & LM Perry (common name Java
361 apple) (Kamada et al., 2018). This ellagitannin probably comes from residues of woody
362 peduncles and leaves that were not correctly removed during cutting of harvested fruits,
363 but its presence in persimmon peels and pulp cannot be completely ruled out.
364
365 **4. Discussion**
366 Although production of persimmon fruits is mainly intended for fresh
367 consumption, many unsuitable fruits must be processed annually to produce new
368 derived products such as persimmon juice. González et al. (2015) reported for the first
369 time an enzyme maceration process for production of persimmon juice at semi
370 industrial scale which has been successfully tested in the juice industry. Jiménez-
371 Sánchez et al. (2015) performed a complete qualitative analytical characterization
372 through HPLC–DAD–ESI-TOF/MS of different persimmon juices produced under

373 different technologies. Additionally Martínez et al. (2017) showed that production of
374 persimmon beverages might open up new uses for discarded fruits. Unfortunately, juice
375 industry generates a serious environmental problem of accumulation and waste disposal
376 after processing. The new agro-industry should become a system where everything is
377 used, trying to eliminate the generation of waste, according to the ZERI (zero emissions
378 researches and initiatives) concept.

379 In relation to the use and revalorization of persimmon by-products generated in
380 the juice production process, by-product A resulting from a macerating pectinase
381 treatment to optimize juice extraction was especially suitable for recovery of valuable
382 bioactive compounds like carotenoids. Furthermore, enzymatic pretreatment of a
383 persimmon slurry was also effective in releasing carotenoids from complex food matrix,
384 thus significantly improving the extraction yield in a similar way to an enzyme-assisted
385 extraction (EAE) non-thermal method (Saini and Keum, 2018). In the other hand, by-
386 product A showed the lowest %MC, what is generally considered favorable for the
387 efficient extraction of carotenoids due to the hydrophobic nature of these. In summary
388 both enzymatic treatment of by-product B and low %MC of by-product A influenced
389 greatly the carotenoid yields obtained.

390 The main extraction methods of carotenoids reported using different persimmon
391 tissues, as natural sources, are solid-liquid extraction (Izuchi et al., 2009; Veberic et al.,
392 2010), accelerated solvent extraction (ASE) (Zaghdoudi et al., 2015), high-pressure
393 treatment (HP) (De Ancos et al., 2000), and supercritical fluid extraction (SFE)
394 (Zaghdoudi et al., 2016). The choice of solvent is always the most critical factor for
395 efficient extraction of carotenoids, and mainly depends on the carotenoid composition
396 of the natural source (Saini and Keum, 2018). Usually, presence of carotenoids with
397 varied levels of polarity makes their simultaneous extraction difficult. Polar solvents

398 such as acetone and ethanol were chosen for extraction of dipolar (lutein and
399 zeaxanthin) and monopolar (β-cryptoxanthin) carotenoids, whereas a mixture of
400 ethanol/acetone/hexane was applied for the simultaneous extraction of polar and non-
401 polar carotenoids (lycopene and carotenes). In general, solvent combinations provide
402 synergistic effects on the extraction of carotenoids.

403 Persimmon by-products A and B showed different %YEs expressed as mg dry
404 extract per 100 g DW. The highest %YEs were exhibited by by-product B and these
405 were approximately 4.9, 11.5, 5.2, and 2.7 times higher than those from by-product A
406 for the solvents ethanol, acetone or the solvent combinations ethanol/acetone and
407 ethanol/acetone/hexane, respectively. Obtained %YEs were higher than those from the
408 agro-industrial by-products grape marc, mango bagasse and peanut skin (Braga et al.,
409 2016). Intermediate %YEs were obtained when extractions from persimmon seed
410 powder were performed with ethanol (4,850 mg/100 g extract) and acetone (2,580
411 mg/100 g extract) as absolute solvents (Akter et al., 2010). According to Babbar et al.
412 (2011), the type of plant residue would be more influential than the solvent system on
413 extraction yield.

414 HPLC has become the method of choice for carotenoid analysis (Rivera and
415 Canela-Garayoa, 2012) and reverse-phase columns like YMC Carotenoid C30 are the
416 most widely used stationary phases for the analysis of these molecules. Chromatograms
417 of carotenoids in persimmon by-product extracts revealed the presence of nine of these
418 compounds which were identified as primary carotenoids, in particular, neoxanthin,
419 violaxanthin, zeaxanthin, lutein, antheraxanthin, cryptoxanthin 5,6-epoxide,
420 β-cryptoxanthin, α-carotene, and β-carotene. All of them except cryptoxanthin 5,6-
421 epoxide and α-carotene have been extracted and identified in saponified extracts from
422 flesh of persimmon fruits cv. Sharon grown in Spain (De Ancos et al. (2000). The

423 presence of α-carotene in the extracts from by-product A could be due to the high
424 maturity degree of fruits used usually for juice processing. β-cryptoxanthin and β-
425 carotene represented 49.2% and 13.2% of the total carotenoid content determined in the
426 acetone extract from by-product A. Additionally, the high content of both carotenoids
427 give to this extract an important provitamin A value (De Ancos et al., 2000; Saini and
428 Keum, 2018). Carotenoids which contain an unsubstituted β-ionone ring can be
429 converted "in vivo" into vitamin A, developing the same biological effects.

430 Carotenoids have potential health benefits and some of them have been
431 attributed to their antioxidant activity (Seifried et al., 2017). According to Jamova and
432 Valko (2013) carotenoids effectively scavenge peroxyl radicals and act predominantly
433 as antioxidants. The growing interest in the substitution of synthetic food antioxidants
434 by natural ones has fostered research on plant sources and the screening of inexpensive
435 or residual materials from agricultural industries for extracting new antioxidants (Moure
436 et al., 2001). Although polyphenols are the major plant compounds with antioxidant
437 activity, various studies (González et al., 2015; Mena et al., 2011; Tezcan et al., 2009)
438 have reported differences relating to the phenolic compounds contribution to
439 antioxidant capacity assays. Our data pointed out that TPC did not greatly participate in
440 antioxidant activity of persimmon by-product extracts obtained herein whereas the total
441 carotenoid content did.

442 On the other hand, HPLC-MS is a powerful method in phenolic analysis (Zhang
443 et al., 2017). Characterization of polyphenols, including simple polyphenols (phenolic
444 acids and flavonoids) and polymerized flavan-3-ols (tannins or procyanidins) in diverse
445 persimmon juices has been reported (Jiménez-Sánchez et al., 2015). None studies
446 however, have been done on the polyphenolic profile of persimmon by-products,
447 derived for juice processing. A total of 26 phenolic compounds belonging to different

chemical classes were tentatively characterized in ethanol and acetone extracts derived from persimmon by-product A. Industrial processing of persimmon fruits, together with the high degree of maturity of these would favor detection of this number of phenolic compounds.

5. Conclusion

Two by-products derived from the industrialization of persimmon juice were used for carotenoid extraction in order to revalue these agro-industrial residues. This study showed that the by-product A resulting from a macerating pectinase enzyme treatment to optimize juice extraction was especially suitable for recovery of valuable carotenoids. In this by-product, with the acetone extract, twenty two phenolic compounds belonging to different chemical classes were identified. The high content of carotenoids which contain an unsubstituted β-ionone ring gave to this extract an important provitamin A activity and capacity to develop similar biological effects. Sequence of processing persimmon peels and pulp by a macerating pectinase enzyme followed by the solid-liquid extraction of carotenoids from the resulting by-product using absolute acetone as solvent could be an efficient method to obtain a dried extract that could be used as a nutraceutical ingredient.

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643 **Figure captions**

644 **Fig. 1.** Flow diagram of industrial production of persimmon juice.

645

646 **Fig. 2.** (A) Extraction yields (mg/100 g DW) and (B) total carotenoid contents (mg/100
647 g extract) of persimmon extracts obtained from by-product A (▬) and B (▭) using
648 different solvents or solvent combinations.

649

650 **Fig. 3.** HPLC-DAD Chromatograms of carotenoids in different extracts from
651 persimmon by-product A: (A) Ethanol extract, (B) Acetone extract, (C) Ethanol/acetone
652 (50:50 v/v) extract, and (D) Etanol/acetone/hexane (25:25:50 v/v/v) extract. Peaks are
653 labeled as follows: 1 = neoxanthin, 2 = violaxanthin, 3 = zeaxanthin, 4 = lutein, 5 =
654 antheraxantin, 6 = β-criptoxanthin 5,6-epoxide, 7 = β-criptoxanthin, 8 = α-carotene, and
655 9 = β-carotene.

656

657 **Fig. 4.** Individual carotenoid contents (mg/100 g extract) of persimmon extracts
658 obtained from (A) by-product A and (B) by-product B using different solvents or
659 solvent combinations.

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Table 1. Total phenolic compounds (TPC) and antioxidant activity assays (ABTS and ORAC) in different extracts from persimmon by-product A derived from juice processing

Organic Solvents and Solvent Combinations	TPC (mg GAE/100 g extract)	ABTS (mM TE/100 g extract)	ORAC (mM TE/100 g extract)
Ethanol	86.64* ± 5.23 b	123.37 ± 11.73 a	302.10 ± 8.77 b
Acetone	101.28 ± 6.53 a	138.98 ± 14.59 a	454.63 ± 11.65 a
Ethanol/Acetone	48.88 ± 3.84 c	11.61 ± 3.36 b	239.27 ± 10.60 c
Ethanol/Acetone/Hexane	54.79 ± 5.76 c	3.36 ± 0.29 c	210.00 ± 3.73 d

*Values are reported as mean ± standard deviation (SD). Means followed by different lower-case letters are significantly different ($P < 0.05$) according to the Tukey´s multiple range test.

Table

Table2. Identified compounds by HPLC-MS in the ethanol extract derived from persimmon by-product A.

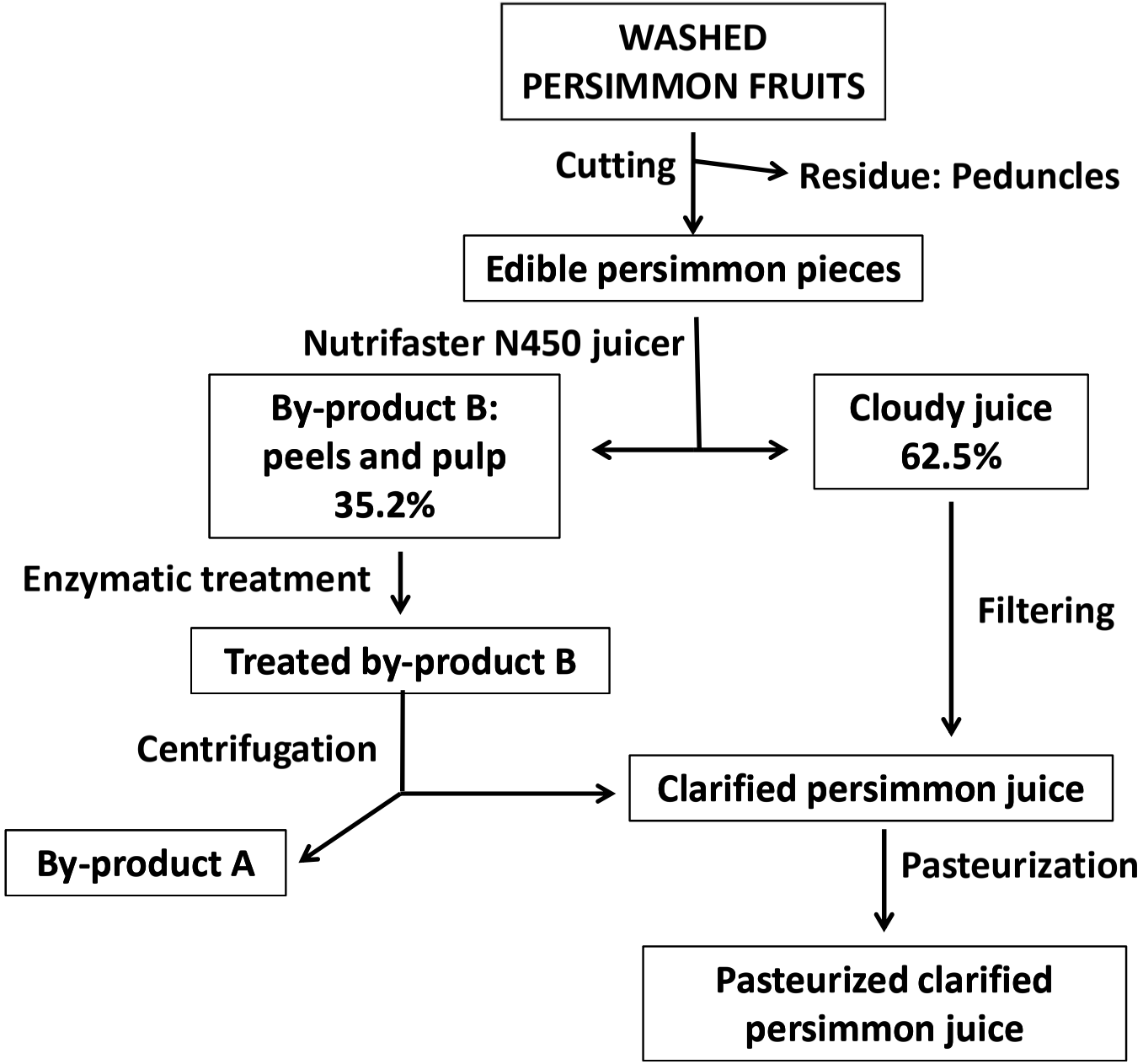
Peak	RT	*m/z* (min)	Proposed compound	Molecular formula
1	13.3	478.4	Quercetin-glucuronide	$C_{21}H_{18}O_{13}$
2	15.2	759.0	Quercetin-3-O-(2'-rhamnosyl)-rutinoside	$C_{33}H_{40}O_{20}$
3	17.4	630.6	Gentisoyl glucoside	$C_{13}H_{16}O_9$
4	19.5	462.6	Kaempferol-glucuronide	$C_{21}H_{18}O_{12}$
5	20.8	446.5	Apigenin-7-O-glucuronide	$C_{21}H_{18}O_{11}$
6	21.8	608.7	Diosmetin-7-rutinoside	$C_{28}H_{32}O_{15}$
7	28.1	326.7	p-coumaroyl- glucoside	$C_{15}H_{18}O_8$
8	29.4	460.6	Verbascoside	$C_{29}H_{36}O_{15}$
9	31.3	592.7	Epicatechin-(4beta->8)-gallocatechin	$C_{30}H_{26}O_{13}$
10	32.2	552.8	Ligstroside derivative I	$C_{25}H_{32}O_{12}$
11	33.1	610.6	Rutin	$C_{27}H_{30}O_{16}$
12	34.2	581.1	Naringenin-7-O-rutinoside	$C_{27}H_{32}O_{14}$
13	34.8	965.5	Castalagin derivative II	$C_{42}H_{29}O_{27}$
14	36.0	612.9	Dehydrated tergallic C-glucoside	$C_{27}H_{19}O_{17}$

Table

Table 3. Identified compounds by HPLC-MS in the acetone extract derived from persimmon by-product A.

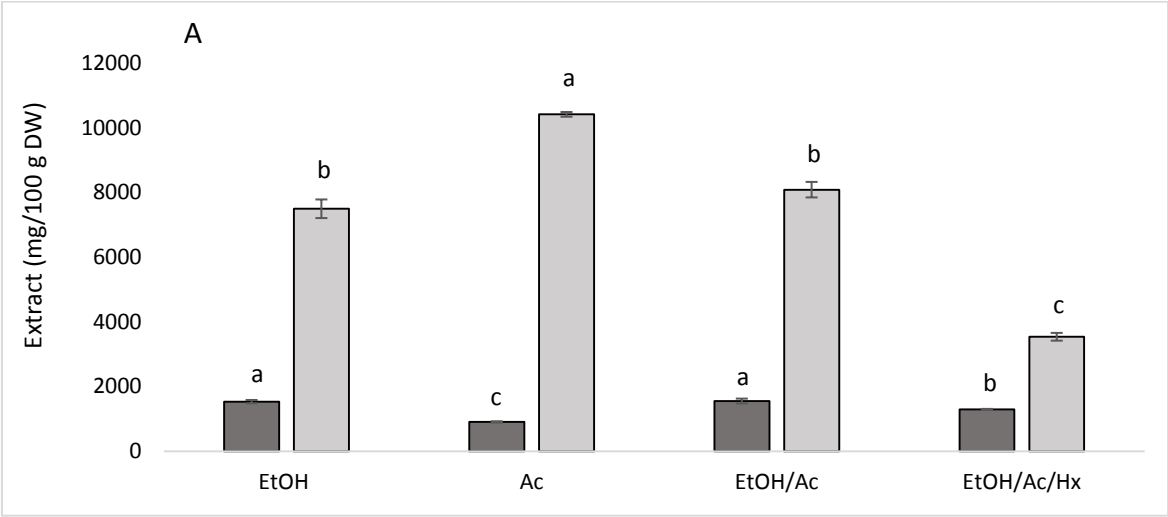
Peak	RT (min)	*m/z*	Proposed compound	Molecular formula
1	11.8	584.6	Ellagic acid dimer $-H_2O$	$C_{28}H_{10}O_{16}$
2	13.3	478.4	Quercetin-glucuronide	$C_{21}H_{18}O_{13}$
3	15.2	759.0	Quercetin-3-O-(2'-rhamnosyl)-rutinoside	$C_{33}H_{40}O_{20}$
4	19.4	596.7	Eriodictyol-7-rutinoside (Eriocitrin)	$C_{27}H_{32}O_{15}$
5	19.5	462.6	Kaempferol-glucuronide	$C_{21}H_{18}O_{12}$
6	20.8	446.5	Apigenin-7-O-glucuronide	$C_{21}H_{18}O_{11}$
7	21.8	608.7	Diosmetin-7-rutinoside	$C_{28}H_{32}O_{15}$
8	21.9	644.7	Digalloyl-diglucose	$C_{26}H_{32}O_{20}$
9	24.1	300.5	4'-Methylkaempferol (Kaempferide)	$C_{16}H_{12}O_6$
10	28.1	326.7	p-coumaroyl-glucoside	$C_{15}H_{18}O_8$
11	29.0	328.7	Vanillic acid 4-hexoside	$C_{14}H_{18}O_9$
12	29.4	460.6	Verbascoside	$C_{29}H_{36}O_{15}$
13	29.9	326.7	p-coumaroyl-glucoside	$C_{15}H_{18}O_8$
14	31.3	592.7	Epicatechin-(4beta->8)-gallocatechin	$C_{30}H_{26}O_{13}$
15	31.5	963.3	Castalagin derivative I	$C_{42}H_{27}O_{27}$
16	31.9	576.8	Epicatechin dimmer (Procyanidin B2)	$C_{30}H_{26}O_{12}$
17	32.2	552.8	Ligstroside derivative I	$C_{25}H_{32}O_{12}$
18	33.4	764.5	7-methylquercetin-3-galactoside-6''-rhamnoside-3''-rhamnoside (Xanthorhamnin B)	$C_{34}H_{42}O_{20}$
19	34.6	655.1	Campneoside I	$C_{30}H_{38}O_{16}$
20	34.8	965.5	Castalagin derivative II	$C_{42}H_{29}O_{27}$
21	36.0	396.8	5,7-dihydroxy-2-(1-methylpropyl)isopropyl-chromone-8-β-D-glucoside	$C_{22}H_{30}O_{10}$
22	36.2	308.6	Kaempferol 3-O-β-D-rutinoside	$C_{27}H_{30}O_{15}$
23	36.3	628.3	6,8-Di-C-β-D-glucopyranosylapigenin (Vicenin 2)	$C_{27}H_{30}O_{15}$

Figure


WASHED PERSIMMON FRUITS
Cutting
Residue: Peduncles
Edible persimmon pieces
Nutrifaster N450 juicer
By-product B: peels and pulp 35.2%
Cloudy juice 62.5%
Enzymatic treatment
Filtering
Treated by-product B
Centrifugation
Clarified persimmon juice
By-product A
Pasteurization
Pasteurized clarified persimmon juice

Figure

Fig. 2


A
Extract (mg/100 g DW)
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8000
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b
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a
a
b
b
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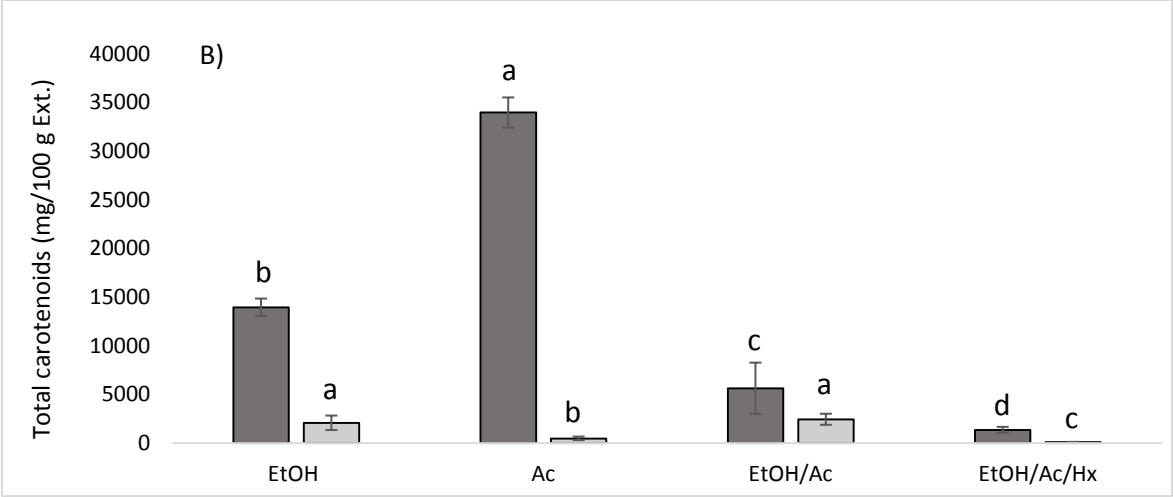
EtOH/Ac
EtOH/Ac/Hx


B)
Total carotenoids (mg/100 g Ext.)
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25000
20000
15000
10000
5000
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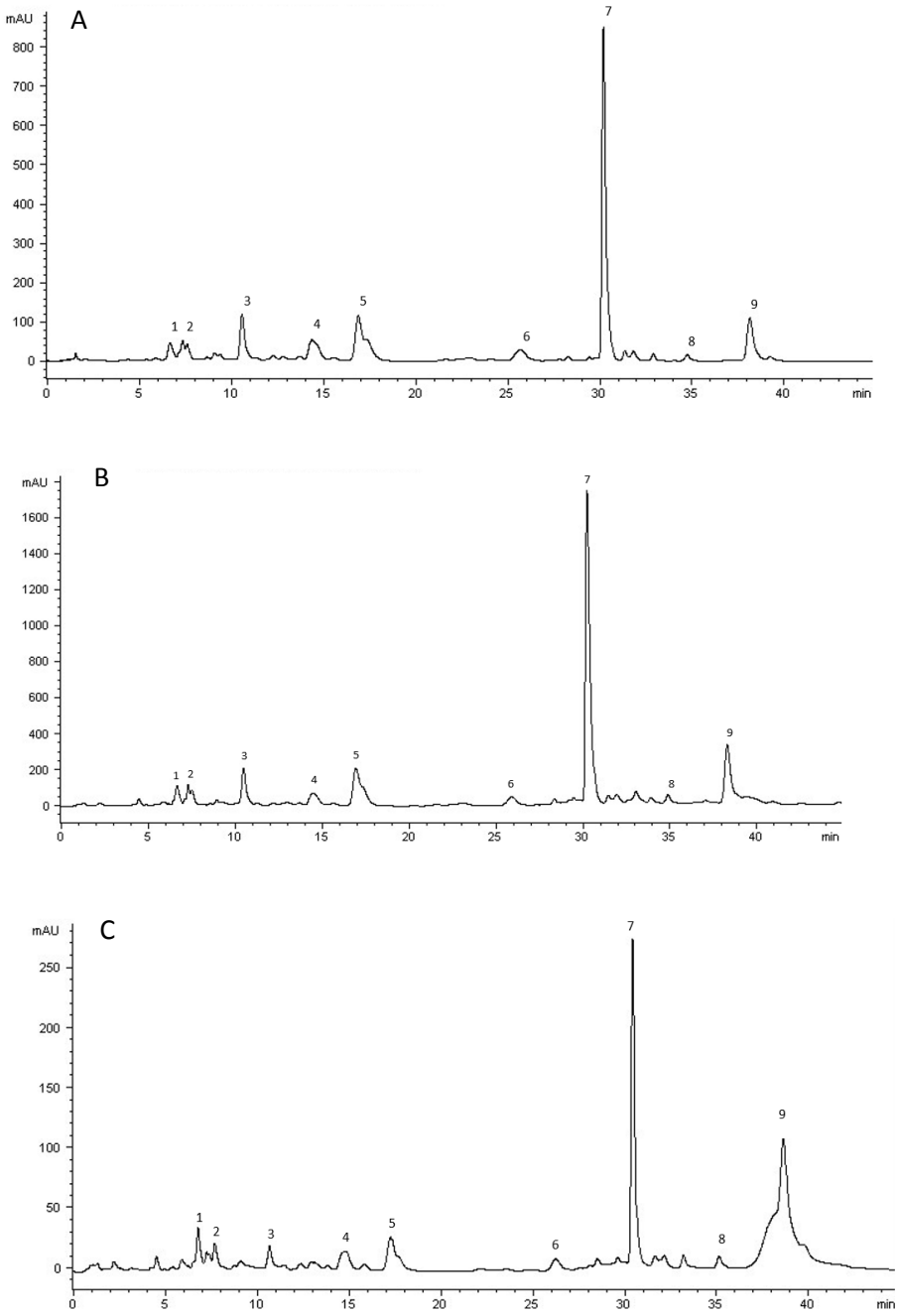
a
b
c
a
d
c
EtOH
Ac
EtOH/Ac
EtOH/Ac/Hx

Figure

Fig. 3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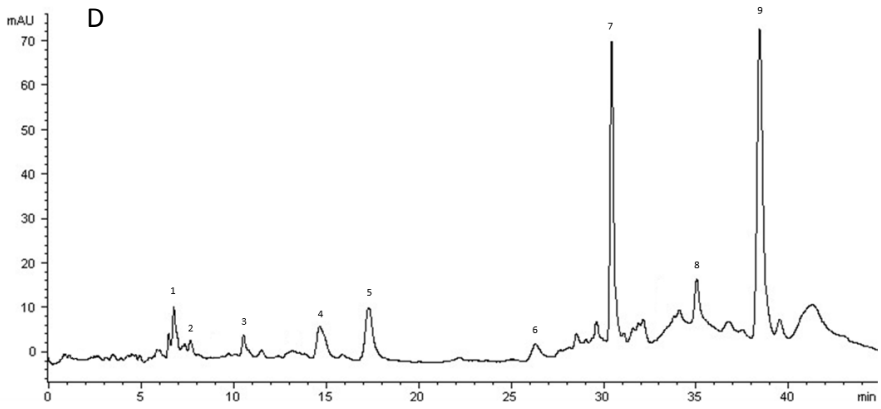
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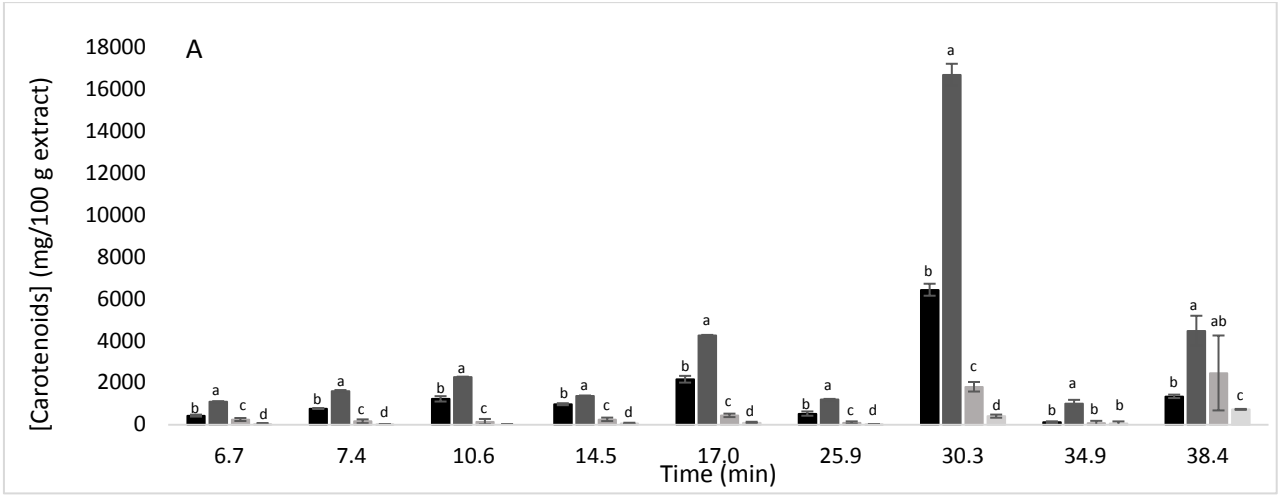
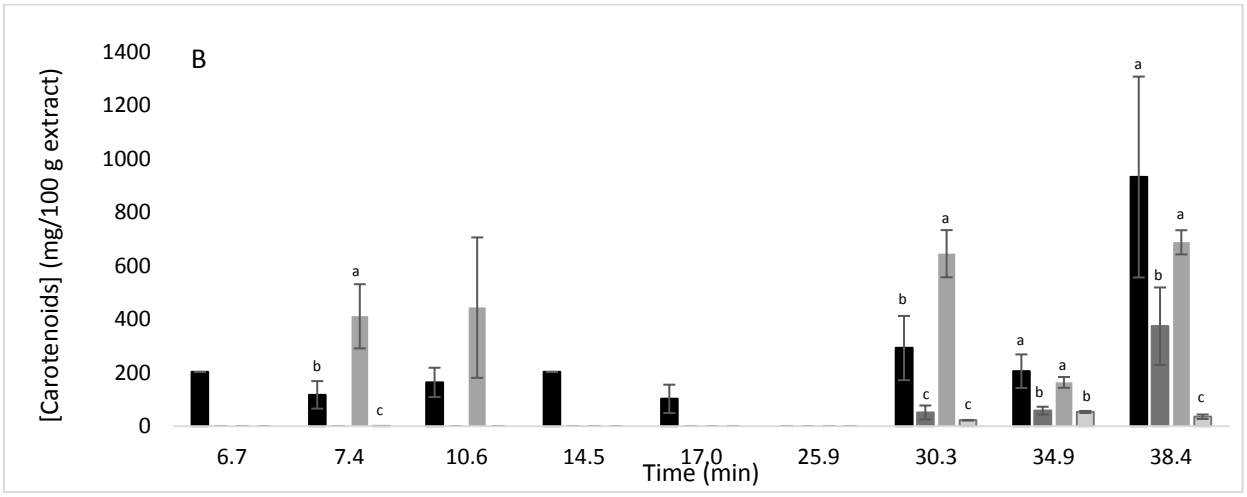
Figure

Fig. 4


A
[Carotenoids] (mg/100 g extract)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
6.7
7.4
10.6
14.5
17.0
25.9
30.3
34.9
38.4
Time (min)


B
[Carotenoids] (mg/100 g extract)
1400
1200
1000
800
600
400
200
0
6.7
7.4
10.6
14.5
17.0
25.9
30.3
34.9
38.4
Time (min)

***Declaration of Interest Statement**

Declaration of interests

X The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: