Determination of Color, Antioxidant Activity and Phenolic Profile of Different Fruit Tissue of Spanish 'Verde Doncella' Apple Cultivar

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Keywords: *Spectrophotometric methods; Phenolic compounds; Antioxidant activity, 'Verde Doncella', FRAP, HPLC-DAD.*

ABSTRACT

The polyphenolic profile and antioxidant activity of peel, pomace, and juice of ‘Verde Doncella’, a Spanish apple cultivar is presented. Phenolic profile of the worldwide cultivated, ‘Red Delicious’ cultivar was used for comparison. Flavanols, hydroxycinamic acids, flavonols, phloridzin, procyanidin B2, and gallic acid were quantified by HPLC. Larger concentrations of polyphenolics were found in the peel, which is in agreement with the Total Phenolic Content (TPC) and antioxidant activity (FRAP) values. ‘Verde Doncella’ expressed lower concentrations of flavanols and quercetin derivates in peel, pomace, and juice when compared to ‘Red Delicious’. ‘Verde Doncella’ was richer in p-coumaric acid and procyanidn B2 in the peel.

INTRODUCTION

Production area of ‘Verde Doncella’ (Malus domestica), a lesser-known, high market value Spanish apple cultivar, is mainly located in the Aragón region, in northeastern Spain. ‘Verde Doncella’ has a relatively long history in this area, stretching back as far as the 19th century (*1*). The fruit possess a pinkish-yellow color and is highly appreciated by consumers due to its juicy, sweet, and aromatic characteristics. Since the 1950s, important transformations in the Aragón agricultural sector have led to the abandonment of primitive agricultural practices in favor of mechanical-based production. These changes have resulted in the replacement of traditional cultivars with others from diverse origins, to increase demand and production. However, in the last decade, an increasing trend to reintroduce local varieties into the marketplace, products reflecting the local region has been observed (*1*).

The general perception that apples are good for human health, together with the consumer's increasing demand for functional foods, has encouraged researchers to study in depth the polyphenolic profiles and antioxidant properties of many apple cultivars. It is well known that apples are one of the most important natural sources of polyphenols, exhibiting antioxidant activity, which can potentially prevent chronic diseases (*2*, *3*).

During the past few years, a lot of research has been devoted to polyphenols, their occurrence in apples (*4*-*9*) and apple derivates (by-products) (*10*-*14*). These studies have contributed to elucidate the major polyphenolic groups and many individual polyphenolic compounds in a variety of cultivars. According to the studies mentioned above, the major phenolic groups that are present in different apple cultivars belong to the hydroxycinnamic acids, flavanols, flavonol anthocyanins, and dihydrochalcons families. With respect to individual compounds, the major apple phenolics are chlorogenic acid, quercetin glycosides, procyanidins and phloridzin. Distribution of these compounds vary considerably among apple cultivars, and seem to be regulated by environmental and post-harvest factors, including fruit season, fruit maturity, light exposure, storage and processing (*15*).

Major phenolics are well characterized in commercially important cultivars such as 'Red Delicious,' Golden Delicious, Fuji and Granny Smith, but little or no data is available for traditional, secondary varieties specific to small production areas such as 'Verde Doncella'. To the best of our knowledge, only one study carried out more than twenty years ago (*16*), has analyzed the phenolic composition in 'Verde Doncella' apples. In this study, four major groups

of compounds (catechins, procyanidins, hydroxycinnamic acid esters and flavonoid glycosides) in the peel, pomace, and juice of five apple cultivars, were quantified using HPLC.

The lack of information with respect to phenolic composition and antioxidant properties for 'Verde Doncella', has motivated the present work. This paper therefore provides a preliminary insight into the phenolic profile (including color measurements and quantification of major phenolics), and antioxidant activity (FRAP) for 'Verde Doncella'. For comparison purposes, the present paper also includes data for 'Red Delicious' apples.

MATERIALS AND METHODS

Chemicals

Folin-Ciolcalteu reagent, sodium carbonate anhydrous, gallic acid monohydrate, 2,4,6-tris(2-pyridyl)-s-triazine, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, sodium acetate trihydrate, iron (III) chloride hexahydrate, procyanidin B2, chlorogenic acid, (+)-catechin, (+)-epicatechin, kaempferol, caffeic acid, quercetin, quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-rhamnoside, p-coumaric acid, phloridzin and rutin hydrate were all obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Sodium hydroxide 0.1 mol/L, hydrochloric acid 1.0 mol/L, malic acid, sodium fluoride, and iron (II) sulfate 7 hydrate were all obtained from Panreac Química S.A.U. (Barcelona, Spain). LiChrosolv methanol for liquid chromatography and acetic acid (glacial) anhydrous GK for analysis were obtained from Merck KGaA (Darmstadt, Germany).

Plant material

The apple cultivars evaluated in this study were *Malus domestica* 'Verde Doncella' and *Malus domestica* 'Red Delicious.' All fruits were commercially harvested in 2010, in an orchard belonging to Frutas Villalengua S.L., located in Zaragoza, (Spain). Apples remained in the suppliers packaging cartons and were cold stored at 2-3ºC, for two weeks until analysis.

Color measurements

Apple cartons were removed from cold storage and allowed to acclimate to room temperature for one hour. Colorimetric measurements were performed according to a previously published group article (*17*) on each apple of both cultivars (measuring peel only) using a Instrument System Spectroradiometer IS CAS 140 (Instrument System, München, Germany) with a TOP 100 probe with an AF Nikkor 200 mm 1:4 lens. The spectroradiometer equipment was controlled by ISCOLOR software (Version 2.53, 1996. Instrument Systems Optische Messtechnik GmbH; München, Germany). Illumination was supplied by a 12V-100W projection lamp (type 6834, Royal Philips Electronics, Amsterdam, The Netherlands) attached to a DC power supply (Diamond Antenna, San Marcos, CA, USA). Illumination equipment was operational for 40 min until light spectrum stabilized. White standard was calibrated using a Spectralon® reflectance standard (NIST certified, Labsphere Inc., North Sutton, NH, USA). Apples were measured on a rotating sample platform. Reflectance spectra were measured every 4 s allowing the apple to revolve 360º. Approximately 200 measurements were collected around the latitude of each apple in 4 s, averaged into one measurement. Spectra were measured between 380 and 900 nm every 1 nm. From these spectra, CIELAB (CIE 2004) coordinates L*, a*, b*, C* and h_{ab} were calculated with the CIE64 Standard Observer and the D65 Illuminant.

Sample processing (Phenolics extraction from peel, pomace and juice)

Fresh apple samples from 'Red Delicious' and 'Verde Doncella' cultivars were peeled with a hand peeler (1-2 mm thickness). Apple pomace and juice were collected after processing the remaining apples through a juicer (Sammic, Azkoitia, Spain). Apple peel, pomace, and juice

were processed separately. Five g for each sample was added into sterile 50 mL conical centrifuge tubes. Approximately 10 mL of an 80% aqueous methanol extraction solution containing sodium fluoride in order to slow oxidation was added to each centrifuge tube. The tubes were shaken for 30 min and then stored at -32ºC for 24 hours. After 24 hours, sample solutions were centrifuged at 2600 g for 40 min at 0ºC ± 1ºC and then filtered. In order to optimize sample clarification, a total of 13 different filters (in terms of pore size and supplier) were tested to filtrate the supernatant. Additionally, several methods of filtration (syringe, gravity, vacuum) were assayed as shown in **Table 2**. The supernatant was finally filtered through a Pall Life Sci, Corp. 0.45µm Acrodisc syringe filter and the filtrate was stored at -32 ºC prior to analysis. These extracts (for apple peel, pomace and juice) were employed in further chemical analysis (TPC, FRAP and HPLC).

Dry weight

Apple dry weight was determined gravimetrically, based on sample weight loss after being heated in an oven at 38ºC for several days (*18*). Samples were dryed in labeled brown paper bags.

Total Phenolic Content (TPC)

Total phenolic content was determined by a modified Folin-Ciocalteu method (*19, 20*). Briefly, 1 mL aliquot of peel, pomace or juice extract was mixed with 5 mL of Folin-Ciocalteu reagent. After 30 seconds and before 8 min, 4 mL of 7.5% sodium carbonate (Na_2CO_3) was added into

156 volumetric flasks. Flasks were incubated in the dark for 60 min at room temperature. Absorbance
157 was measured at 760 nm against a blank extraction solution (80% aqueous methanolic solution
158 with NaF) in an UV/Visible spectrophotometer (model 6506 from Jenway). The standard curve
159 was prepared with gallic acid (0 to 200 mg/L solutions) in 80% methanol. Total phenolic content
160 of samples were expressed in Gallic acid equivalents (GAE) (mg/100 g). Experiments were
161 performed in triplicate.
162
163 *Antioxidant activity: Ferric Reducing Ability of Plasma/Antioxidant Power (FRAP) Assay*
164
165 The FRAP method was modified from protocol (*21*). This method is based on the reducing
166 power of an antioxidant, which will reduce the ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}); the
167 latter will form a blue-violet complex (Fe^{2+}/TPTZ) which will increase the absorption at 595 nm.
168 The FRAP reagent (150 µL) and 20 µL of apple extract (peel, pomace or juice) or standard
169 (Trolox), were added into each well of a 96-well TPP (TPP AG, Trasadingen, Switzerland) tissue
170 culture plate. Plates were then read at 595 nm using a Tecan GENios multifunction micro plate
171 reader (Tecan Trading AG, Männedorf, Switzerland). Replications were made in triplicate of
172 each treatment. Standard Curves were prepared for each plate. The antioxidant capacity is
173 mentioned as Trolox equivalents (µmol eq. Trolox /100 g).
174
175 *Determination of phenolic compounds by HPLC*
176
177 Phenolics were identified and quantified with a HPLC system from Agilent Technologies (1200
178 Series) equipped with a quaternary pump, a degasser, a thermostatic auto-sampler, and a UV-

Diode Array detector. Injection volume for each apple extract (peel, pomace or juice) was 10 μL. Chromatographic separation was performed using a Zorbax SB-C18 column (150 mm x 4.6 mm i.d.; particle size 3.5 μm). The binary phase was performed according to a modified Tsao & Yang (2003) procedure. Solvent A consisted of 6% acetic acid in 2mM sodium acetate (final pH 2.55, v/v) and solvent B, was pure acetonitrile. All solvents were filtered and degassed through a 0.45-μm nylon filter before analysis. Flow rate was set at 0.6 mL/min for a total run time of 48 min. The system was run with a gradient program: 0-15% B in 27 min, 15-30% B in 9 min, 30-50% B in 3 min and 50-100% B in 3 min. A post-run of 6 min at initial conditions for equilibrium was also performed. This program permitted the analysis of the major apple phenolics in a relatively short chromatographic run (≅ 30 min). Phenolics were detected at 280, 320, 360, and 520 nm (**Figures 1 and 2**).

Chromatograms and UV-Vis spectra were acquired with Chemstation software (Agilent technologies, Santa Clara, California, USA). Phenolics identification was achieved by comparing retention times and UV-Vis spectra with available standard reference compounds. Unknown peaks were tentatively identified by comparison with known polyphenol group profiles of similar apple cultivars previously described in the literature (*7, 22*). Concentration of phenolics was determined by interpolating in pure compound standard curves. All samples were prepared and analyzed in triplicate.

Data Analysis

The values obtained in the analysis of TPC, antioxidant capacity and quantitative data derived from HPLC analysis were subjected to analysis of variance (ANOVA) using GraphPad Prism (Version 5.00, GraphPad Software, La Jolla, Ca, US). When significance was observed ($p \leq 0.05$) a Tukey's test was performed for separation of means. Additionally, the relationship between the total phenolics (measured by both TPC and HPLC) and the antioxidant activity were examined by Pearson correlations.

RESULTS AND DISCUSSION

Color

CIELAB color coordinate measurements are presented in **Table 1**. Previous publications have reported measured CIELAB color coordinates in apples (*23, 24*). However in each experiment, color was measured using a hand held pistol, recording individual random points of a sample. In this experiment, samples were placed on a rotating platform (360º) and evaluated with a fixed camera, recording constant color value measurements (n=200) during a single revolution. Use of a rotational platform allowed samples to be read homogenously and precisely, avoiding possible errors related to light position source or measurement angle. Presented color measurements of 'Red Delicious' are consistent with other investigations measuring color of the same cultivar (*24, 25*). Color parameters of 'Verde Doncella' are reported for the first time.

Optimization of extract filtration for absorbance-based measurements

During preliminary tests it was observed that phenolic extractions with methanol provided turbid supernatants with small particles in suspension. Such turbidity presented a problem for further spectrometric measurements, as it lead to unstable and high absorbance values. Therefore, an optimization effort was made to select a filter that could provide greater clarity for the supernatant, while still being efficient and fast (to limit sample oxidation). **Table 2** lists the 13 filters assayed, the type of filtration (vacuum, gravity and syringe), the pore size and the average absorbance obtained after juice filtration. Vacuum filtration resulted in rapid sample recovery; however, vacuum produced more turbid extracts (and therefore greater absorbance values) when compared to gravity and syringe filtration. On the other hand, gravity filtration was slow and the time required in collecting enough sample filtrate risked increasing sample oxidation. Taking into account both quality of absorbance measurements and filtration time, the Pall Life. Sci. Corp Acrodise Syringe Filter filter was chosen.

Total Phenolic Content (TPC)

When comparing between cultivars (Table 3), the most outstanding result is that peel from 'Red Delicious' contained a TPC (12.7 mg/GAE/ g DW) more than twice as large as the TPC of 'Verde Doncella' (13 mg/GAE/ g DW). The TPC obtained from 'Red Delicious' peel in our work corresponded well with previously reported studies (*2*). On the contrary, only few

247 differences were found between ‘Red Delicious’ and ‘Verde Doncella’ when comparing TPC for
248 pomace and juice samples, as no significant differences were observed, respectively, between
249 means.
250 For both varieties, TPC varied significantly between collected peel and pomace. TPC values for
251 apple peel provided the largest values, which is in agreement that phenolics will accumulate in
252 dermal tissues of plant bodies, thus increasing TPC (*7*). Previous studies (*3*);(*26*) explained that
253 TPC in peel was greater than in juice or pomace due to the presence of phenolic compounds such
254 as anthocyanins and quercetin glycoside molecules, found only in the peel region. Included in
255 TPC is phloridzin, a dihydrochalcone that is up to three times more concentrated in the skin than
256 in the flesh (*4*). With regard to ‘Verde Doncella’, TPC values were also greater in peel samples
257 when compared to pomace and juice samples. The single study that we have found reporting data
258 from ‘Verde Doncella’ has been conducted by Perez-Ilzarbe and co-workers (*16*). In this study,
259 the phenolic compounds in flesh, juice and skins of five apple varieties (Starking red, Reineta,
260 Golden Delicious, ‘Verde Doncella’ and Granny Smith), were identified by HPLC. Major
261 compounds quantified were cathechins, procyanidins, hidroxycinnamic acids and flavonoid
262 derivates. The study concluded that the phenolic content showed different patterns depending on
263 the part and cultivar of the fruit, highlighting that ‘Red Delicious’ polyphenols concentrations are
264 significantly higher than ‘Verde Doncella’ in peel, pomace and juice, a result in agreement with
265 our data.
266
267 *Antioxidant activity: Ferric Reducing Ability of Plasma/Antioxidant Power (FRAP) Assay*
268

269 Protocols used were based on studies by Benzie & Strain (*27*, *28*) with some modifications. To
270 prepare the FRAP reagent, these studies used a mixture of three solutions (TPTZ, Acetone
271 buffer, and $FeCl_2 \sum 6H_2O$). In every publication describing FRAP preparation, ethanol or acetone
272 was used to dissolve TPTZ powder, followed by water. In our study, neither water nor ethanol
273 was fully able to dissolve TPTZ; however, by using methanol, a more polar solvent, better results
274 were obtained. Antioxidant properties of apple extracts were evaluated to identify their capacity
275 to reduce iron from ferric (Fe^{+3}) to ferrous (Fe^{+2}). Antioxidant results are presented in **Table 3**
276 for 'Red Delicious' and 'Verde Doncella' apple peel, juice and pomace samples.
277
278 Antioxidant activity measured from peel appeared greater than pomace for both cultivars, which
279 is in accordance with previously observed TPC contents. The United States Department of
280 Agriculture (2010) reported Oxygen Radical Absorbance Capacity (ORAC) values of 'Red
281 Delicious' with skin greater than 'Red Delicious' without skin. This Institution also reported
282 'Red Delicious' ORAC values greater than all other fresh apple varieties tested, a result in
283 accordance with our TPC data.
284
285 Significant differences were found between antioxidant activity in apple peel and juice in both
286 varieties. 'Red Delicious' peel extract displayed significantly greater antioxidant activity (143
287 μmol eq. Trolox) when compared to 'Verde Doncella' (52 μmol eq. Trolox); this result was
288 consistent with the TPC contents found in both cultivars. On the contrary, antioxidant activities
289 in both apple juices were not significantly different (6.6 μmol eq. Trolox found in both cultivars).
290 Antioxidant activity data presented in our study is in agreement with data published by other
291 authors (*29*).

Determination of phenolics by HPLC

The characteristic HPLC chromatographic profile of apple samples in 'Red Delicious' and 'Verde Doncella' cultivars are presented in **Figures 1 to 4**. Of the four wavelengths (λ) tested for separating apple peel phenolic compounds, λ monitored at 280, 320, and 360 nm yielded UV-Spectra similar to pure compounds tested for detecting hydroxybenzoic acid derivatives, flavan-3-ols, dihydrochalcone, and hydroxycinnamic acid derivatives. Tsao and Yang (*7*) reported similar results when analyzing 'Red Delicious' apple peel. They also reported that the variation in wavelength provides advantages for simultaneous detection of major polyphenolics in fruit.

'Verde Doncella' chromatograms from peel samples contained a greater number of peaks when compared to 'Red Delicious'. In regard to 'Verde Doncella', a total of four minor peaks were found after phloridzin (**Figure 4**). As expected, HPLC profiles were more complex (in terms of number of compounds and peak area) for peel than for pomace and juice in both cultivars. The flavanol epicatechin (peak number 10), and the dihydrochalcone phloridzin (peaks 24 in 'Red Delicious' and 25 in 'Verde Doncella'), were the greatest peaks in the chromatographic profile of both cultivars. These results are in agreement with previous studies (*7*, *22*) that pointed out epicatechin and phloridzin as the most abundant compounds in apple peel.

A total of 14 compounds belonging to the five major families of phenolic compounds (flavanols, hydroxycinnamic acids, flavonols, dihydrocalcones and procyanidins were determined by HPLC.

315 Method sensitivity was achieved by using wavelengths at the maximum UV absortion (λ_{max}) for
316 different families of polyphenols. All standards gave high linearity within the calibration range.
317 Data with the optimum λ used for measurements, and the mean concentrations of compounds in
318 peel, pomace and juice samples are presented in **Table 4**.
319
320 ***Apple Peel***. As shown in **Figures 3** and **4**, the chromatographic profile of apple peel was more
321 complex in terms of the number of compounds and peak areas. Although different phenolic
322 distribution patterns can be observed among apple cultivars, it is known that apple peel has
323 substantially higher phenolic content and antioxidant activity than other fruit parts. For example,
324 Boyer and Liu (*2*) reported that apple peel contains the most phytochemical compounds
325 including procyanidins, catechin, epicatechin, chlorogenic acid, phloridzin, and quercetin
326 conjugates.
327 Epicatechin arose as the major phenol in both varieties, although 'Red Delicious' content (273
328 μg/g DW) was much higher than the one found in 'Verde Doncella' (135 μg/g DW). Other
329 compounds presenting relatively high concentrations for both cultivars were catechin,
330 chlorogenic acid and quercetin-3-glucoside. Two compounds presented a greater concentration
331 in 'Verde Doncella'; p-coumaric acid (reaching a value of 59 μg/g DW) and procyanidin B2
332 (118 μg/g fresh apple). On the contrary, the two other hydroxycinnamic acids were more
333 concentrated in 'Red Delicious'. Quercetin derivates were almost exclusively found in the peel
334 of both cultivars. However, major differences were observed between their contents as 'Red
335 Delicious' presented a total concentration of flavonols (333 μg/g fresh apple), more than twice as
336 large as those found in 'Verde Doncella' (135 μg/g fresh apple). Data clearly illustrates that even
337 if apple peel represents a minor percentage (around 10 %) of the whole fruit weigh, it is a major

source of phenolic compounds. Total phenol content was by far greater in the peel, with 1173 and 894 μg/g DW for 'Red Delicious' and 'Verde Doncella', respectively, which highlights the significance of apple peel as a polyphenol source in both varieties.

Apple pomace. Pomace chromatograms contained fewer peaks when compared to peel samples. 'Red Delicious' chromatograms included 21 peaks, while 'Verde Doncella' had 16 peaks. Phenolic compounds quantified as having the greatest concentration in apple pomace were flavanols (catechin and epicatechin), chlorogenic acid and procyanidin B2. With respect to quercetin derivates, only quercetin-3-galactoside and quercetin-3-O-rutinoside could be quantified in the pomace of 'Red Delicious', whereas no quercetin derivate could be detected in 'Verde Doncella' pomace. Burda et al. (*8*) previously reported that quercetin glycosides were found only in peel samples, after testing skin and flesh samples. Schieber et al. (*6*) reported the presence of quercetin 3-rhamnoside in dried apple seeds (nearly twice as much as the next quercetin glycoside). Despite our processed pomace samples included the seeds, quercetin-3-rhamnoside could not be detected in any of the pomace samples tested for either cultivar. Other compounds found in smaller quantities in both cultivar pomaces included: phloridzin and gallic acid. It is important to point out that the total content of flavanols, hydroxycinnamic acids, flavonols, phloridzin, procyanidin B2 and gallic acid content was always less in 'Verde Doncella' than in 'Red Delicious'.

Apple Juice. Total phenolics were much higher in 'Red Delicious' (89 μg/g FW) than in 'Verde Doncella' juice (58 μg/g FW). 'Red Delicious' juice contained greater polyphenol compound concentrations when compared to 'Verde Doncella' juice (87 μg/g *vs.* 58 μg/g). Valles et al. (*30*)

reported Spanish varieties had fewer polyphenol compounds, namely epicatechin, phloridzin, procyanidin B2, and trimer and tetramer procyanidins when compared to English apple varieties, results that are reflected by our data. Interestingly, no p-coumaric acid was found in juice (nor in pomace) of 'Verde Doncella', although a relatively high content of this compound was present in the peel. Catechin and epicatechin, together with chlorogenic acid and procyanidin B2 were identified as polyphenol compounds containing greater concentrations in juices from both cultivars. Previous studies (*14, 22, 31*) have identified catechin, epicatechin, chlorogenic acid, caffeic acid, and phloridzin in apple juices and ciders

Relationship between total phenolics and antioxidant activity

The relationship between the total phenolics (measured by both TPC and HPLC) and the antioxidant activity were examined by Pearson correlations. 'Red Delicious' peel had the greatest antioxidant activity according to the FRAP method, results which are consistent with the TPC values found for this cultivar. The FRAP activity of peel, pomace, and juice of both cultivars showed positive linear correlations with TPC (r=0,99) and total phenolics determined by HPLC (r=0.96 for 'Red Delicious'; r=0.99 for 'Verde Doncella'). When calculated against the major groups of polyphenols, the FRAP values were found to have the best linear correlation with the flavanols (r=0.99) and quercetin derivates (r=0.98) for 'Red Delicious,' and 'Verde Doncella' (r=0.95 and r=0.99, respectively). This assay clearly showed therefore that flavanols and flavonols (quercetin derivates) were the most important contributors to the antioxidant activity of both apple cultivars.

CONCLUSIONS

This study provides a database for color CIELAB coordinates, qualitative and quantitative phenolic composition, and antioxidant activity of 'Verde Doncella,' a valuable apple cultivar from northeast Spain that has received little to no attention in previous works focused on apple phenolic composition. 'Verde Doncella' results were compared to data from 'Red Delicious', a worldwide-cultivated variety.

Our results highlight that the phenolic distribution patterns as well as the antioxidant activity were quite different among cultivars. 'Verde Doncella' demonstrated lower TPC and total phenolics values measured by HPLC, especially with respect to total flavanols and quercetin derivates in the three parts of the fruit evaluated (peel, pomace and juice). These observations agreed with the low antioxidant activity values acquired for this variety. For both cultivars, the qualitative and quantitative distribution of phenolic compounds varied significantly between the peel, pomace and juice. At the individual compound level; flavanols and flavonols (quercetin derivates) were the most important contributors to the antioxidant activity of both apple cultivars. The high polyphenolic potential and antioxidant activities of 'Verde Doncella' in apple pomace, comparable to those of 'Red Delicious,' point out the possible health benefits in the consumption of this variety. This study shows that 'Verde Doncella' cultivar presents an interesting polyphenolic profile. The high total phenol content, especially p-coumaric acid and procyanidin B2 in peel as well as phloridzin in pomace, make this apple cultivar a valuable source of natural antioxidants.

ACKNOWLEDGEMENTS

This study was supported by the projects AGL2009-59 08501 (Programa Nacional de Proyectos de Investigación Fundamental), co-financed by the European Social Fund and by the Departamento de Ciencia, Tecnología y Universidad del Gobierno de Aragón, Research Group "Foods from Plant Origin". Fellowship funding JAE-Predoc/CSIC was supported by the Ministerio de Ciencia e Innovación (MICCIM).

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C-2: Include couple of recent references from Int. J. Food Properties.
C-3: In order to save printing space, include 4 figures into one as attached samples?

Table 1. Color coordinates obtained in the initial characterization of apples. The presented valuescorrespond to the average ± standard deviation of 200 measured points per sample.

Cultivar	L	a	b	C	h
'Verde Doncella'	76.4±3.9	0.0±3.0	49.8±2.2	49.9±2.3	90.1±3.4
'Red Delicious'	51.2±5.6	31.5±3.7	33.0±3.0	45.8±1.3	46.3±5.7

Parameter L* indicates brightness or lightness (0 = black, 100 = white)
a* indicates chromaticity on a green (-) to red (+) axis
b* indicates chromaticity on a blue (-) to yellow axis (+).
The hue is an angle in a color wheel of 360°, with 0°, 90°, 180° and 270° representing the hues red-purple, yellow, bluish-green and blue respectively, while Chroma is the intensity or purity of the hue (McGuire, 1992)

511 **Table 2**. Filters commercial name, type of filtration, pore size and average absorbance of filtrate
512 extracts assayed for filtration optimization.
513

Filter name	Type of filtration	Pore size (µm)	Average absorbance ± Standard Deviation*
1.Albet 9 cm 88663	Gravity; vacuum	25	0.43±0.01
2.Albet 11 cm DP140110	Gravity	15.5	0.22±0.01
3.Albet 13 cm	Gravity; vacuum	25	0.55±0.01
4.Filter-Lab 1250	Gravity	12	0.25±0.02
5.Filter-Lab M2BL0045142	Gravity	0.45	0.16±0.03
6.Machery-Nagel Co. 9 cm MN640d	Gravity; vacuum	2 to 4	0.17±0.05
7.Pall Life Sci. Corp. Acrodisc Syringe Filter	Syringe	0.2	0.20±0.01
8.Pall Life Sci. Corp. Acrodisc Syringe Filter	Syringe	0.45	0.16±0.03
9.Selex coffee filters Nº 4	Gravity	40	0.70±0.08
10.Schleicher & Schuell 0860 Rundfilter	Gravity; vacuum	7 to 12	0.26±0.01
11.Whatman Nº 1	Gravity	11	0.34±0.03
12.Whatman Nº 2	Gravity	8	0.30±0.01
13.Whatman Nº 4	Gravity	20 to 25	0.33±0.02

514 * Each filter was tested in triplicate (n=3)
515

Table 3. Mean and standard deviation (n=3) of total phenolic content (TPC) and antioxidant activity of 'Verde Doncella' and 'Red Delicious' fruit extracts using Folin-Ciocalteu and FRAP methods*. Data for peel and pomace is expressed in per gram dry weight (DW) apple whereas data for juice is expressed per gram fresh weigh (FW) apple

Parameter	VD Peel	RD Peel	VD Pomace	RD Pomace	VD Juice	RD Juice
TPC (mg GAE / g)	12,7±0,4 b	28±0,7 c	3,65±0,1 a	3,9±,2 a	0,89±0,04 A	0,86±0,05 A
Antioxidant activity (mg GAE / g)	52±1,1 b	143±7,4 c	21±05 a	24±1,2 a	6,6±0,3 A	6,6±0,4 A

* Within columns, values followed by the same letter were not significantly different ($p < 0.05$).

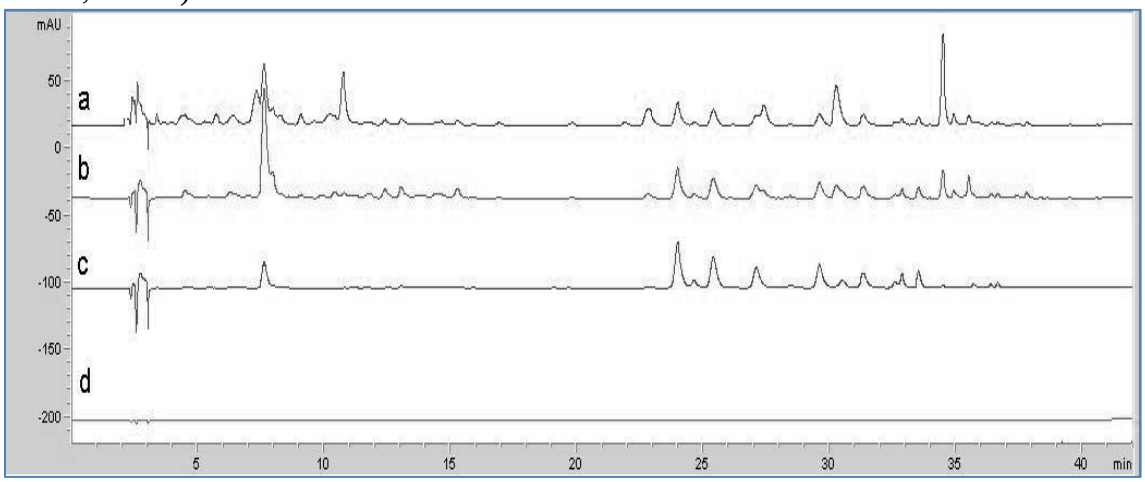
Table 4. Mean concentrations (μg/g fresh apple) and standard deviations (n=3) of individual and total polyphenols determined by HPLC. Data for peel and pomace is expressed in per gram dry weight (DW) apple whereas data for juice is expressed in per gram fresh weigh (FW) apple

Compound	Optimum λ (nm)	VD Peel	RD Peel	VD Pomace	RD Pomace	VD Juice	RD Juice
		μg/g DW apple				μg/g FA	
Catechin	280	118±7,1 c	160±6,0 d	56±5,6 a	80±4,3 b	19±1.4 A	25±1.1 A
Epicatechin	280	135±6,5 c	273±45 d	38±1,9 a	83±12,7 b	14±3.6 A	22±1.2 B
Total flavanols		253±9,4 c	433±67 d	94±7,2 a	163±8,3 b	**33±4.9 A**	**47±1.9 B**
Chlorogenic acid	320	94±1,8 c	133±10 d	26±5,9 a	40±3,0 b	14±3.6 A	20±1.5 B
Caffeic acid	320	19±7,1 ab	73±15 c	6±4,1 a	31±6,3 b	3.3±0.4 A	5.5±0.5 B
p-coumaric acid	320	59±19,4 c	27±1 b	nd	6±3,7 a	nd	3.9±1.6
Hydroxycinamic acids		171±27,6 c	233±46 c	31±8,1 a	77±10,7 b	**17±4.9 A**	**29±2.8 B**
Quercetin	360	49±6,5 a	58±7 a	nd	nd	nd	nd
Quercetin-3-galactoside	360	12±0,6 b	93±5 c	nd	3±1,3 a	nd	nd
Quercetin-3-glucoside	360	42±5,3 a	52±8 a	nd	nd	nd	nd
Quercetin-3-rhamnoside	360	12±1,8 a	29±3 b	nd	nd	nd	nd
Quercetin-3-O-rutinoside (rutin)	360	18±4,7 a	87±8 b	nd	13±1,7 a	nd	nd
Total flavonols		135±14,1 b	333±39 c		17±2,0 a	**nd**	**nd**
Phloridzin	280	51±4,1 c	73±1 d	22±0,6 b	13±1,7 a	2.3±0.9 A	1.2±0.3 A
Procyanidin B2	280	118±8,8 d	80±2 c	13±0,9 a	25±1,3 b	6.2±0.2 A	8.0±0.1 B
Gallic acid	280	14±8,8 ab	21±7 b	9±3,1 a	10±1,3 a	60 B	3.1±0.9 A
HPLC total phenols		**894±133 c**	**1173±267 d**	**169±37,5 a**	**420±58 b**	**58 A**	**89 B**

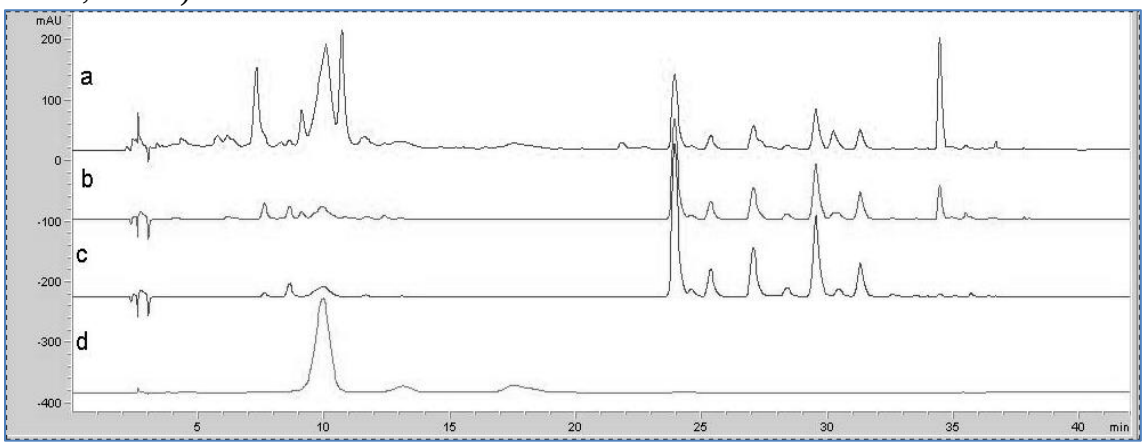
nd = not detected

Figure 1. HLPC chromatograms.

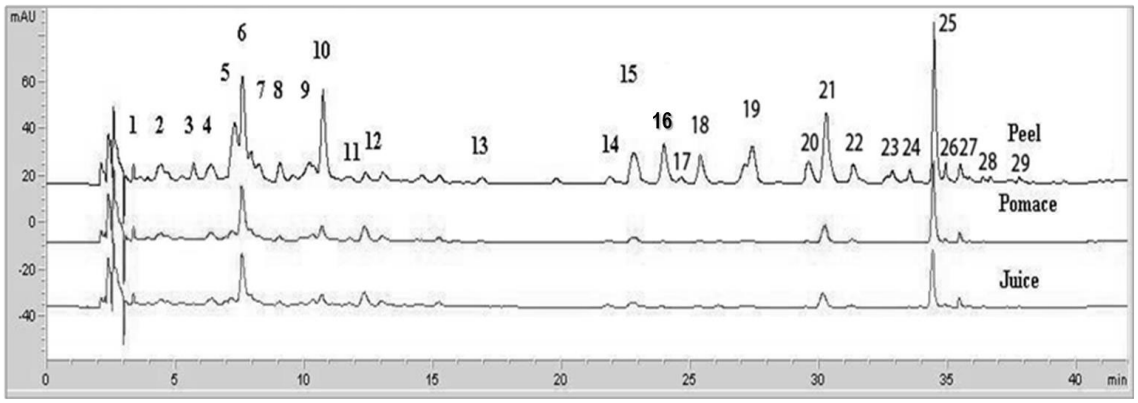
a) 'Verde Doncella' apple samples (peel) measured at different wavelengths (a=280, b=320, c=360, d=520).



b) 'Red Delicious' apple samples (peel) measured at different wavelengths (a=280, b=320, c=360, d=520).

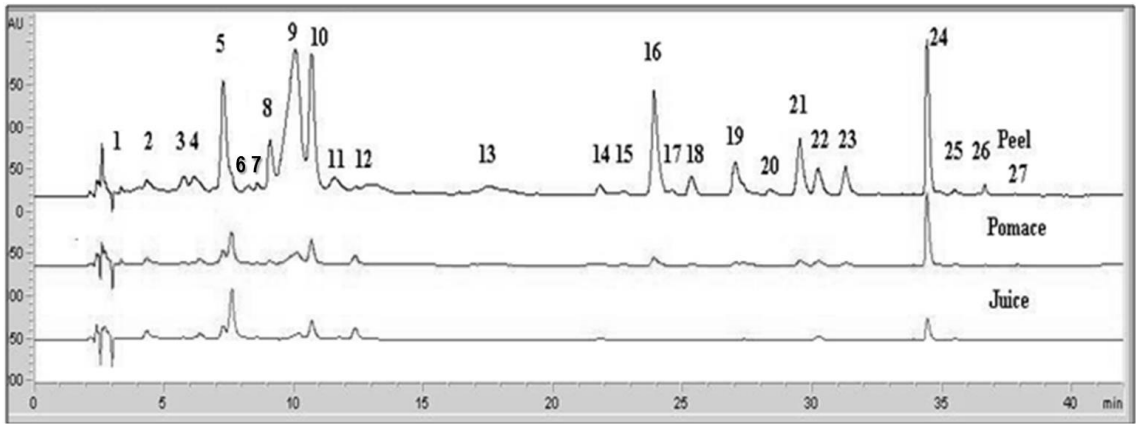


c) Peel, pomace and juice extracts from 'Verde Doncella' read at 280 nm.



(1) Gallic Acid; (2) Procyanidin B1; (3) Unknown procyanidin dimer; (4) Catechin; (5) Procyanidin B2; (6) Chlorogenic Acid; (7) Unknown procyanidin dimer; (8) Caffeic Acid; (9) anthocyanin; (10) Epicatechin; (11) Cyanidin-3-rutinoside; (12) Unknown procyanidin dimer; (13) p-Coumaric Acid; (14); Unknown procyanidin dimer; (15) 3-hydroxyphloretin 2-xyloglucoside; (16) Quercetin 3-galactoside; (17) Rutin; (18) Quercetin 3-glucoside; (19) Quercetin derivative; (20) Unknown phloretin derivatve; (21) Phloretin 2´xyloglucoside; (22) Quercetin 3-rhamnoside; (23) Unknown phloretin derivative; (24) Unknown phloretin derivative; (25) Phloridizin; (26) Unknown; (27) Hyperin; (28) Avicularoside; (29) Quercetin.

d) Peel, pomace and juice extracts from 'Red Delicious' read at 280 nm.



(1) Gallic Acid; (2) Procyanidin B1; (3) Unknown procyanidin dimer; (4) Catechin; (5) Procyanidin B2; (6) Chlorogenic Acid; (7) Cyanidin-3-galactoside; (8) Caffeic Acid; (9) Unknown procyanidin dimer; (10) Epicatechin; (11) Cyanidin-3-rutinoside; (12) Procyanidin dimer; (13) p-Coumaric Acid; (14); Procyanidin dimer; (15) 3-hydroxyphloretin 2´-xyloglucoside; (16) Quercetin 3-galactoside; (17) Rutin; (18) Quercetin 3-glucoside; (19) Quercetin 3-xyloside; (20) 3´-hydroxyphloretin 2´glucoside; (21) Quercetin 3-arabinoside; (22) Phloretin 2-xyloglucoside; (23) Quercetin 3-rhamnoside; (24) Phloridizin; (25) Unknown; (26) Quercetin 3-α-L-arabofuranoside; (27) Quercetin.