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Analysis of Polyphenols Content and Antioxidant Capacity from Hybrids Mandarin Peel

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Abstract: Mandarin cultivars (*Citrus reticulata*) represent 22% of the total citrus fruit crops. Mandarin peels are an abundant source of natural flavonoids and other antioxidants. To determine the polyphenols content and antioxidant capacity from hybrid mandarins peel, 33 samples of hybrid mandarins (´Clemenvilla`, ´Nadorcott` and ´Ortanique`) of the province of Valencia (Spain), were selected. Fresh mandarin peel extracts were prepared by Ultrasound Assisted Extraction (400 W, 80% v/v duty cycle, 40 °C) at 30 min employing ethanol 50% (*v*/*v*) as solvent in 1:10 (*w*/*v*) solid-liquid ratio. C18 cartridges (200 mg) were employed for the Solid Phase Extraction clean-up process and Ultra-Performance Liquid Chromatography system coupled with a Quadrupole Time-Of-Flight Mass Spectrometer was used to identify and quantify the polyphenols. ´Clemenvilla` and ´Ortanique` showed the highest antioxidant capacity by DPPH and TEAC, respectively. For these three hybrids, the main polyphenol present in samples was hesperidin, being higher in ´Nadorcott` peel (72 ± 7.0 μg/g). After, narirutin was higher in ´Ortanique` and ´Nadorcott` (33 ± 6.3 and 31.8 ± 6.8 μg/g, respectively) and rutin was it in ´Clemenvilla` peel (7.3 ± 3.8 μg/g). Results suggest that mandarin peels are an important source of polyphenol compounds with high antioxidant capacity.

Keywords: mandarin peel; polyphenols, hesperidin; antioxidant capacity.

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1. Introduction

The citrus fruits are one of the principal crops worldwide, being mandarin cultivars (*Citrus reticulata*) the 22% of the total citrus fruit crops [1]. These fruits are well accepted by the consumers because of its sweetness flavors and easy peeling [2]. Mandarin fruit residues (peel, seeds, and pulp) are usually discarded without regard to potential nutritional and commercial value. Mandarin peels makes up approximately 35-40% of the weight [3] and are an abundant source of natural flavonoids [4] and other antioxidants.

This study addressed the polyphenol characterization from three varieties of hybrids mandarin peel (´Clemenvilla`, ´Nadorcott` and ´Ortanique`) that are not widely studied. Ultrasound Assisted Extraction (UAE), a Solid Phase Extraction (SPE) clean-up process and Ultra-Performance Liquid Chromatography system coupled with a Quadrupole Time-Of-Flight Mass Spectrometer (UPLC-QTOF-MS/MS) analysis were carried out to determine and quantify the polyphenols from hybrid mandarin peel.

2. Materials and Methods

2.1. Plant Materials and Extraction Method

33 samples of hybrid mandarins (X ´Clemenvilla`, X ´Nadorcott` and n=X ´Ortanique`), procured by citrus farmers of the province of Valencia (Spain), were selected.

Fresh mandarin peel extracts were prepared according to the method reported by Anticona et al. [5] by UAE. 6 g of peels were placed in a beaker glass with ethanol-water (50:50, *v*/*v*) as solvent in a solid-liquid ratio of 1:10 (*w*/*v*). The extraction was assisted by an ultrasonic processor QSONICA Q500 (Newtown, CT. U.S.A.) with these following conditions: 400 W, 80% *v*/*v* duty cycle, 40 °C at 30 min. The extracts were centrifuged (4000 r.p.m., 4 °C, 5 min) and the supernatants were filtered by a membrane filter Whatman no. 1 with a pore size of 11 μm (Whatman International Ltd., UK) and collected to be stored at −20 °C in dark conditions, until use. The procedure is described in Figure 1.

2.2. Chemical Analysis Methods

Total polyphenols (TP) and total flavonoids (TF) content were determined according to the methods described by Anticona et al. [5]. For TP, 3 mL of anhydrous sodium carbonate (Na_2CO_3) solution (2%, w/v) and 100 μL of Folin-Ciocalteau reagent (1:1, v/v) were added to an aliquot of 100 μL of diluted sample. The mixture was incubated for 1 h at room temperature. The absorbance was measured at 765 nm using a UV/VIS Lambda 2 spectrophotometer (Perkin Elmer, USA). Results were expressed as mg of gallic acid equivalent (GAE)/100 g fresh weight (FW) of peel. The TF determination was carried out mixing 100 μL of appropriately diluted samples with 1088 mL of ethanol (30%, v/v). 48 μL of sodium nitrite ($NaNO_2$) solution (0.5 mol/L) was added and the mix was vortexed. After 5 min of reaction, 48 μL of aluminum chloride hexahydrate ($AlCl_3.6H_2O$) (0.3 mol/L) was added. The mixture was vortexed and allowed to react for 5 min at room temperature. Then, 320 μL of sodium hydroxide (NaOH) (1 mol/L) was added and mixture was vortexed again. The absorbance was measured at 510 nm and the results were expressed as mg of catechin equivalents (CE)/100 g fresh weight (FW) of peel.

The antioxidant capacity by 2,2-diphenyl-1-picrylhydryzyl (DPPH) assay were applied according to the method described by Anticona et al. [5]. The DPPH-coloured radical was used measuring the initial absorbance at 515 nm. The reaction was begun by adding 50 μL of suitable dilution of sample to 1.45 mL of DPPH radical (0.06 mM). After being incubated during 30 min at room temperature, the final absorbance was measured. In case of the Trolox equivalent antioxidant capacity (TEAC) assay the method described by Zulueta et al. [6] was employed, with modifications for the final reaction tested. 25 mL of ABTS radical (ABTS•+) (7 mM) was prepared with 440 μL of potassium persulphate $K_2S_2O_8$ (140 mM) and allowed to stand in darkness at room temperature for 12 – 16 h. The solution was diluted with ethanol until an absorbance of 0.70 ± 0.02 reached at 734 nm and 30 °C. The absorbance of 2 mL of formed ABTS•+ was recorded as the initial absorbance and 100 μL of appropriately diluted samples were added. The mixture was incubated for 3 min and the final absorbance was measured. In both assays (DPPH and TEAC) the percentage of inhibition (% I) was calculated using the following formula (Equation 1):

$$\% I = [(A0 - A1)/A0] \times 100 \quad (1) \qquad (1)$$

where, A0 is the absorbance of the control, A1 is the absorbance in the presence of sample. Results were expressed as mM Trolox-equivalent (mM TE).

2.2. Chromatographic Analysis

Before the chromatographic analysis, 5 mL of sample were placed on C18 cartridges (200 mg) for the SPE clean-up process according to the method described by Gonzales et al. [7] with some modifications. An UPLC-QTOF-MS/MS was used to identify and quantify the main polyphenols in samples.

The UPLC-QTOF-MS/MS analysis was performed on an LC SCIEX system equipped with a UPLC C18 column, 50 × 2.1 mm, (Waters, USA) applying the following elution binary gradient at a flow rate of 0.4 mL/min: 0–5 min, isocratic 70% A (water/formic acid, 99.9/0.1 [*v*/*v*]), 30% B (methanol/formic acid, 99.9/0.1 [*v*/*v*]); 5–12 min, linear from 30 to 95% B; 12–18 min, isocratic 95% B; 18–18.5 min, linear from 5 to 70% A; 18.5–25 min, isocratic

70% A. The injection volume was 5 µL. The compounds were detected from m/z 100–950 in negative ion mode in a transfer time of 100 ms. Automated calibration was performed using an external calibrant delivery system. The MS used an information dependent acquisition method with the survey scan type (TOF-MS) and the dependent scan type (product ion) using -30V of collision energy. Data was evaluated using the qualitatively evaluated using the PeakViewTM software. Relative quantification was performed using Multiquant 3.0.3 software.

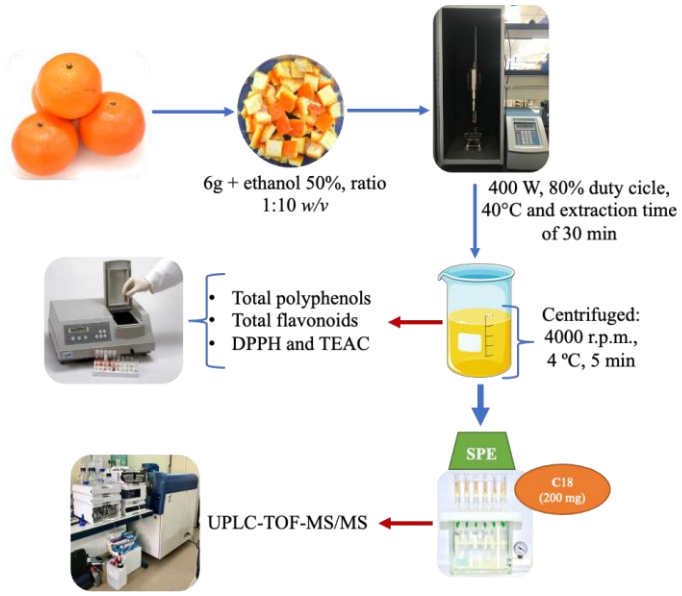

Figure 1. Mandarin peel extraction and polyphenol analysis

3. Results and Discussion

3.1. Bioactive Compounds

The TP and TF content varied according to each hybrid mandarin variety ($p < 0.05$). The TP content was higher in ´Ortanique` samples compared to ´Clemenvilla` and ´Nadorcott` peels (Table 1). These results differ to the values obtained by Safdar et al. [3] who ranged from 2439 to 3248 mg GAE/100g in mandarin peel powder treated by UAE. Also, Nipornram et. al. [8] obtained 14899 mg GAE/100g of TPC in peel powder of *C. reticulata* Blanco cv. Sainampueng. These differences are due to the structure of samples analyzed, because in our study fresh peels were employed. In this line, Londoño-Londoño et al. [9] observed greater differences between the TP content of fresh peel and peel powder of *C. reticulata* samples obtained by UAE.

Flavonoids are the principal bioactive compounds in citrus peel [10]. ´Clemenvilla` samples had the highest values of TF content compared to ´Nadorcott` and ´Ortanique` (Table 1). Ho & Lin [11] obtained a total of 790 mg CE/100 g of extract of *C. reticulata* peel powder being the principal difference in the concentration of TF the structure characteristics of samples analyzed.

Table 1. Total polyphenol and flavonoids content determined in hybrid mandarin peels.

Bioactive compound	Clemenvilla	Nadorcott	Ortanique
TP (mg GAE/ 100 g FW ± SD)	828.4 ± 95.8[a]	724.2 ± 43.0[a]	1155.2 ± 171.3[b]
TF (mg CE/ 100 g FW ± SD)	89.6 ± 15.5[a]	70.9 ± 12.5[a]	71.7 ± 17.8[a]

[1] a-b: different letters in the same row indicate that there are statistically significant differences ($p < 0.05$) between the values of each variety. TP: total polyphenols; GAE: gallic acid equivalent; FW: fresh weight; SD: standard deviation; TF: total flavonoids; CE: catequin equivalent.

3.2. Antioxidant Capacity

DDPH and TEAC assays were employed to assess the antioxidant capacity of hybrid mandarin peels. There are useful methods to be applied in fruits samples to determine the antioxidant capacity and is recommended the employ of two or more methods [12]. As is observed in Figure 2 ´Clemenvilla` and ´Ortanique` extracts showed the highest antioxidant capacity by DPPH (14 ± 3.8 mmol Trolox/100 g) and TEAC (32 ± 3.8 mmol Trolox/100 g) assays, respectively. Also, the values of mmol TE/100 g obtained by DPPH were lower than the values of TEAC assay. This is similar with the results of antioxidant capacity in whole ´Murcott` mandarin samples observed in a study of Gironés-Vilaplana et al. [13] to DPPH (2.5 mmol TE/100 g) and TEAC (6.47 mmol TE/100 g). The different values in total antioxidant capacity obtained by the assays employed reflect the difference in the ability of bioactive compounds to reduce the DPPH and ABTS radicals in this type of *in vitro* assays. The main difference is that the DPPH assay is more sensitive to hydrophobic compounds while TEAC assay is more sensitive to hydrophilic antioxidants like polyphenols [14]. In this sense, the mmol TE values in samples assessed by TEAC assay were in the same order than the mg GAE values (TP): ´Ortanique` > ´Clemenvilla` > ´Nadorcott`. The results obtained by TEAC assay were higher than the values determined by Montero-Calderon et al. [15] (3.97 ± 0.15 mmol TE/100 g) in samples of orange peel treated by UAE (400 W, 30 min, 50% ethanol). Also M'hiri et al. [16] showed lower TEAC values from orange peel extracts by ultrasounds.

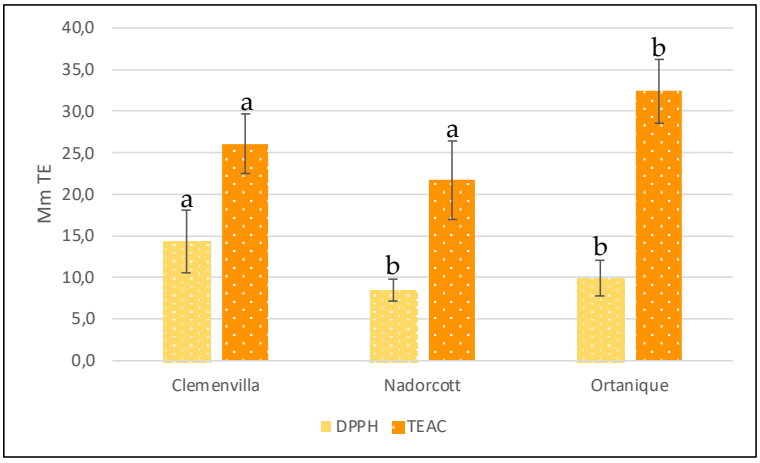

Figure 2. Differences of antioxidant capacity assessed by DPPH and TEAC assays in hybrid mandarin peels. a-b: different letters in the same color indicate that there are statistically significant differences ($p < 0.05$) between the values. TE: Trolox equivalent.

3.3. Identification and Quantification of Polyphenols by Ultra-Performance Liquid Chromatography System Coupled with a Quadrupole Time-Of-Flight Mass Spectrometer Analysis

Principal polyphenol composition of mandarin peel extracts is observed in Table 2. Fayek et al. [17] indicated that UPLC-QTOF-MS/MS is a useful technique to analyze the phenolic composition in citrus peels. The main polyphenol presents in the hybrids was hesperidin, being higher in ´Nadorcott` peel (72±7.0 μg/g). According to this, Nipornram et al. [8] and Hayat et al. [18] determined that hesperidin is one of the major compounds in mandarin peel. However, Zhao et al. [19] observed in their study that nobiletin is the main polyphenol, followed by hesperidin. Slightly higher concentration of hesperidin from mandarin peel extract was reported by Safdar et al. [3] (84.41 μg/g). In second position in amount detected appears narirutin in ´Ortanique` and ´Nadorcott` (33±6.3 and 31.8

± 6.8 μg/g, respectively), and rutin in ´Clemenvilla` (7.3±3.8 μg/g). In case of narirutin amounts, a notable difference is observed in Clemenvilla samples, being lower than ´Nadorcott` and ´Ortanique` peels. Deepest studies are necessary to explain these differences. Lower concentrations of rutin (1.0 μg/g) were obtained by Zhao et al. [19] in clementine peel extracts. In relation to ferulic acid and 4-hidroxibenzoic acid, ´Clemenvilla` and ´Nadorcott` exhibit the higher concentrations. However, higher concentrations of ferulic acid were observed by Safdar et al. (3) in ´Kinnow` mandarin peels (42.56 μg/g).

Table 2. Polyphenol compounds identified and quantified in hybrid mandarin peels by UPLC-QTOF-MS/MS.

Compound [1]	Molecular formula	[M-H]⁻ *m/z* (-)	Clemenvilla	Nadorcott	Ortanique
4-hidroxibenzoic acid	$C_7H_6O_3$	137.02442	4.1 ± 10.7^{a}	1.9 ± 0.8^{b}	2.1 ± 1.2^{b}
Rutin	$C_{27}H_{30}O_{16}$	609.14611	7.3 ± 3.8^{a}	5.6 ± 3.3^{ab}	6.9 ± 2.2^{ab}
Ferulic acid	$C_{10}H_{10}O_4$	193.05063	1.5 ± 0.7^{a}	6.8 ± 0.9^{b}	2.2 ± 0.5^{c}
Narirutin	$C_{27}H_{32}O_{14}$	579.17193	4.3 ± 2.6^{a}	31.8 ± 6.8^{b}	33.1 ± 6.3^{b}
Hesperidin	$C_{28}H_{34}O_{15}$	609.18249	63.7 ± 10.7^{a}	72.3 ± 7.0^{b}	63.7 ± 6.8^{a}

[1] Concentrations are expressed in μg/g FW of peel. a-c: different letters in the same row indicate that there are statistically significant differences ($p < 0.05$) between the values of each variety.

4. Conclusion

Results suggest that there are significant differences in the content of TP, TF, and antioxidant capacity according to the varieties analyzed. Finally, hesperidin is the major phenolic compound in hybrid mandarin peels and, narirutin and rutin were identified and quantified in samples analyzed. Analyzed mandarin peels are an important source of polyphenol compounds with high antioxidant capacity.

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