Comparison of phenolic compounds profile and antioxidant properties of different sweet cherry (*Prunus avium* L.) varieties

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Abstract

In the present work, three Spanish local varieties of *Prunus avium* (L.), as well as two foreign varieties were studied. The content of total phenols, flavonoids, anthocyanins, glucose and fructose of methanolic extracts from ripe fruits of each variety were analysed. A phytochemical profile of these cultivars was performed by UHPLC-qTOF-MS. The employed chromatographic method allowed a clear and rapid separation of the three main phenolic compound groups present in the extracts: hydroxycinnamic acids, anthocyanins and flavonoids. In addition, the extracts DPPH• radical scavenging ability, as well as their capacity to affect xanthine /xanthine oxidase system, were determined. Finally, variations in ROS intracellular concentrations in HepG2 cell line cultures treated with cherry extracts were measured through DCFH-DA assay. All extracts showed a significant inhibitory effect on the xanthine/xanthine oxidase system. Differences between *in vitro* and in cell culture results evidence the interaction among the phenolic compounds of the extract.

Keywords: *Prunus avium*, Polyphenols, Antioxidant activity, Xanthine oxidase, Anthocyanins

1. Introduction

Prunus avium L. is a tree belonging to the *Rosaceae* family. Their fruits are popular and attractive edible products, valued for consumed unprocessed as well as used to make products such as juice, jams, and alcoholic beverages. Taste, colour, sweetness, sourness and firmness are important quality characteristics that can influence consumer acceptance. Furthermore, intake of sweet cherry has been associated with beneficial health effects (Nawirska-Olszańska *et al*., 2017). These fruits are rich in nutrients and antioxidant compounds, constituting an example of a food whose consumption is thought to prevent chronic and degenerative diseases (Martini *et al*., 2017). The phenolic content of cherries contributes to these beneficial health effects. The intake of polyphenols has been associated with a decrease in cardiovascular diseases and cancer risk (Tresserra-Rimbau *et al*., 2014). In this respect, these fruit extracts exhibit free radical scavenging activities, and in consequence help to prevent cell oxidative injury, exhibiting anti-inflammatory and antitumoral properties. In addition, sweet cherry consumption is associated with a lower risk of gout attacks and arthritis as well as gout-related pain reduction (Singh *et al*., 2015). Other potential health properties of sweet cherries are the ability to reduce blood pressure, control of body weight, diabetes, and prevention of Alzheimer's disease (Kent *et al*., 2016; Wu *et al*., 2014).

Anthocyanins are the main phenolic compound group present in sweet cherries. These substances are responsible for the red skin and flesh colour of these fruits. For consumers, the dark red colour of cherries is an important indicator of maturity and quality, and is directly correlated with anthocyanin content (Liu *et al.*, 2011). 3-*O*-glucoside and 3-*O*-rutinose of cyanidin are the major anthocyanins, although 3-*O*-rutinose of peonidin or pelargonidin appear in lower amounts (Martini *et al.*, 2017). Flavan-3-ols and flavonols are also important phenolic substances present in *P. avium* fruits, epicatechin and quercetin-3-*O*-rutinose being the main compounds of these classes (de Pascual-Teresa *et al.*, 2000). Finally, hydroxycinnamic acids and hydroxybenzoic acid derivatives have also been identified in sweet cherries (Wang *et al.*, 2017).

Antioxidant activity and phenolic composition are genotype dependent and are influenced by climatic factors (Fanidis *et al.*, 2010). Therefore, in order to avoid variations due to environmental conditions, this study was carried out with five different varieties from the Jerte Valley (Spain). In this location, cherries are grown in a traditional way on terraces carved out of the high mountainsides with a favourable microclimate. When they are at optimum ripeness, they are hand-picked using chestnut baskets, following age-old traditions, which preserve their freshness, quality attributes and level of health promoting compounds (Correia *et al.*, 2017). Many *P. avium* varieties are cultivated in this valley, from the most commercial ones: Van and Sunburst, cherries of large size and dark skin colour; to native varieties, such as Jarandilla, Pico Colorado, and Navalinda, smaller than the commercial varieties. These last two local varieties are certified cherries (bearing the quality seal of Protected Denomination of Origin) and valued by consumers for their taste and high quality. Sixty percent of local variety production is destined for international markets, so they can be found in many countries, especially in Europe.

In recent years, the screening of crude extracts using techniques such as LC-MS (liquid chromatography–mass spectrometry) has become a powerful tool for chemical identification due to its high specificity, separation efficiency and structural information capability. In addition, high-resolution mass spectrometric methods based on qTOF-MS (Quadrupole Time of Flight Mass Spectrometry) have been widely used for qualitative studies since they have many advantages, such as a large amount of structure-related information, and the exact mass and fragmentation pattern is presented in a highly sensitive and convenient manner (Ganzera & Sturm, 2018).

The profile and content of phenolic compounds in cherry cultivars have been studied extensively and important contributions regarding the components present in these fruits have been attentively reported.

Their phytochemical composition, focusing on the phenolic fraction, has been investigated using both, targeted and non-targeted approaches, through LC-ESI-MS experiments and many investigations aimed at the identification of phenolic compounds in cherries have been performed (Nawirska-Olszańska *et al*., 2017; Wang *et al*., 2017; Casedas *et al*., 2016). These studies have found significant differences in chemical composition between different cultivars.

The aim of the present work is to make a comparative study of the above-mentioned cherry varieties in order to evaluate differences in their phenolic composition, their nutritional potential and health qualities. Glucose and fructose concentration, as well as total phenol, flavonoid and anthocyanin content, which are primarily responsible for the antioxidant effect of these fruits, were quantified. The main phenolic compound profiles of each cherry cultivar were obtained using ultra high performance liquid chromatography coupled to a quadrupole-time-of-flight mass spectrometry (UHPLC/qTOF-MS). Main anthocyanin and flavonoid compounds were quantified. The antioxidant activity of methanol extracts of the five mentioned cherry varieties was analysed and related to their chemical composition. In this respect, ability of the extracts to scavenge DPPH• radical and their effects on the xanthine/xanthine oxidase system were analysed. Likewise, the effect of the different cherry varieties on intracellular HepG2 cell culture ROS levels was determined in normal culture and under hydrogen peroxide-induced oxidative stress.

2. Material and methods

2.1. Chemicals and reagents

Purified water was obtained using the Milli-Qplus185 system (Millipore, Billerica, MA, USA). LC–MS grade methanol (MeOH) was purchased from Honeywell. Standards of chlorogenic acid (5-*O*-caffeoylquinic acid), cyanidin-3-*O*-glucoside and quercetin-3-*O*-glucoside were obtained from Extrasynthesis Phytochemicals (Genay Cedex, France). Formic acid was purchased from Aldrich (St. Louis, MO). Standards stock solutions were prepared in methanol (10 mg/L).

2.2. Plant material and preparation of extracts

Fruits from five different varieties of *P. avium*: Navalinda, Jarandilla, Pico Colorado, Van and Sunburst from the Jerte Valley (Spain) were harvested in June of 2015 at their commercial ripening stage. For each variety, about 500 g of fruits were sampled randomly from 15 trees from different areas of each crop field. Fruits were stored at -20 ºC until use.

25 g of stoning fruits of each variety were ground with liquid nitrogen using a mortar and then extracted using 250 mL of methanol with 0.1% of commercial HCl (37% w/w). Samples along with solvent were kept in a beaker and sonicated four times at room temperature using an ultrasonic bath for 10 minutes each time (Ultrasons Selecta 40 KHz). During the process the extracts were protected from light. After centrifugation (3500 rpm for 5 min), supernatants were concentrated in a rotavapor at 40 °C, and stored at 4 °C until use. Extract yield was 13.82% (w/w) for Navalinda; 14.72% for Jarandilla; 17.10% for Pico Colorado; 20.38% for Van; and 12.03% for Sunburst.

2.3. Determination of total phenolic content

The Folin-Cicalteau reagent was used for the determination of total phenolic content in the cherry extract samples (Muñoz Mingarro *et al.,* 2015). Briefly, 5 µL of cherry extracts at concentrations between 10 and 0.625 mg/mL were mixed with 80 µL of 10% Folin–Ciocalteau reagent, and after five minutes 160 µL of 7.5% Na_2CO_3 were added and mixed. Absorbance was measured, after 30 min, at 765 nm. Quantification was done on the basis of a standard curve of gallic acid. All tests were performed in triplicate. Results were expressed as mg of gallic acid equivalents (GAE) in 100 g of fresh cherries.

2.4. Determination of total flavonoid content

The total flavonoid content of the extracts were determined by aluminium chloride colorimetric method (Muñoz Mingarro *et al*., 2015), performing all measurements in triplicate. The absorbance was measured at 510nm. Results were expressed as mg of epicatechin equivalents (EE)/100 g of fresh cherries.

2.5. Determination of total anthocyanin content

The total anthocyanin content was determined using the pH differential spectroscopic method (Giusti & Wrolstad, 2001). 500 µL of each extract diluted in a 10 mg/mL concentration with methanol plus 0.1% commercial HCl were mixed with 7 mL of two different buffers: 0.2 M KCl pH 1.0 and 1 M CH_3COONa pH 4.5. After incubation for 15 minutes at room temperature, absorbance values were measured at 510 and 700 nm. All extracts were analysed in triplicate.

Absorbance differences were then calculated as follows:

$$Abs = [(Abs510-Abs700)pH1.0-(Abs510-Abs700)pH4.5].$$

Anthocyanin concentration was estimated following the equation below:

C (mg/L) = [Abs/ε*L]*MW*DF* 10^3

Where: ε is cyanidin-3-*O*-glucoside molar extinction coefficient = 26900 L/mol*cm; MW is cyanidin-3-O-glucoside molecular weight = 449.2 g/mol; L is the cuvette optical path length = 1 cm; and DF is the dilution factor.

The total anthocyanin content was expressed as mg of cyanidin-3-*O*-glucoside equivalents (CGE)/100 g of fresh cherries.

2.6. Determination of cherries colour

The chromatic characteristics of studied freeze fruits were measured in 20 cherries of each variety with a Konica Minolta CM-3500d spectrophotometer, with 8 mm diameter measurement area, following CIE (Commision International de l´Eclairage) system. D65 was used as standard illuminant, which attempts to portray standard illumination conditions in open air. The observer angle was 10º. CIE colour space is defined by three coordinates: L*: lightness black (opaque)/white (transparent) coordinate; a*: red/green coordinate, positive values indicate redness, and negative ones greenness; b*: yellow/blue coordinate, positive values indicate yellowness and negative values blueness (Gonçalves et al., 2007). Hue angle, which represents the dominant colour as perceived by an observer, was calculated as arctan (b*/a*). Chroma was obtained following the formulae (a*2+b*2)1/2 (Jha, 2010). Each measurement is the average of three measurements performed equidistant points of each cherry.

2.7. Determination of glucose and fructose content

Glucose and fructose extract concentrations were quantified through a commercial glucose/fructose enzyme kit (Megazyme, catalog no. K-FRUGL; Xygen Diagnostics Inc., Burgessville, Ontario, Canada), according to supplier specifications. Determinations were measured in triplicate. Results were expressed as g of glucose or fructose in 100 g of fresh cherries.

2.8. Sample preparation and LC-qTOF-MS analysis

Samples were dissolved in methanol by adding 1000 µL/10 mg of dried sample and followed by vortex mixing for 5 min.

Samples were analysed on a 1290 Infinity series UHPLC system coupled through an electrospray ionisation source (ESI) with jet stream technology to a 6550 iFunnel qTOF-MS system (Agilent

Technologies, Waldbronn, Germany). For the separation, an injection volume of 2 µL was introduced to a reversed-phase column (Zorbax Eclipse XDB-C18 4.6 × 50 mm, 1.8 µm, Agilent Tech.) and kept at 40°C. The system was operated at 0.5 mL/min flow rate consisting of solvent A, water with formic acid (FA) at 0.1%, and solvent B, methanol, as mobile phases. Gradient started at 2% B (0-5 min), later a linear gradient from 2 to 50% B (5-10 min), then up to 95% B (10-18 min), kept at 95% B for 2 min (18-20 min), and returned to starting conditions in 1 min to finally keep the re-equilibration at 2% B until 25 min. Detector was operated in full scan mode (*m/z* 50 to 2000) in positive and negative ESI mode at a scan rate of 1 scan/s. Accurate mass measurement was assured through an automated calibrator delivery system that continuously introduced a reference solution containing masses of *m/z* 121.0509 (purine) and *m/z* 922.0098 (HP-921) in positive ESI mode; whereas *m/z* 112.9856 (TFA) and *m/z* 922.009798 (HP-921) were introduced as a reference solution in negative ESI mode. The capillary voltage was ± 4000 V for positive and negative ionisation mode. The source temperature was 225 °C. The nebulizer and gas flow rates were 35 psig and 11 L/min respectively, using a fragmentor voltage of 75 V and a radiofrequency voltage in the octupole (OCT RF Vpp) of 750 V. For the study, MassHunter Workstation Software Data Acquisition version B.07.00 (Agilent Technologies) was used for control and acquisition of all data. LC-qTOF-MS data processing was performed in MassHunter Qualitative Analysis (Agilent Technologies) Software version B.08.00.

Cyanidin-3-*O*-Glucoside and Quercetin-3-*O*-rutinoside were quantified by means of direct calibration with pure standards. Direct calibration was assayed in duplicate at least four concentrations, covering the expected values.

2.9. *In vitro* radical scavenging activity

2.9.1. DPPH test

The *in vitro* scavenging free radical activity was tested towards a stable free radical α, α-diphenyl-β-picrylhydrazyl (DPPH•), which present violet coloration (absortion band 517 nm). The colour turns from purple to yellow when DPPH• radical is reduced by an extract antioxidant compound. The protocol described by Acero & Muñoz-Mingarro (2012) was used to determine extracts scavenging activity. Results were expressed as the mean IC_{50} of the three replicates.

2.9.2. Superoxide anion scavenging assay

193 Xanthine oxidase catalyses the oxidation of xanthine and hypoxanthine to uric acid, generating
194 superoxide anions. These ROS cause the nitroblue tetrazolium (NBT) reduction. The superoxide anion
195 scavenging capacity of the extracts was measured in triplicate by colorimetric determination, following
196 Morales *et al*. (2011) protocol. Results are shown as the NTB reduction inhibition in comparison to control.

197 **2.10. Cell culture radical scavenging activity**

198 **2.10.1. Cell culture**

199 Human hepatocarcinoma HepG2 (ECACC-85011430) was obtained from the European Collection of
200 Cell Cultures (Health Protection Agency, UK). Cell line was maintained in EMEM (Eagle´s Minimum
201 Essential Medium) which was supplemented with 2 mM glutamine, 1% non-essential amino acids, 10%
202 foetal bovine serum (FBS) (HyClone, Logan, UT, USA), and 1% antibiotics (10. 000U of penicillin and 10
203 mg/mL of streptomycin) and incubated at 37 °C under a 5% CO_2 atmosphere. During ROS assays, cells
204 were incubated in medium supplemented with 1% FBS to prevent the interaction between serum
205 components and the phenolic compounds of the extracts that may result in cytotoxic artefacts (Long &
206 Halliwell, 2011).

207 **2.10.2. Intracellular ROS measurement**

208 Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay was used for the detection of intracellular
209 oxidative species (Muñoz Mingarro *et al*., 2015). In 96-well plates 8000 HepG2 cells per well were seeded
210 in 150 µL of EMEM medium supplemented with 1% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 1%
211 non-essential amino acids, and 2 mM L-glutamine. Plates were kept in an incubator for 24 h. Cells were
212 washed and incubated for 30 min with DCFH-DA (20 µM) at 37 °C. To assess the antioxidant activity,
213 different concentrations of the extracts dissolved in EMEM medium supplemented with 1% FBS were
214 added. At this time measurement of fluorescence began at an emission wavelength of 530 nm and an
215 excitation wavelength of 485 nm. DCFH fluorescence was then measured for 90 min, every 15 min. Results
216 are given as percentage of fluorescence related to control after 90 min of treatment.

217 In order to study the effect of extracts over cells under oxidative stress, a new plate was prepared as
218 described above. After 24 h cells were incubated with increasing concentrations of the extracts dissolved
219 in EMEM medium supplemented with 1% FBS. After 24 h cells were washed with PBS (pH 7.4) and
220 incubated with DCFH-DA (20 µM) in PBS at 37 °C. After 30 min, 200 µM H_2O_2 were added as an oxidative

stress inducer. Measurement of fluorescence began at the time H_2O_2 was added. The results were expressed as a percentage of fluorescence related to stressed control, after 90 min of stress induction (H_2O_2 addition).

2.11. Statistical analysis

Colour characteristics (L*, a*, b*, Hue angle and Chroma), glucose and fructose concentration, total phenolic, flavonoid and anthocyanin content, *in vitro* scavenging activity results, as well as intracellular ROS data were analysed for statistical significance ($P < 0.05$) by ANOVA followed by Bonferroni's test.

Dependent variables were compared using a linear Pearson correlation, in order to identify relationship among them ($P<0.05$).

3. Results

3.1. Determination of total phenolic, flavonoid and anthocyanin content

There are significant differences between cultivars in these three colorimetric measures. Figure 1 shows that the Van cultivar contains the highest amounts of total phenolic and anthocyanin content, with values of 284.48 ± 3.07 GAE mg/100 g of fresh cherries, and 52.75 ± 2.42 mg of CGE/100 g of fresh cherries respectively. With regard to flavonoid content, Van and Pico Colorado have similar concentrations of 180.51 ± 3.80 and 186.83 ± 0.52 EE mg/100 g of fresh cherries respectively, with no significant differences. Sunburst cultivar by contrast, showed the lowest levels of all the analysed variables.

3.2. Determination of colour

The chromatic parameters of fruits L*, a*, b*, chroma and hue angle are shown in Table 1. Significant differences were found in all parameters between some cherry varieties. It is noticeable that chroma differences are statistically significant between all varieties ($P>0.05$). Regarding L*, we can observe that cherries with the darkest colour, Van cherries, showed significantly lower values. Values of L*, are in accordance with values of a* and b* and, in consequence, with chroma and hue angle.

3.3. Determination of glucose and fructose content

Concentrations of glucose and fructose in cherry extracts are presented in Table 2. Significant differences in the content of both sugars were found between cultivars, except for Navalinda and Pico Colorado, which showed similar amounts of glucose and fructose. Van is the cherry with the highest content of sugars, while Jarandilla showed the lowest concentration in both monosaccharides. Glucose concentrations ranged from 2.61 to 6.52 g/100 g of fresh cherries, while fructose concentrations moved

from 3.18 to 6.89 g/100 g of fresh cherries. Fructose appeared in higher concentrations than glucose in all cultivars.

3.4. LC-qTOF-MS analysis

The chromatographic method was performed using optimized conditions as a result of the study of the complex composition of the sample, and our previous knowledge. The final method, described in the material and methods section (2.8), provided a very efficient separation in less than 25 min. This method enables a rapid and extraordinary efficient separation of the three principal groups in cherry extracts, allowing easy identification of the principal peaks obtained amidst others from less concentrated compounds.

After the separation, the detection by ESI-qTOF-MS allowed us to obtain the accurate masses of the analytes, which led to the study of the most significant compounds present in the samples. Eight main compounds were identified, some of them, by comparison of the retention times and spectra with reference standards. The remaining compounds were tentatively identified by matching the accurate masses (± 5 ppm error) of the detected molecular ions and their in-source fragmentation patterns. These patterns were obtained from those compounds previously reported in the literature, and after comparison against online databases as FOODB (http://foodb.ca)) and by using the tool developed by our University CEU massmediator (http://ceumass.eps.uspceu.es/mediator), searching against METLIN (http://metlin.scripps.edu) and HMDB (http://hmdb.ca).

The mass spectrum data along with peak assignments for the most abundant identified compounds are described in Table 3.

Compounds **1**-**9** were studied extensively because they represented those with the highest concentrations within the chromatogram. These principal compounds present in our samples, can be classified into three main groups of phenolic compounds, ordered by retention time: hydroxycinnamic acids, anthocyanins and flavonoids.

Compounds (**1**) and (**2**). High amounts of chlorogenic acids were detected in the Extracted Ion Chromatogram (EIC). As it is shown in Figure 2B, two main peaks at tR = 7.7 min and 7.9 min, and two additional small peaks at tR = 8.9 min and 9.6 min were registered. They produced a molecular ion $[M-H]^-$ at *m/z* 353 which was consistent with the molecular formula of $C_{16}H_{18}O_9$. *O*-caffeoylquinic (CQA), which

was the main quinate derivative found in the cherry cultivars studied, which agreed with previous reported data (Martini *et al*., 2017). Esterification can occur at positions 1, 3, 4 or 5 of the quinic acid moiety, resulting in four positional isomers, Figure 2, which made it difficult to distinguish between them. Only one CQA positional isomer, 5-CQA, was identified by comparison of the retention time and mass spectral data with the commercial standard. Other positional isomers detected were assigned based on in-source fragmentation pattern MS positive-ionisation mode, which involved the cleavage of caffeoyl and quinic acid moieties. Thus, the predominant ion for this peak, in the full MS positive-ionisation mode, was $[M+H]^+$ at *m/z* 355. The fragment at *m/z* 163 $[M+H\text{-}192Da]^+$ (loss of quinic acid unit) corresponded to caffeic acid (CA). The retention time was compared with previously studied caffeoylquinates, and with the described elution order: 3-CQA, 5-CQA, 4-CQA. Compounds (**1**) and (**2**) being the most abundant isomers within the chromatogram, were then identified as 3-*O*-caffeoylquinic (3-CQA) and 4-*O*-caffeoylquinic (4-CQA) respectively. Although, naturally occurring phenolic cinnamic acids occur in the *trans* configuration, exposure to UV light causes phytochemical isomerisation to the *cis* configuration. Thus, geometrical *cis*/*trans* isomers of caffeic acid were also a possibility (Parveen *et al*., 2011).

Compound (**3**). Three peaks were located in the EIC: a high peak at tR = 8.7 min, and two additional small peaks at tR = 9.5 min and tR = 9.8 min. They produced a molecular ion $[M-H]^-$ at *m/z* 337 which was consistent with the molecular formula of $C_{16}H_{18}O_8$. Peaks were tentatively assigned as monoacyl *para*-coumaroylquinic acids (pCoQA) positional isomers (Figure 2B). ESI+-MS of these compounds gave predominant ions at *m/z* 339 $[M+H]^+$ and a base peak at *m/z* 147 $[M+H\text{-}192Da]^+$ (loss of quinic acid unit) which corresponds to *p*CoQA. These results are consistent with previous studies and with the following described elution order: 3-*p*CoQA, 5-*p*CoQA, 4-*p*CoQA. Compound (**3**) was identified as 3-*O*-*p*-coumaroylquinic (3-*p*CoQA), and was the most abundant isomer of this class within the chromatogram (González-Gómez *et al*., 2010).

Compound (**4**) eluted at tR 7.61 min, had a positively charged molecular ion $[M]^+$ at *m/z* 595, yielding by in-source fragmentation base peaks at m/z 449 $[M+H\text{-}146Da]^+$ and *m/z* 287 $[M+H\text{-}2Hex]^+$. They arose from the loss of glycan moiety and suggested that the aglycone was cyanidin. The base peak at *m/z* 449 indicated that both glycosides were connected to different phenolic hydroxyls and the compound was therefore tentatively identified as cyanidin-3,5-*O*-dihexoside (Parveen *et al*., 2011).

Compound (**5**) that eluted at tR 7.61 min, had a positively charged molecular ion $[M]^+$ at *m/z* 449 and was identified, by comparison of the retention time and mass spectral data with commercial standard, as cyanidin-3-*O*-glucoside.

Compound (**6**) that eluted at tR 7.61 min, had a positively charged molecular ion at *m/z* 595. It yielded by fragmentation in-source ions at m/z 449 $[M+H-146Da]^+$, resulting from the loss of a rhamnosyl unit, and at *m/z* 287 $[M+H-2Hex]^+$ arising from the loss of a glucosyl-rhamnosyl unit. These results suggested that the aglycone was cyanidin. In this case, both glycosyls were connected to the same phenolic hydroxyl, Figure 3A, and the compound was therefore tentatively identified as cyanidin-3-*O*-rutinoside.

Compound (**7**) eluted at tR 7.61 min, had a positively charged molecular ion at *m/z* 609, yielding by fragmentation in-source ions at *m/z* 301 $[M+H-2Hex]^+$, which suggested that the aglycone was peonidin. Compound (**7**) was therefore tentatively characterised as peonidin-3-*O*-rutinoside.

Compound (**8**) that eluted at tR 7.61 min, presented a molecular ion at *m/z* 663 $[M+Na]^+$ which might be coherent with isorhamnetin-*O*-glycosylated derivative (Table 3, Figure 3C).

Quercetin-3-*O*-rutinoside (**9**) was identified by comparison of the retention time and mass spectral data with commercial standard. The analysis of MS spectra revealed the loss of 146 Da (rhamnose group) and 162 Da (glucose group) to produce an *m/z* 301 (quercetin aglycone) daughter ion.

3.5. *In vitro* radical scavenging activity

All cultivars showed dose dependent DPPH• radical scavenging activity. Cultivars with the highest content in phenolic compounds, Van and Navalinda, exhibited the greatest scavenging capacity, with IC_{50} of 3.97 ± 0.02 mg/mL and 4.31 ± 0.06 mg/mL respectively. However, these cultivars did not show significant differences (ANOVA-Bonferroni $P<0.05$) in this assay although they present significant differences between their phenolic content. Likewise, the three varieties with lower phenolic concentration values presented the highest IC_{50} for the DPPH• scavenging ability (Jarandilla: 5.30 ± 0.03 mg/mL; Pico Colorado: 9.11 ± 0.09 mg/mL; 5.14 ± 0.04 mg/mL). However, Pico Colorado, the cultivar with the lightest colour, had a phenolic concentration higher than Jarandilla and Sunburst, but a significantly lower antioxidant capacity. These results suggest that there must be important qualitative differences, and not only quantitative ones, between cultivars in relation to phenolic composition. This fact was confirmed in the phytochemical analysis.

The xanthine oxidase enzyme catalyses the hypoxanthine and xanthine oxidation to uric acid, generating ROS during the process. The superoxide anion scavenging capacity of the extracts were measured through NBT reduction inhibition.

Figure 4 shows the percentage of NBT reduction inhibition for each cultivar at each assayed concentration. Results could be a consequence to both an inhibition of xanthine oxidase enzyme and/or a radical scavenging activity of extracts active metabolites. As can be observed in Figure 4, all extracts, even at lowest concentrations, were able to inhibit more than 50% of the NBT reduction. The Navalinda extract effect is particularly noticeable with a 90% inhibition. Pico Colorado again exhibited the lowest scavenging capacity, although it only presented significant differences with other cultivars at the lowest concentrations except with Navalinda, at the highest one.

3.6. Cell culture radical scavenging activity

Figure 5A shows the development of fluorescence measurements of the intracellular ROS study at the assay end point (90 min). As in other assays, the antioxidant effect of Van cultivar is again noticeable. This extract is the only one able to reduce ROS levels in respect to control, at all studied concentrations, showing a dose-response function. The other cultivars exhibited a different trend, similar in all of them. Extracts of Navalinda, Jarandilla, Pico Colorado and Sunburst induced a rise in ROS concentration at the highest concentrations, while at lower doses showed an antioxidant effect reducing the fluorescence percentage respect to control (100%). This trend was specially marked in Navalinda and Pico Colorado.

In order to study the ability of cherry extracts to prevent oxidative stress, cells were pre-treated with the extracts before they were stressed with H_2O_2. All extracts were able to reduce ROS concentration after stress in a dose dependent manner (Figure 5B). Van and Pico Colorado were the most efficient antioxidant extracts in this assay. Both extracts improved redox intracellular status in cells under induced oxidative stress.

4. Discussion

Several epidemiological studies have demonstrated that phenolic compounds diet consumption is related to a risk reduction of heart disease, arthritis, neurodegenerative disorders, and certain cancers among other illnesses (Pacifico *et al*., 2014). Phenolic composition in cherries may differ from one variety to anothers (Hayaloglu & Demir, 2016). For this reason, the study of phytochemical composition, related to

quality and quantity of phenolic compounds in these fruits is highly interesting. Similar studies have been carried out with different sweet cherry cultivars from other countries (Nawirska-Olszańska *et al*., 2017; Martini *et al*., 2017; Mirto *et al*., 2018). However, taking into account that Spain is third in sweet cherry production in Europe, and fifth worldwide, phenolic profile of the cultivars analysed, obtained by UHPLC-qTOF-MS, and their antioxidant activity are very interesting. Moreover, the assayed chromatographic method is a new method developed by the CEMBIO group that allows easy and rapid identification of different phenolic groups.

As shown in Figure 1, there were significant differences in total phenolic concentration between cultivars, ranging from 284.48 to 85.9 mg GAE/100g of fresh cherries. Navalinda and Van are the varieties with higher concentrations of these compounds, while the Sunburst cultivar was the one with the lowest amount of phenols. Nawirska-Oszańska *et al.* (2017) found total phenolic concentrations between 84.96 and 162.21 mg GAE/100 g of fresh cherries in 24 sweet cherry cultivars originating in the Czech Republic. Three of the five studied cultivars, Navalinda, Pico Colorado and Van, exhibited important phenolic quantities related to these authors' data. In the Jerte Valley, cherries are handled with extreme care, and this fact, among others, could affect active principle concentrations (Correia *et al*., 2017). Among the phenolic compounds found in these fruits, hydroxycinnamic and hydroxybenzoic acids, anthocyanins and flavonoids are the main constituents (Liu *et al*., 2011).

There are significant differences between cultivars with respect to anthocyanin and flavonoid content, as expected due to the differences in cherry colour. Van were the darkest cherry (lower L* value, Table 1), whereas Pico Colorado exhibited a red-orange colour both in skin and in flesh, being the lightest cherry of the study, with higher L*, chroma and hue angle. Flavonoids are phenols that give a yellow colour to fruits, therefore the highest b* values were observed in Pico Colorado cherries, which had the highest flavonoid content (186.83 EE mg/100 g of fresh cherries). Pico Colorado is an orange-yellow coloured cherry, the other cultivars being dark red, colour related to anthocyanin skin fruit content (Viljevac *et al*., 2012). There is a negative correlation between anthocyanin content and b* ($r=-0.9017$, $P=0.037$), revealing a relation between anthocyanins and the colour blue. However higher amounts of anthocyanins did not give higher a* values (redness). Similar results have been reported by Gonçalves *et al.* (2007). This phenomenon is related to an "inversion area" described by Eagerman *et al*. (1973), where the increased pigment concentration affects darkness and chroma, and colour scales do not respond linearly to luminous

transmittance. Indeed, Van, which is the darkest cherry, presents the highest anthocyanin concentration and the lowest a* level, while at the other end, Pico Colorado is the lightest, with the lowest anthocyanin content and the highest a* value. On the other hand, in keeping with total phenolic content, the Sunburst genotype showed the lowest amounts of both flavonoids and anthocyanins. The flavonoid concentrations agree with those found by other authors in other cherry varieties (de Souza *et al*., 2014; di Matteo *et al*., 2017). However, flavonoid content for the Van cultivar was more than double those found in the same cultivar by Liu *et al*. (2011). These variations could be due to different harvesting times, sun exposure, management after harvesting, climatic conditions, and/or soil conditions (Faniadis *et al*., 2010).

Nine compounds **1**-**9** were studied extensively as the main components in the extracts. All compounds were classified into three unambiguous groups that eluted clearly into three sets: hydroxycinnamic acids, anthocyanins and flavonoids. For all compounds, the high-resolution and accurate mass data agreed well with the theoretical molecular formulae.

Regarding the hydroxycinnamic acids characterization, caffeoylquinic acids (CQA) and *p*-coumaroylquinic acids (*p*CoQA) eluting from 7.7 min-9.6 min were clearly located on the Base Peak Chromatograms (BPC). Peaks were identified as the well-known 3-CQA (**1**), 4-CQA (**2**), and 3-*p*CoQA (**3**), which represent the most abundant hydroxycinnamates detected in the studied methanol cherry extracts (Table 3 and Figure 2A). The identification procedure was initiated with a full MS negative-ionisation mode which showed the presence of the predominant $[M-H]^-$ molecular ions. Next, we observed that the analysis under MS positive-ionisation mode presented a typical fragmentation pattern. This in-source fragmentation yielded spectral details, which were used for tentative identification of detected hydroxycinnamate derivatives.

In the characterization of anthocyanins, four different peaks eluting from 9 min-10 min were clearly located on the Base Peak Chromatogram (BPC) and in the EIC. They were designated as the most abundant anthocyanins (**4**-**7**) detected in the analysed methanol cherry extracts (Table 3). The bioanalytical method developed produced anthocyanin changes in each sample and allowed us to establish a comparison in the content from the EIC for each significant compound (Figure 3A).

According to previous studies, major anthocyanins of sweet cherries are cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside, while peonidin-3-*O*-rutinoside and pelargonidin are the minor anthocyanins, which agrees with our findings.

Our results are not in agreement with those of González-Gómez *et al.* (2010), who found higher concentrations of anthocyanins, mainly of cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside in Navalinda than in the Van cultivar. Composition of sweet cherry fruits are influenced substantially by the cultivar variety, but also by soil composition, agricultural practices, ripening and climatic conditions. Variations in these factors could explain significant variations in the levels of secondary metabolites.

Among the flavonoids group, isorhamnetin-*O*-dihexoside (**8**) and quercetin-3-*O*-rutinoside (**9**) have been identified as the main compounds belonging to this class in cherry extracts (Table 3 and Figure 3C).

As discussed above, there are important qualitative and quantitative differences with regard to the phenolic components of cherry extracts. For this reason, the antioxidant activity of those extracts cannot be evaluated using just one assay. Each antioxidant assay differs in the way free radicals are generated, the strategy to measure the end point of the inhibition reaction, the antioxidant property that is evaluated, and the method sensibility (Roginsky & Lissi, 2005). Therefore, the use of several antioxidant assays is necessary in order to obtain useful information to compare the antioxidant capability of the different cherry cultivars.

Although, results of the DPPH• radical scavenging capacity of the extracts are not directly related to total phenol content, this correlation had been previously described (Skrzyński *et al.*, 2016). The cultivars with the highest antioxidant ability were Navalinda and Van, in accordance with the highest phenol content. However, Pico Colorado with 200.4 GAE mg/100 g of fresh cherries, higher than the concentrations present in Jarandilla and Sunburst, demonstrated the lowest scavenging capacity. Qualitative differences between extracts could explain these results. Pico Colorado is the variety with the lowest concentrations of cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside and peonidin-3-*O*-rutinose. On the other hand, the Pico Colorado extract was rich in flavonoids, presenting the highest concentration of quercetin-3-*O*-rutinose (Figure 3). These results suggest that anthocyanin content provides better DPPH• radical scavenging activity to extracts than flavonoids. In fact, except for the Sunburst cultivar, we found that there is a good correlation between cyanidin-3-*O*-glucoside concentration and DPPH scavenging activity, which was statistically significant ($r=-0.974$; $P<0.026$). Van and Navalinda did not show significant differences between their DPPH• free radical scavenging activity IC_{50}. Previous *in vitro* studies with different sweet cherry cultivar extracts revealed a correlation between the antioxidant properties of this fruit and the anthocyanin content (Hayaloglu & Demir, 2016). Navalinda and Van showed the highest amounts of total phenols and

anthocyanins, with higher concentrations of cyanidin-3-*O*-glucoside in both cultivars than in the other varieties (29.9 and 32.4 mg/100g of fresh cherries for Navalinda and Van respectively). Results indicate that cyanidin heterosides must play an important role in this antioxidant activity.

Flavonoids and phenolic compounds in general are thought to be antioxidants, not only due to their ability to scavenge free radicals, but also for their capability to inhibit the xanthine oxidase enzyme. Xanthine oxidase catalyses the oxidation of hypoxanthine to xanthine and uric acid, generating ROS during the process. The overproduction of uric acid mediated by this enzyme is associated with gout, cardiovascular diseases, diabetes and nephrolithiasis (Liu *et al*., 2014). Accordingly, cherries are traditionally related to improvements in gout symptoms. Together with the highest amounts of uric acid, xanthine oxidase activity increases ROS production, generating cell oxidative stress, which is associated with inflammation, atherosclerosis, cancer and aging. All these reasons make this enzyme a potential target for the prevention of several illnesses with an oxidative stress origin (de Araújo *et al*., 2013). We determined the NBT reduction inhibition, which is related to a decrease in ROS concentration. This drop in ROS levels could be explained both by an enzyme inhibition and by the scavenging capacity of the extracts. All extracts showed a high capacity to inhibit NBT reduction, higher than 50% even at the lowest concentrations. The most significant effect corresponded to the Navalinda cultivar that was able to inhibit 90% of the NBT reduction at the highest assayed concentration. Again, the variety with the lowest capability in this assay was Pico Colorado, being only significantly lower in respect to the other cultivars at the lowest concentration. Therefore, these results could confirm that the consumption of cherries is associated to a lower risk of gout attack as well as with arthritis and gout-related pain reduction (Singh *et al*., 2015).

Cherry extracts studied possessed a high *in vitro* antioxidant potential due to their high phenolic levels. Bioactive compound antioxidant activity in cells cannot merely be predicted on the basis of *in vitro* studies. The HepG2 cell line constitutes a model that reproduces the human hepatocyte and it has been widely used to assess the effects of various compounds of natural origin (Goya *et al*., 2009). Results from the intracellular ROS direct assay revealed that Van extracts showed statistical differences compared to the control treatment. This extract is able to reduce ROS levels at all assayed concentrations in a dose related manner. The other cultivars presented different trends, all similar: at low concentrations, they demonstrated an antioxidant effect, but at higher concentrations caused ROS levels to rise, mainly in Pico Colorado and Navalinda. Cherries have been selected over the years for their sweetness. Thus, this means they have

been selected due to their glucose and fructose concentrations. High concentrations of these sugars can induce cell oxidative stress in HepG2 cultures and in other *in vivo* and *in vitro* models (Otero *et al*., 2002). Navalinda and Pico Colorado present high amounts of sugars (10.7 and 10.25 g/100 g of fresh cherries of glucose and fructose together respectively). At high extract concentrations, the effects of these sugars should prevail, over phenolic compounds. However, Van contained the highest amounts of glucose and fructose, and did not produce a rise in ROS levels at high concentrations. This result can be explained by higher concentrations of total phenols, mainly anthocyanins in Van cherries than in other varieties. In addition, a pro-oxidant and cytotoxic activity has been described for phenolic compounds under certain conditions such as concentration (Sakihama *et al*., 2002). Mixtures of different phenolic compounds can produce synergistic or antagonistic pro-oxidant effects, not always noticeable in *in vitro* tests, as different and complex mechanisms are involved in these cellular responses (Cotoras *et al*., 2014). For example, quercetin has demonstrated protective effects on cells at low and medium concentrations, but at concentrations over 100 μM showed toxic effects (Dajas, 2012). At high concentrations, this flavonoid can present pro-oxidant or antioxidant abilities depending on multiple factors such as, time of exposure or intracellular antioxidant mechanisms (Dell´Alabani *et al*., 2017). In this regard, Pico Colorado extract is the one with highest quercetin-3-*O*-rutinoside concentration, this fact together with high concentrations of glucose and fructose could explain the intracellular ROS rise in cells treated with high concentrations of this extract.

ROS cytotoxic effect has been reported across different cell types (Uttara *et al*., 2009). However, ROS also act as molecular signals in physiological processes such as cell proliferation, migration or cell survival (Rhee *et al*., 2000; Stone & Yang, 2006; Veal *et al*., 2007). While deleterious effects of ROS have been well documented, beneficial consequences are undervalued (Huang & McNamara, 2012). Therefore, these results are not easy to evaluate, and more studies are required in order to discern if the effect of these cherry extracts over ROS concentrations is helpful or not for cell physiology.

With regard to the oxidative stress protection effect of the extracts, all extracts were able to reduce the oxidative stress generated by the oxygen peroxide at the highest assayed concentrations. However, the Van cultivar demonstrated the greatest ability surprisingly together with Pico Colorado. These varieties are the ones with the highest flavonoid concentrations, with high extract concentrations of quercetin-3-*O*-rutinose (0.99 and 0.88 mg/100 g of fresh cherry respectively). Results suggest that flavonoids are the

compounds responsible for this capability, with a strong correlation between flavonoid content, except again for Sunburst, and oxidative stress protection effect, which was statistically significant ($r= -0.953$; $P<0.047$). These results agree with those of González-Gómez *et al*. (2010) who also found a significant correlation between flavonoid derivative compounds and antioxidant capacity of cherry extracts.

Anthocyanins and flavonoids have a stronger relationship with the antioxidant activity of cherry extracts than hydroxycinnamic acids. However, synergistic interactions among antioxidant compounds could determine the antioxidant ability of these fruits, as has been discovered using artificial simplified phytocomplexes (Comisso *et al*., 2017). Metabolic transformations as well as bioactive compound interactions should affect the bioavailability and activity of phenols. These modifications and interactions should increase or reduce the *in vitro* analysed antioxidant activity of certain foods (Fernández-Panchón *et al.*, 2008).

5. Conclusions

Hydroxycinnamic acids, anthocyanins and flavonoids are the main components of all of the analysed methanol sweet cherry extracts. In all performed assays, all extracts showed interesting antioxidant activity. However, the effect of sweet cherries on the xanthine/xanthine oxidase system is very evident, and allows us to relate cherries with the prevention of several illnesses. According to our results, the varieties with higher total phenol amounts demonstrated better antioxidant activity. However, results also indicated the importance of the synergistic interactions among phenolic compounds. The *in vitro* antioxidant activity of the extracts did not exactly agree with the effect of these fruits in cell culture. In this sense, although anthocyanins seemed to be the main phenolic group responsible for the *in vitro* antioxidant activity, cell culture results clearly show that flavonoids, are also important compounds. Therefore, more *in vivo* antioxidant assays must be performed in order to understand the effects of cherry compounds in cell metabolism.

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Table 1. Chromatic characteristics of cherries. The results are expressed as mean ± S.E (n=20). Different letters indicate statistically significant differences between cultivars for each variable (ANOVA-Bonferroni, $P < 0.05$).

	Chromatic coordinates				
	L*	**a***	**b***	**Hue angle**	**Chroma**
Navalinda	31.7 ± 1.1 *a*	20.2 ± 0.92 *a*	10.26 ± 0.78 *a*	0.47 ± 0.03 *a*	22.67 ± 0.93 *a*
Jarandilla	26.4 ± 1.2 *b*	16.22 ± 0.65 *b*	6.52 ± 0.56 *b*	0.38 ± 0.02 *b*	17.49 ± 0.76 *b*
Pico Colorado	33.10 ± 0.10 *a*	20.56 ± 0.74 *a*	15.98 ± 0.68 *c*	0.66 ± 0.02 *c*	26.04 ± 0.79 *c*
Van	21.59 ± 0.64 *c*	6.49± 0.94 *c*	1.62 ± 0.43 *d*	0.24 ± 0.03 *d*	6.70 ± 1.01 *d*
Sunburst	25.18 ± 1.25 *b*	14.4 ± 2.14 *d*	5.63 ± 1.41 *b*	0.37 ± 0.06 b	15.50 ± 2.41 *e*

673 **Table 2.** Glucose and fructose concentrations (mg/100 g of fresh cherries) of different sweet cherry
674 varieties. Values are expressed as the mean ± standard deviation. Different letters indicate statistical
675 differences between varieties for each sugar (ANOVA, Bonferroni (P<0.05).

	Navalinda	Jarandilla	Pico Colorado	Van	Sunburst
Glucose	4.92 ± 0.30 *b*	2.61 ± 0.10 *a*	4.87 ±0.10 *b*	6.52 ± 0.39 *c*	3.70 ± 0.21 *d*
Fructose	5.78 ± 0.32 *b*	3.18 ± 0.15 *a*	5.38 ± 0.31 *b*	6.89 ± 0.27 *c*	4.44 ± 0.05 *d*

676

677 **Table 3.** Groups of the main phenolic compounds identified by LC-ESI-Q-TOF-MS in cherry fruits methanol
678 extracts.

peak	compound	t_R (min)	chemical formula	monoisot. mass	*m/z* (experimental) + ve [a,b]	*m/z* (experimental) - ve [a]	annotation level [c]
Hydroxycinnamic acids							
(**1**)	3-*O*-Caffeoylquinic acid (neochlorogenic acid)	7.7	$C_{16}H_{18}O_9$	354.0951	$[M+Na]^+$ = 377.0842 $[M+H]^+$ = 355.1016 $[M+H-192]^+$ = **163.0395**	$[M-H]^-$ = **353.0629**	(2)
(**2**)	4-*O*-Caffeoylquinic acid	7.9					
(**3**)	3-*p*-Coumarylquinic acid	8.6	$C_{16}H_{18}O_8$	338.100	$[M+Na]^+$ = 361.0888 $[M+H]^+$ = 339.1068 $[M+H-192]^+$ = **147.0445**	$[M-H]^-$ = **337.0697**	(2)
Anthocyanins							
(**4**)	Cyanidin-3,5-*O*-dihexoside	9.1	$C_{27}H_{31}O_{15}^+$	595.1663	$[M]^+$ = 595.1663 $[M+H-Hex]^+$ = **449.1078** $[M+H-2Hex]^+$ = 287.0546	no ionisation	(2)
(**5**)	Cyanidin-3-*O*-glucoside	9.2	$C_{21}H_{21}O_{11}^+$	449.0772	$[M]^+$ = **449.1076** $[M+H-Hex]^+$ = 287.0548	no ionisation	(1)
(**6**)	Cyanidin-3-*O*-rutinoside	9.3	$C_{27}H_{31}O_{15}^+$	595.1663	$[M]^+$ = **595.1667** $[M+H-Hex]^+$ = 449.1077 $[M+H-2Hex]^+$ = 287.0553	poor ionisation $[M-H]^-$ = **593.1096**	(2)
(**7**)	Peonidin-3-*O*-rutinoside	9.7	$C_{28}H_{33}O_{15}^+$	609.182	$[M]^+$ = **609.1815** $[M+H-CH_3]^+$ = 595.1652 $[M+H-Hex-CH_3]^+$ = 449.1060 $[M+H-2Hex]^+$ = 301.1254 $[M+H-2Hex-H_2O]^+$ = 282.2788	no ionisation	(2)
Flavonols							
(**8**)	Isorhamnetin-*O*-hexoside	9.9	$C_{28}H_{32}O_{17}$	640.1639	$[M+Na]^+$ = 663.1557	--	(2)
(**9**)	Quercetin-3-*O*-rutinoside	11.0	$C_{27}H_{30}O_{16}$	610.1534	$[M+Na]^+$ = 633.1416 $[M+H]^+$ = 611.1612 $[M+H-Hex]^+$ = 465.1025 $[M+H-Hex]^+$ = 303.0502	$[M-H]^-$ = 609.1036	(1)

679

680 *a* the *m/z* values for the base peaks are given in bold type.
681 *b* Neutral losses of hexose moiety (Glu: -162 Da), (Rham: -146 Da), Neutral losses of quinic acid: -192 Da.
682 *c* (1) Compounds identified by direct comparison of their retention times and accurate masses with commercial standards.
683 (2) Compounds identified by comparison with the data reported in literature and online database.
684

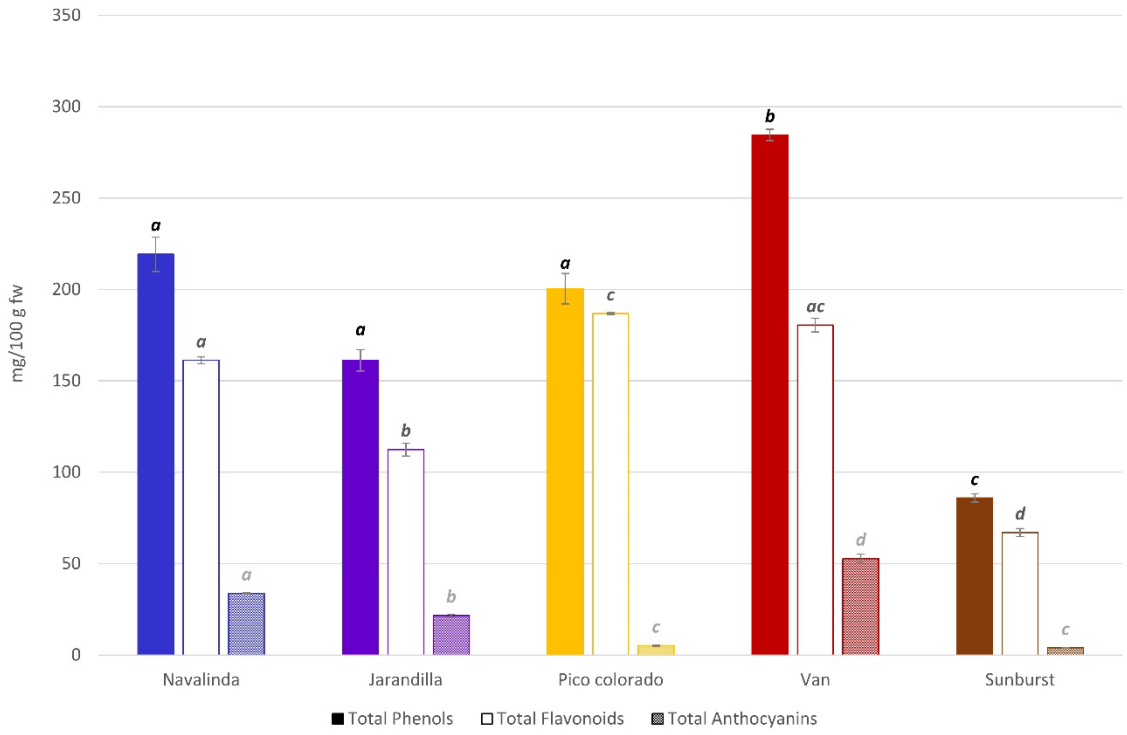
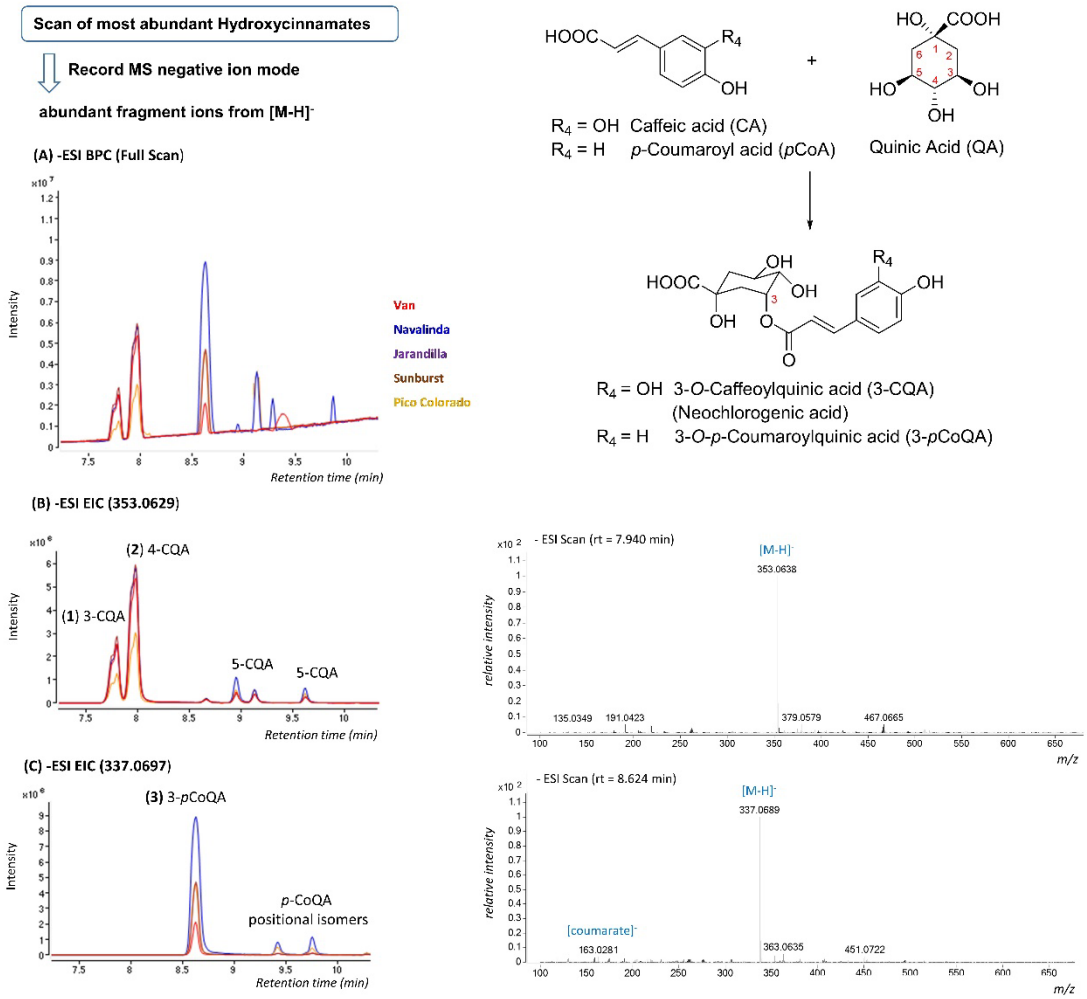
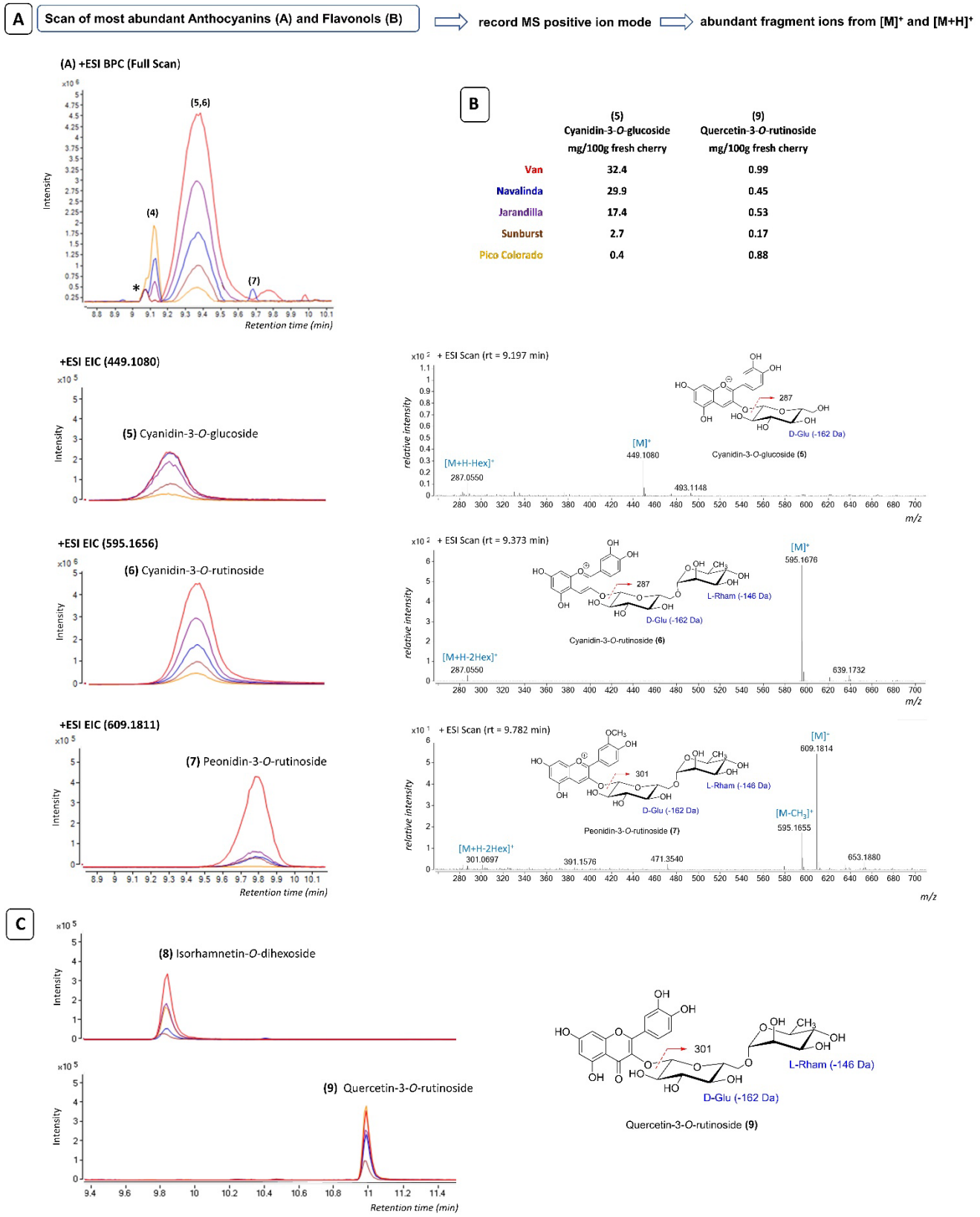


Figure 1. Total phenolic, total flavonoid and total anthocyanin content of different cherry cultivars. Total phenolic content is expressed as GEA mg/100 g of fresh cherries; total flavonoids as EE mg/100 g of fresh cherries; total anthocyanins as CGE mg/100 g of fresh cherries. The results are expressed as mean ± S.E. of n = 3. Different letters indicate statistically significant differences between cultivars for each variable (ANOVA-Bonferroni, $P < 0.05$).



692

693 **Figure 2.** Screening of hydroxycinnamic acids from cherry fruits by LC-qTOF-MS with negative ionisation
694 mode. (A) Overlaid Base Peak Chromatograms (BPC) and (B and C) Extraction Ion Chromatograms (EIC).
695 **(1)** to **(3)** refer to the assigned compounds by matching them with databases and literature reported data
696 and by comparison with commercial standards, see Table 3. Left: Enlarged LC-MS chromatogram from 7.0
697 min to 10.5 min. Right: MS spectra and chemical structures for some compounds, containing one
698 hydroxycinnamate moiety, present in analysed samples.


A
Scan of most abundant Anthocyanins (A) and Flavonols (B)
record MS positive ion mode
abundant fragment ions from [M]+ and [M+H]+
(A) +ESI BPC (Full Scan)
(5,6)
(4)
(7)
*
Intensity
Retention time (min)
B
(5) Cyanidin-3-O-glucoside mg/100g fresh cherry
(9) Quercetin-3-O-rutinoside mg/100g fresh cherry
Van 32.4 0.99
Navalinda 29.9 0.45
Jarandilla 17.4 0.53
Sunburst 2.7 0.17
Pico Colorado 0.4 0.88
+ESI EIC (449.1080)
(5) Cyanidin-3-O-glucoside
+ ESI Scan (rt = 9.197 min)
[M]+ 449.1080
[M+H-Hex]+ 287.0550
493.1148
Cyanidin-3-O-glucoside (5)
D-Glu (-162 Da)
287
relative intensity
m/z
+ESI EIC (595.1656)
(6) Cyanidin-3-O-rutinoside
+ ESI Scan (rt = 9.373 min)
[M]+ 595.1676
[M+H-2Hex]+ 287.0550
639.1732
Cyanidin-3-O-rutinoside (6)
L-Rham (-146 Da)
D-Glu (-162 Da)
+ESI EIC (609.1811)
(7) Peonidin-3-O-rutinoside
+ ESI Scan (rt = 9.782 min)
[M]+ 609.1814
[M-CH3]+ 595.1655
[M+H-2Hex]+ 301.0697
391.1576
471.3540
653.1880
Peonidin-3-O-rutinoside (7)
301
C
(8) Isorhamnetin-O-dihexoside
(9) Quercetin-3-O-rutinoside
Retention time (min)
Quercetin-3-O-rutinoside (9)

699

Figure 3. A: Screening of anthocyanins from cherry fruits by LC-qTOF-MS with positive ionisation mode. Overlaid Base Peak Chromatograms (BPC) and Extraction Ion Chromatograms (EIC) obtained from the analysis of methanol cherry extracts. (**4**) to (**7**) refer to the assigned compounds by matching with databases and literature reported data and by comparison with commercial standards, see Table 3. (*****) solvent impurities. Enlarged LC-MS chromatogram from 8.5 min to 10.5 min. MS spectra and chemical structures for some compounds, containing one anthocyanidin moiety, present in analysed samples. **B**: Concentrations (mg/100 g fresh cherries) of cyanidin-3-O-glucoside (5) and quercetin-3-O-rutinoside (9) found in the different sweet cherry varieties. **C**: Screening of flavonoids from cherry fruits by LC-qTOF-MS with positive ionisation mode. Overlaid Extraction Ion Chromatograms (EIC) obtained from the analysis of methanol cherry extracts. (**8**) and (**9**) refer to the assigned compounds by matching with databases and literature reported data and by comparison with commercial standards, see Table 3. Enlarged LC-MS chromatogram from 8.5 min to 12.0 min.

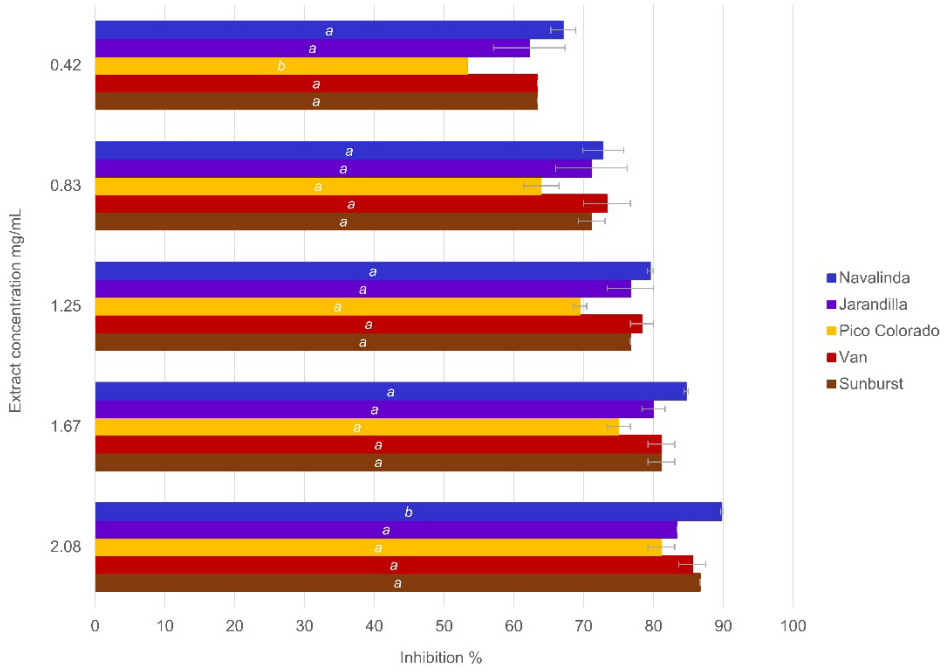
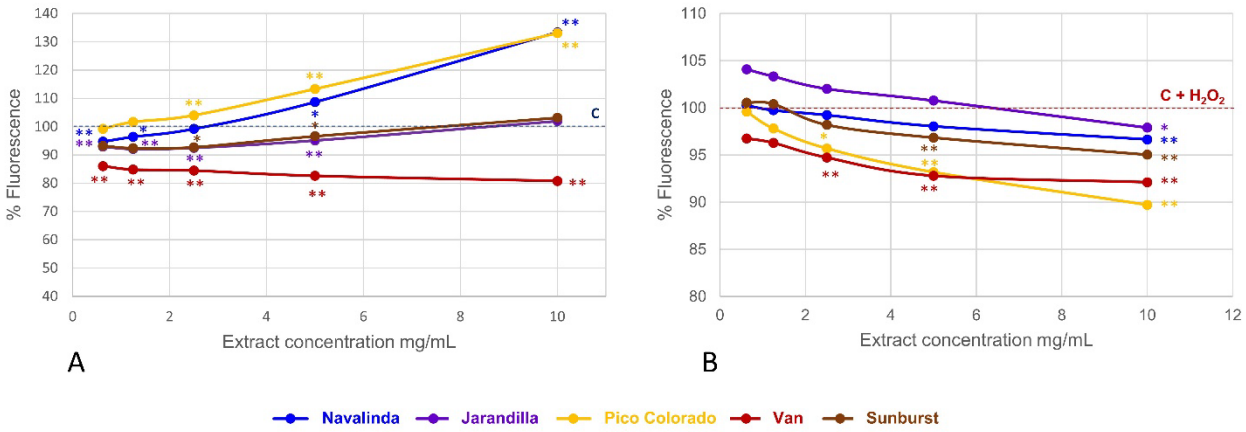


Figure 4. Superoxide scavenging capacity of cherry extracts at different concentrations measured as inhibition percentage of NBT reduction. The results are expressed as mean ± S.E. of n = 3. Different letters indicate statistically significant differences between cultivars for each concentration (ANOVA-Bonferroni, $P < 0.05$).



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718

719 **Figure 5. A**: Intracellular ROS concentration after 90 minutes of treatment. Results appear as a percentage
720 of fluorescence with respect to control (100%). **B**: Effect of different cherry extracts on intracellular ROS
721 concentration in HepG2 cell line culture stressed with H_2O2. Results appear as a percentage of
722 fluorescence with respect to control with H_2O_2 (100%) after 90 min after cells were stressed. * indicates
723 statistically significant differences between control and treatment. ANOVA-Bonferroni, *: $P < 0.05$; **:
724 $P<0.01$.

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