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Currants and strawberries as bioactive compound sources: determination of antioxidant profiles with HPLC-DAD/MS

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Summary

Among plant foods, berry fruit shows a high antioxidant capacity. Medical research has pointed out the medicinal properties of certain pigmented polyphenols, such as flavonoids, anthocyanins, tannins and other phytochemicals, which are mainly found in the skin and seeds of the berries. The aim of this work was to contribute to the study of the nutraceutical features of some berry fruit (currants, gooseberries and strawberries). The different antioxidant compound contents of the fresh fruit of different cultivars and selections of *Ribes* spp. and *Fragaria* x *ananassa* Duch have been analyzed. The fruit of 29 cultivars from 3 different species of *Ribes* spp. and 5 strawberry cultivars have been analysed by High Performance Liquid Chromatography coupled to a UV/Vis detector and a mass detector (MS) to identify and quantify the main antioxidant compounds.

As far as the *Ribes* spp. cultivars are concerned, the presence of a high content of phenolic compounds has been confirmed, and they represent therefore an important source of antioxidant compounds. Moreover, the results have shown that the considered cultivars and selections of strawberries are good sources of bioactive compounds, especially phenolic substances. The results of this study could contribute to offer new insights into the nutraceutical aspects of the considered berry fruit species.

Introduction

The word "berry" has two meanings: one based on a botanical definition, the other on the common commercial identification. True (botanical) berries are fleshy fruit with a cartilaginous endocarp full of seeds. Black currants, red currants and gooseberries are true berries because they are many-seeded epigynous berries (WESTWOOD, 1993). Other berries are not real berries according to the scientific definition. Blueberries, instead, can be defined as a fake epigynous berry. Strawberries are not berry fruit but either are multiple fruits developed from a dialycarpous ovary (on aggregate of drupes: *Rubus*, on aggregate of achenes: *Fragaria*) (SPICHIGER et al., 2002). The common term "berry fruit" includes different fruits, such as strawberry (*Fragaria* spp.), blueberry (*Vaccinium* spp.), currant and gooseberry (*Ribes* spp.), raspberry and blackberry (*Rubus* spp.). "Berry fruit", "soft fruit" and "small fruit" are synonymous terms for the above mentioned species, and they are used indifferently. In this work, the term "berry fruit" is used to indicate all the analyzed species.

It is well known that a healthy diet includes the consumption of fruit and vegetables. Many medical and epidemiological studies have shown an inverse relationship between fruit consumption and the incidence of coronary and heart diseases, as well as certain cancers (RICE-EVANS et al., 1997; BUB et al., 2003; VANAMALA et al., 2006). Among plant foods, berry fruits show a high antioxidant capacity. Medical research has pointed out the medicinal properties of certain pigmented polyphenols, such as flavonoids, anthocyanins, and tannins and other phytochemicals, mainly located in the skins and seeds of berries. The nutritional content and antioxidant activity are

used to distinguish several berries in a new category of functional foods called "superfruits", a rapidly-growing multi-billion dollar industry that began in 2005 and which has been identified by DataMonitor as one of the top 10 food categories for growth in 2008 (FOOD U.S.A. navigator, 2007).

Phenolic compounds are abundant in highly colored berry fruit, and due to their popularity and consumption, these berry fruits serve as one of the most important dietary sources of phenolics (KAHKONEN et al., 2001). Berry fruits are reported to contain a wide variety of phenolics, including hydroxybenzoic and hydroxycinnamic acid derivatives (phenolic acids), anthocyanins, flavonols, flavanols, condensed tannins (proanthocyanidins) and hydrolyzable tannins (MACHIEX et al., 1990). The chemical characteristics, that are, nature, size, structure, solubility, degree and position of glycosylation, the type of esterification or polymerization and conjugation of phenolics with other compounds can influence their bioavailability, absorption, distribution, metabolism and excretion in humans (AHERNE and O'BRIEN, 2002; HOLLMANN, 2001). Phenolic components, as mentioned above, can range from simple molecules, such as phenolic acids, to highly polymerized compounds, such as tannins. The phenolic compounds found in berries can be divided into two principal categories: flavonoid compounds and non-flavonoid compounds, which include the so called phenolic acids, tannins and coumarins.

Vitamin C is also an important antioxidant berry fruit constituent. Vitamin C is a highly effective antioxidant in humans, which act by lowering oxidative stress, a substrate for ascorbate peroxidase. It is also an enzyme cofactor for the biosynthesis of many important biochemicals, and it acts as an electron donor for eight different enzymes. The term vitamin C is usually used as the generic descriptor of all the components of fruit that exhibit the biological activity of ascorbic acid. In fruit, vitamin C is assumed to be the sum of the ascorbic acid (AA) and dehydroascorbic acid (DHAA) contents. These two substances are readily oxidized, especially when exposed to elevated temperatures, some divalent cations (e.g. copper and iron), oxygen, alkaline pH, light or degradative enzymes. Ascorbic acid is a necessary human nutrient, and its biological functions are centered on its antioxidant properties in the biological system. Furthermore, it prevents common degenerative processes (SMIRNOFF et al., 2000). Ascorbic acid is the principal biologically active form, but dehydroascorbic acid also exhibits biological activity, since it can easily be converted into ascorbic acid in the human body. Therefore, it is important to measure both ascorbic and dehydroascorbic acid.

The aim of this work was to contribute to the study of the nutraceutical features of some berry fruit (currants, gooseberries and strawberries), by analyzing the fresh fruit of different cultivars and selections of *Ribes* spp. and *Fragaria* x *ananassa* Duch.

Materials and methods

Plant material
Currant and gooseberry samples of 29 cultivars from three different species of *Ribes* spp. (Tab. 1a) have been harvested at commercial

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Tab. 1a: *Ribes* spp. cultivars

Ribes spp. cultivars			
Ribes nigrum L. (black currant)	*Ribes rubrum* L. (red currant)	*Ribes grossularia* L. (goosberry)	*Ribes* x *nidigrolaria* (hybrids)
Andega	Augustus	Rokula	Jogranda
Ben Lomond	Blanka field A		Jostine
Ben more	Erden		
Ben sarek	Gloire de Sablons		
Black Down	Jonker Van Tets		
Black reward	Junifer		
Burga	Red poll		
Geant de Boskoop	Red start		
Invigo	Red win		
Noir de Bourgogne	Roodneus		
Royal de Naples	Rotet		
Tenah	Rovada field A		
Wellington	Werdavia		

Tab. 1b: *Fragaria x ananassa* L. cultivars and selections

Fragaria x *ananassa* L.
163M88
148M6
150M61
29K55
160M77

ripeness, according to the maturity stage of the cultivars. All the cultivars were collected from a germplasm repository in the Piedmont Region, Italy. A protocol usually applied for raspberries (Zafrilla et al., 2001) and strawberries (Allende et al., 2007) has been modified for the currants and gooseberries.

Five strawberry genotypes (*Fragaria* x *ananassa* Duch.) (Tab. 1b) were collected from a greenhouse on a Spanish farm (Huelva, Andalusia, Spain) under Mediterranean climatic conditions. This fruit was harvested at the commercial maturity stage. Three replications of 10 g of fruit were selected and frozen for each genotype in liquid nitrogen and kept at -80 °C until analyzed, while other three replications of 10 g of fruit where immediately analyzed for their vitamin C content.

Extraction of phenolic compounds

The phenolic compounds were extracted as described by Allende et al. (2007). A lyophilized powder sample (0.77 g) of each sample was homogenized with 25 mL of an extraction solution (acetone/water/acetic acid, 70:29.5:0.5, v/v/v) using an Ultra Turrax (Ika, Staufen, Germany) for 1 min on ice, and this was followed by sonication in a bath-type ultrasonic cleaner (5510E-MTH, Branson Ultrasonic Corporation, U.S.A.) for 15 min. The homogenates were then centrifuged at 3,200 rpm for 10 min in a JP Selecta Centronic centrifuge. The acetone was evaporated at 35 °C using a rotary evaporator under vacuum. After evaporation of the acetone, the residue was flushed through a Sep-Pak C-18 cartridge, previously activated with methanol, and then with water and air. The phenolic compounds were absorbed onto the column, while sugars, acids and other water-soluble compounds were eluted with 10 mL of water. The phenolic compounds were then recovered with approximately 8 mL of methanol. The methanol was evaporated at 35 °C under vacuum

and recovered in 1 mL of extraction solution, filtered through a 0.45 μm Nylon filter (Millex HV13, Millipore, Bedford, MA) and directly analyzed by High Performance Liquid Chromatography (HPLC).

Analysis of phenolic compounds using reverse-phase HPLC with Diode Array Detector (DAD) and Tandem Mass Spectrometry (MS-MS)

Fifty μL samples of the extracts were analyzed using an HPLC system (Merck Hitachi, Tokyo, Japan) equipped with a model L7100 pump and a model L-7455 photodiode array UV/Vis detector. The samples were injected using an autosampler (model L-7200). Compound separations were achieved on a 250 mm x 4 mm i.d., 5 μm reversed phase LiChrocart C18 column (Merck, Darmstadt, Germany) with water/formic acid (H_2O:CH_3COOH) (95:5, v/v) (A) and methanol (B) used as mobile phases. The linear gradient started with 3 % B, at 5 min 5 % B, at 10 min 8 % B, at 15 min 13 % B, at 19 min 15 % B, at 47 min 40 % B, at 64 min 65 % B, at 66 min 98 % B, at 69 min 98 % B and at 70 min 3 % B. The flow rate was 1 mL/min, and chromatograms were recorded at 280, 320, 360 and 510 nm. Anthocyanins were quantified through comparisons with an external standard of cyanidin 3-glucoside at 510 nm, flavonols as quercetin 3-rutinoside at 360 nm, hydroxycinnamic acid derivatives as chlorogenic acid at 320 nm, and flavan-3-ols as catechins at 280 nm (all these markers were from Sigma, St. Louis, MO). The results were expressed as mg per 100 g fresh weight. The phenolics identification was carried out considering their UV spectra, molecular weights, and their MS-MS fragments.

Electrospray mass spectrometric analyses were performed using an HPLC system equipped with a DAD detector and mass detector in series consisting of a G1312A binary pump, a G1313A auto-sampler, a G1322A degasser, a G1315B photodiode array detector, and an ion trap mass spectrometer equipped with electrospray ionization (ESI), operating in the negative ion mode, and controlled by software (v.4.0.25) from Agilent Technologies (Waldbronn, Germany). The heated capillary and the voltage were maintained at 350 °C and 4 kV, respectively. Mass scan (MS) and daughter (MS-MS) spectra were measured from m/z 100 to ~1500. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, and the collision energy was set at 50 %. The mass spectrometry data were acquired in the negative mode for all of the phenolic compounds.

The phenolic compounds in the fruit extracts were identified by their

UV spectra, which was recorded by means of a diode-array detector and HPLC-MS, and, wherever possible, through chromatographic comparisons with authentic pure standards.

Analysis of proanthocyanidins: phloroglucinol protocol

The analysis of proanthocyanidins was performed as described by KENNEDY et al. (2001). The proanthocyanidins (PAs) of 11 samples belonging to genus *Ribes* were made to react with a solution of 0.1 N HCl in MeOH, containing 5 g/L phloroglucinol and 10 g/L ascorbic acid at 50 °C for 10 min, and then combined with 1.2 mL of aqueous sodium acetate to stop the reaction.

The phloroglucinol adducts were analyzed by means of reversed-phase HPLC. The used column was a LiChrocart C18 (particle size 5μm, 250 mm x 4 mm i.d., Merck, Darmstadt, Germany), protected by a guard column containing the same material. The method utilized a binary gradient with a mobile phase containing 1 % v/v aqueous acetic acid (mobile phase A) and methanol (mobile phase B). The eluting peaks were monitored at 280 nm. The elution conditions were 1.0 mL/min; 5 % B for 10 min, a linear gradient from 5 to 20 % B for 20 min, a linear gradient from 20 to 40 % B for 25 min. Then the column was washed with 90 % B for 10 min and reequilibrated with 5 % B for 5 min before the next injection. The proanthocyanidin cleavage products were estimated using their response factors in order to calculate the apparent mean degree of polymerization (mDP), the sum of all subunits (flavan-3-ol monomers and phloroglucinol adduct, in moles) was divided by the sum of all flavan-3-ol monomers (in moles).

Extraction and analysis of vitamin C

The ascorbic acid (AA) and dehydroascorbic acid (DHAA) contents were determined according to ZAPATA and DUFOUR (1992), with some modifications. The analysis was just carried out with fresh strawberry samples.

10 g of fresh fruit was homogenized for 30 s in 10 mL of the extraction solution (0.1 M citric acid, 2 mM ethylene diamine tetraacetic acid (EDTA) disodium salt and 4 mM sodium fluoride in methanol – water 5:95, v/v) on a Ultraturrax T-25 (Jauke and Kundel IKA, Dabortechnik, Staufen, Germany). The homogenates were then filtered through cheesecloth, centrifuged at 10,500 rpm for 5 min at 2-5 °C, filtered through an activated C18 Sep-Pak cartridge (Waters, Milford, MA) and finally filtered through a 0.45 μm filter. Then, 250 μL of 1,2-phenylenediamine dihydrochloride (OPDA) solution (35 mg/100 mL) was added to 750 μL of extract for dehydroascorbic acid derivatization in the fluorophore 3-(1,2-dihydroxyethyl)furo[3,4-*b*]quinoxaline-1-one (DFQ). After 37 min in darkness, the samples were analysed by means of HPLC.

The ascorbic and dehydroascorbic acids were evaluated using an HPLC system (Merk Hitachi, Tokio, Japan), equipped with an L-6000 pump coupled to a D-2500 variable-wave-length UV detector. 20 μL samples were injected into a reversed-phased Kromasil 100 C18 column (250 mm × 4 mm i.d., 5 μm particle size; Tecnokroma, Barcelona, Spain) with an ODS C18 guard precolumn. The mobile phase was methanol/water (5/95, v/v), 5 mM cetrimide and 50 mM KH_2PO_4 at pH 4.5. The flow rate was kept at 0.9 mL/min. The detector wavelength was initially set at 348 nm, and after being DFQ eluted, it was manually shifted to 261 nm for ascorbic acid detection. The ascorbic and dehydroascorbic acids were identified and quantified through a comparison with the pattern areas from AA and DHAA. The AA and DHAA standards were purchased from Sigma-Aldrich (Steinheim, Germany). The vitamin C content was calculated by adding the ascorbic and dehydroascorbic acid contents, and the results were expressed as mg per 100 g fresh weight.

Statistical analysis

All the data were subjected to one-way analysis of variance (ANOVA) to compare the means, using SPSS 18.0 software (Chicago, IL), and significant differences between samples were calculated according to the HDS Tukey multiple range test at $p<0.05$ (probability level). The data shown are the mean values (n=3) ± standard deviation.

Results

Characterization and quantification of the phenolic compounds

The combination of the data with mass spectra (MS) data allowed a tentative identification to be made of the conjugated forms, as obtained in previous studies (HE, 2000; SCHIEBER et al., 2001).

Strawberries

Fifteen compounds were identified and quantified in the HPLC-DAD chromatograms of the strawberry selections. The molecules were classified as different phenolic compounds (Tab. 2): phenolic acids (p-coumaric and ellagic acid); flavonols (quercetin, kaempferol); ellagitannins and anthocyanins (pelargonidin and cyanidin derivatives) (Fig. 1). The peaks were identified by comparing the UV-visible spectra with those of the available standards. Further support for this identification was obtained from their MS-MS analyses. The lower peaks, without the typical spectral characteristic of the standard, remained unidentified.

The phenolic contents were different for the five strawberry selections, with a mean of 63.46 mg/g fresh fruit. The concentration ranged from 43.15 mg/100 g fresh fruit (163M88) to 75.53 mg/100 g (160M77), showing statistically significant differences (P<0.05) between the selections.

Ellagitannins

The ellagitannins only showed characteristic UV spectra for an absorption maximum below 280 nm. Four peaks of ellagitannins were found in all the strawberry extracts, except for the 148M6 selection which only had three peaks. Peak 2 had [M–H]⁻ at m/z 935 and the main MS^2 fragments at m/z 633 and m/z 301; it was identified as galloyl-bis-HHDP-glucose. Peak 4 had [M–2H]²⁻ at m/z 935, corrsponding a mass of 1869. The MS/MS spectrum had an M⁻ at m/z 1869 which fragmented to produce m/z 1567 (M – 302, a loss of a hexahydroxyphenoyl (HHDP) unit), 1265 (M – 302, a further loss of HHDP), 1103 (M – 162, a loss of a glucosyl group), 933 (M – 170, a loss of a gallic acid) and 631 (M – 302, a loss of HHDP). This peak was identified as sanguiin H-6 or agrimoniin, which is a dimer of casuarictin/potentillin. Peak 5 had a λ_{max} of 250 nm and, when subjected to acid hydrolysis, yielded ellagic acid. The mass spectrum of this compound was complicated. It showed a [M–2H]²⁻ at m/z 1401. The main products were m/z 2019, 1869, 1567 (a loss of HHDP), 1402, 935, 897, 633 (a loss of tri-HHDP-galloyl-glucose). This peak was identified as lambertianin C, which is a trimer of casuarictin/potentillin. The late-eluting ellagitannin compound (peak 9) remained unknown and requires further identification.

The most abundant phenolic compounds found in strawberries were ellagitannins, with an average of 30.77 mg/100 g, and they showed statistically significant differences (P<0.05). The selections containing the highest level of total ellagitanins were 29K55 and 160M77, with 37.94 and 34.94 mg/100 g, respectively.

The most important group within the ellagitanins was sanguiin-H6, which on average reached 59 % of the total ellagitannins.

Tab. 2: Phenolic compound contents of the strawberry selections. Results are expressed as mg per 100 g_{FW} (mean ± SD, n=3). Values with different letters showed statistically significant differences.

Compound	Strawberry selections				
	148M6	150M61	160M77	163M88	29K55
Anthocyanins					
Cyanidin 3- glucoside	0.88± 0.08	1.47±0.09	1.21±0.14	0.65±0.04	1.39±0.04
Pelargonidin 3-glucoside	18.98±1.6	24.39±1.08	24.25±2.04	10.40±0.07	21.22±2.3
Pelargonidin 3-rutinoside	2.35±0.21	1.48±0.01	1.03±0.13	0.42±0.03	0.88±0.12
Pelargonidin 3- acetylglucoside	nd	6.40±0.28	6.36±0.21	3.11±0.11	5.37±0.61
Total	**22.21 b**	**34.28a**	**32.85a**	**14.59c**	**28.87a**
Flavonols					
Quercetin 3-glucoronide	1.03±0.02	1.18±0.06	0.56±0.02	0.83±0.01	1.71±0.11
Unknown	0.40±0.05	nd	nd	nd	nd
Kaempferol 3-glucoside	0.07±0.01	0.18±0.01	0.10±0.01	nd	0.13±0.02
Total	**1.50b**	**1.36b**	**0.66c**	**0.83c**	**1.84a**
Ellagic acid					
Ellagic rhamnoside	0.63±0.02	0.59±0.04	0.50±0.03	0.51±0.01	0.61±0.05
Ellagic rhamnoside	0.74±0.04	0.76±0.04	0.46±0.02	0.56±0.02	0.57±0.06
Total	**1.37a**	**1.34a**	**0.95c**	**1.07bc**	**1.19ab**
Hydroxycinnamic acids					
p-coumaric acid					
4-glucoside	**4.07±0.48a**	**4.49±0.32a**	**4.13±0.42a**	**0.33±0.04b**	**3.48±0.4a**
Ellagitannins					
Galloyl bis HHDP gluc.	nd	4.18±0.94	4.28±0.18	3.29±0.29	5.11±0.73
Sanguiin -H6	16.34±0.34	15.28±1.89	21.06±0.84	17.79±0.51	20.27±1.2
Lambertianin C	5.59±0.04	5.57±1.07	5.44±0.55	1.64±0.06	7.84±1.13
Unknown	4.15±0.32	3.56±0.56	4.15±0.21	3.61±0.06	4.72±0.05
Total	**26.08b**	**28.58b**	**34.94a**	**26.33b**	**37.94a**
Total phenolic compounds	**55.23b**	**70.07a**	**75.53a**	**43.15c**	**73.31a**

Anthocyanins

The anthocyanins found in strawberries were glycosides of cyanidin (λ_{max} at 512 nm) and of pelargonidin (λ_{max} at 495 nm). Peak 6 in the DAD-chromatogram had an absorption maximum at 512 nm, as well as molecular ions at m/z 447, with fragments at m/z 285 (a loss of hexose) and was identified as cyanidin 3-glucoside. Three peaks (7, 8 and 14) had absorption maxima at 495 nm as well as MS fragmentation ions at m/z 269, and were identified as derivatives of pelargonidin. Peak 7, with [M–H]$^-$ at m/z 431 and a subsequent loss of 162 amu (hexose), was pelargonidin 3-glucoside. Peak 9, with [M–H]$^-$ at m/z 577 and a loss of 308 amu (deoxyhexose-hexose), was assigned as pelargonidin 3-rutinoside. One less polar pelargonidin derivative, peak 14, had [M–H]$^-$ at m/z 473 as well as a loss of 204, and was assigned as pelargonidin 3-acetylglucoside.

The total anthocyanins ranged from 14.59 mg/100 g (163M88) to 34.28 mg/100 g (150M61), with an average of 26.56, and showed statistically significant differences ($P<0.05$). The 150M61, 160M77 and 29K55 selections showed the highest concentrations of all studies. The lowest concentration was observed in 163M88.

Tab. 2 shows the single concentrations of the four major antho-cyanins (i.e. Cy 3-gluc, Pg 3-gluc, Pg 3-rut and Pg 3-acetylgluc) in the five strawberry selection extracts.

As far as the anthocyanins distribution is concerned, Pg 3-glucoside was the predominant compound in the strawberries, representing 71 % of the total mean amount of anthocyanins, and this was followed by Pg acetylglucoside, Pg 3-rutinoside and finally Cy 3-glucoside.

Flavonols

The flavonols detected in the strawberries were derivatives of quercetin and kaempferol. The flavonols were classified on the basis of the shape of their UV-visible spectra, with an absorption maximum at about 352 nm for quercetin glycosides and at about 344 nm for kaempferol glycosides. The shift in the UV maximum from 352 to 344 nm is due to the lack of additional hydroxyl groups attached to ring B in kaempferol, compared to quercetin. Three peaks were detected in the UV-chromatogram, at t_R 42, 48 and 50 min, respectively. The peak 10 had a λ_{max} of 365 nm. The mass spectrum had an m/z 477 molecular ion that yielded an M-176 (cleavage of a glucoronosyl unit) fragment ion at m/z 301, indicating the presence of quercetin-3-glucuronide. Peaks 13 was identified as a kaempferol derivative, due to its UV-spectrum and MS^2 fragmentation ion at m/z 285 in negative MS mode; this peak with [M–H]$^-$ at m/z 447 lost hexose (162 amu) during fragmentation, and was assigned as kaempferol 3-glucoside. Peak 15 was identified as flavonol, due to its UV-spectrum, but no further data could be obtained from LC-MS analysis for its tentative identification.

The total flavonol contents ranged from 0.66 (160M77) to 1.84 (29K55) mg/100 g; the total levels were also statistically different
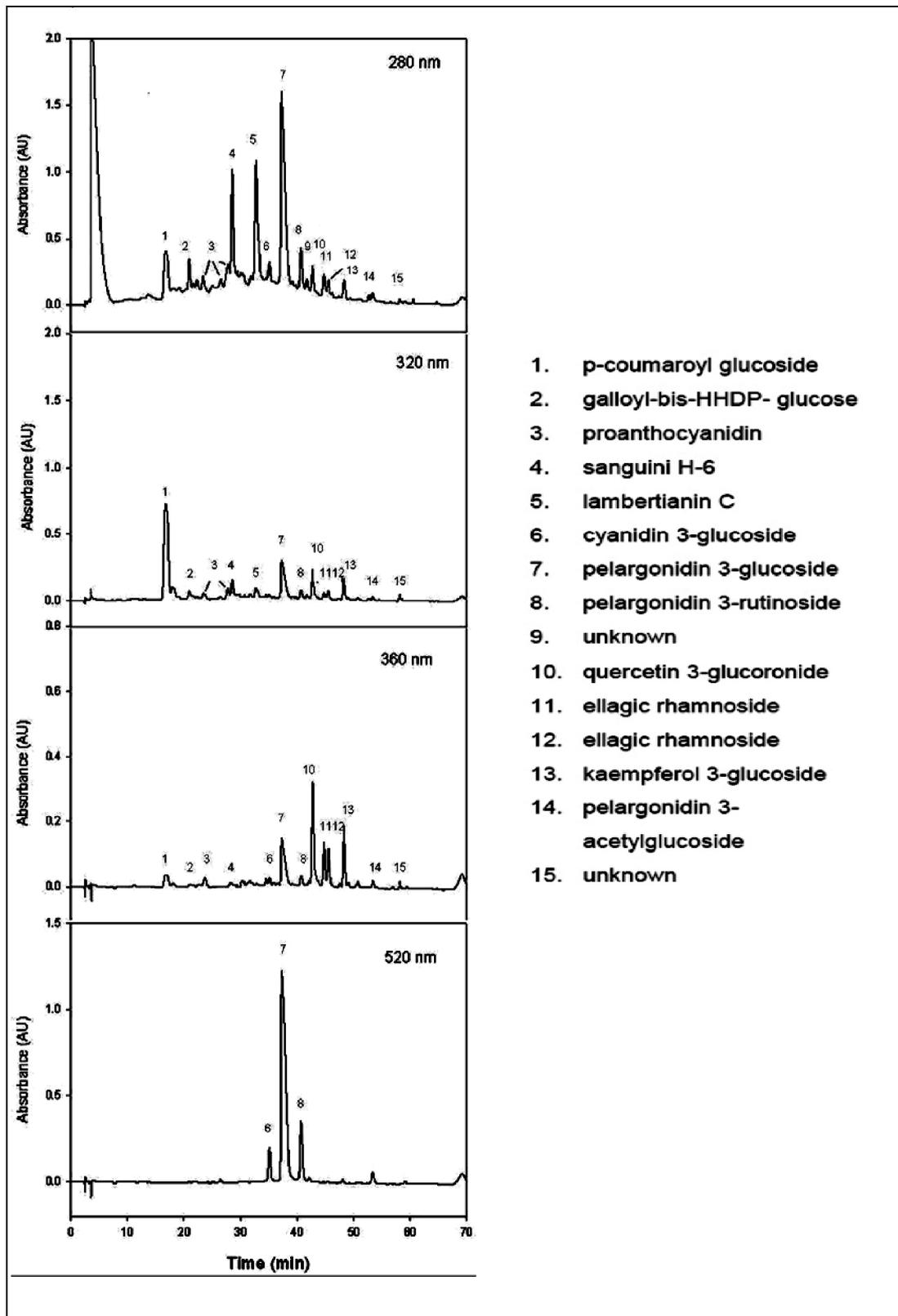
Fig. 1: Chromatographic patterns of strawberry phenolic compounds obtained at 280, 320, 360 and 520 nm.
Peak assignment is provided on the right side.

(P<0.05). Quercetin 3-glucoronide was the most abundant flavonol in these strawberry samples, with a concentration of 85.5 % of the mean total flavonol content. Quercetin 3-glucoronide was the only flavonol compound found in the 163M88 selection, while the 29K55 selection showed the highest values of this compound (1.71 mg/ 100 g).
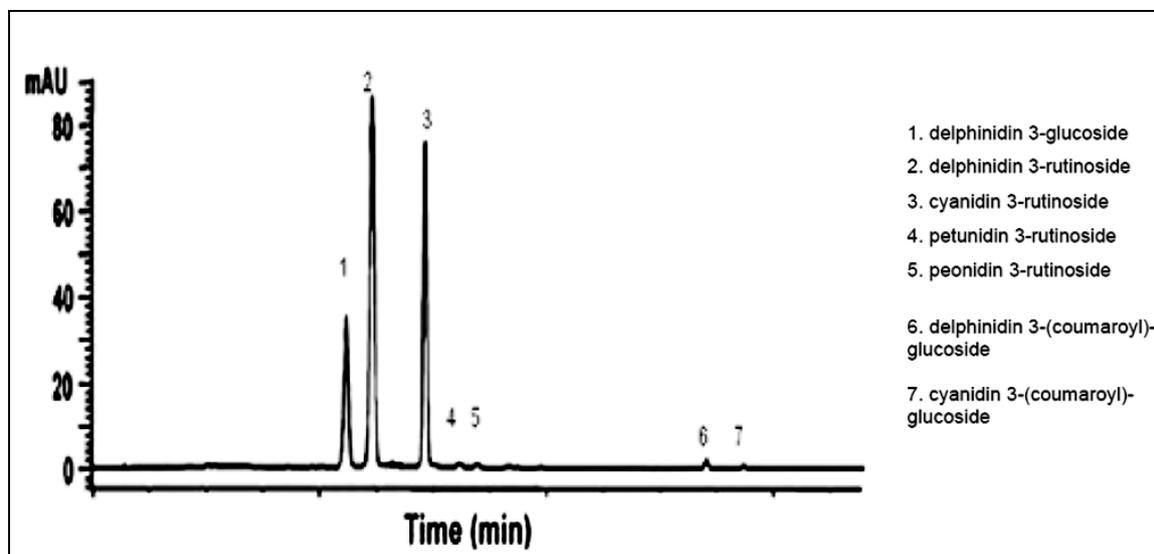
Fig. 2: HPLC separation of anthocyanins detected at 520 nm from black currant (cv Royal de Naples). Peak assignment is provided on the right side.
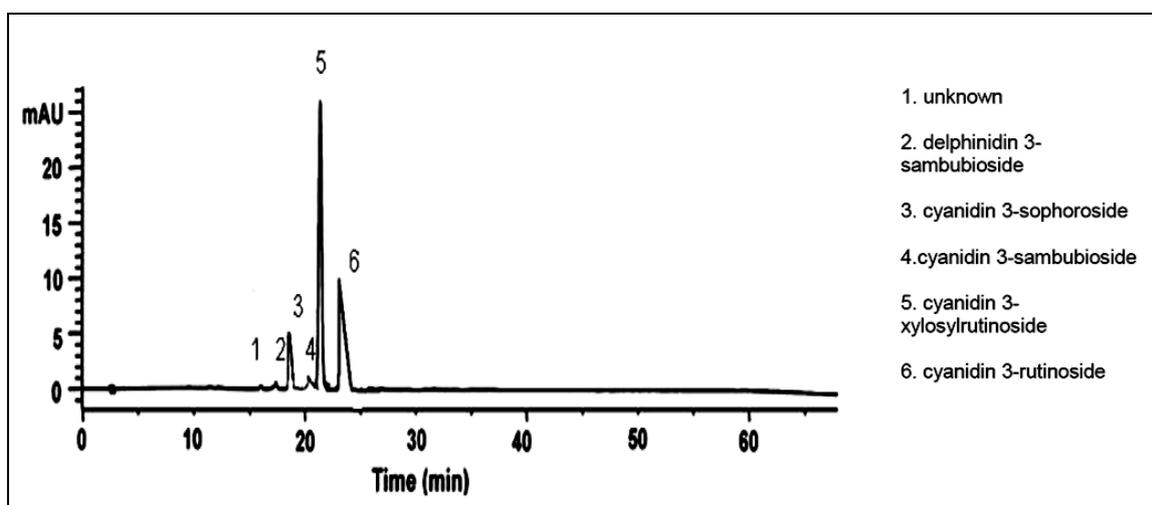
Fig. 3: HPLC separation of anthocyanins detected at 520 nm from red currant (cv Red poll). Peak assignment is provided on the right side.

Ellagic acid

Ellagic acid and its glycosides were distinguished by means of their characteristic UV-visible spectra with absorption maxima at 254 and 360-368 nm, respectively. Two ellagic acids were detected in the strawberry selections at 45 min and 45.4 min. Peaks 11 and 12 had $[M–H]^-$ at m/z 447. The MS^2 products of the ions were at m/z 301, with further fragmentation, being characteristic for ellagic acid. These peaks were identified as ellagic acid rhamnoside. The ellagic acid levels were between 0.95 (160M77) and 1.37 mg/100 g (148M6), with a mean value of 1.19 mg/100 g, and showed statistically significant differences ($P<0.05$).

Hydroxycinnamic acids

The most important peak in the UV chromatogram obtained at 320 nm was for the hydroxycinnamic acids: peak 1 was identifed as a p-coumaric ester (absorption maximum at 310 nm) (t_R 17 min).

This peak had $[M–H]^-$ at m/z 487 with MS^2 fragments at m/z 325 (loss of hexose). This compound was identified as a hexose ester (p-coumaric acid 4-glucoside).

P-coumaric acid glucoside was the only hydroxycinnamic acid found in the analyzed strawberry samples; its content varied from 0.33 mg/100 g (163M88) to 4.49 mg/100 g (150M61), and showed statistical differences ($p<0.05$) with a mean of 3.30 mg/100 g.

Finally, the peaks indicated with number 3 were recognized as generic procyanidins. The identification of this class of compounds was based on the chromatographic behaviour, UV-vis spectra and a comparison with literature. Quantification and identification of the single peaks were not carried out.

Currants and goosberries

The protocol usually used for the identification of phenolics in raspberries (ZAFRILLA et al., 2001) and in strawberries (ALLENDE

et al., 2007) was adapted for currants and gooseberries. Unfortunately, it was not possible to provide the complete chromatographic pattern of the phenolic compounds. In this study, only the anthocyanin compound data were clear and have been reported.

Anthocyanins
The HPLC analyses of 29 different cultivars of *Ribes* spp. led to the identification of 7 anthocyanins in *Ribes nigrum* L., 6 in *Ribes rubrum* L., 3 in the hybrids and 2 in *Ribes grossularia* L.

Ribes nigrum L.
Peaks 1 and 2 were identified as two delphinidins (Fig. 2), the first as delphinidin 3-glucoside, with [M⁻] at *m/z* 463 and a loss of 162 amu (hexose) and the second one as delphinidin 3-rutinoside with [M⁻] at *m/z* 609 and a loss of 308 amu (deoxyhexose-hexose) upon fragmentation.
Peak 3 was identified as cyanidin-3-rutinoside. The mass spectrum of peak 4 revealed a small molecular ion at *m/z* 623 M⁻ and a major fragment ion at *m/z* 315; this corresponded to petunidin 3-rutinoside (M_w 623), and the fragment ion [M – 308], which originated from the loss of rutinoside moiety, resulted in petunidin (M_w 317). The mass spectrum of peak 5 showed significant signals corresponding to the peonidin 3-rutinoside molecular ion at *m/z* 607 M⁻ and a major fragment ion at *m/z* 299. Peak 6 revealed *m/z* 609 and *m/z* 301. These masses corresponded to delphinidin 3-(6- coumaroyl)-glucoside and its characteristic loss of *m/z* 308, the coumaroyl glucoside moiety, which forms delphinidin. The last identified anthocyanin component was cyanidin 3-(6-coumaroyl)-glucoside, which shows *m/z* 593 M⁻ and 285, corresponding to a loss of coumaroyl glucoside.
The anthocyanin content in black currant ranged from 47.03 (cv Ben Sarek) to 661.81 mg/100 g (cv Invigo) with a mean value of 313.37 mg/100 g, and showed statistically significant differences between groups (P<0.05). The most abundant anthocyanins found in these cultivars of *Ribes nigrum* L. were delphinidin 3-rutinoside and cyanidin 3-rutinoside with a mean value of 133.60 and 113.14 mg/100 g, respectively.

Ribes rubrum L., *Ribes grossularia* L. and hybrids
The first peak in the *Ribes rubrum* L. cultivars was identified as anthocyanin, due to its UV-spectrum, but no further data were obtained for its tentative identification from the LC-MS analysis (Fig. 3). Peak 2 showed a shorter t_R than those of the other peaks. It had a delphinidin aglycon (fragment *m/z* 301 M⁻) and a molecular weight of 595. The fragment loss was 294, which could be due to a sambubiose residue. This anthocyanin was identified as delphinidin 3-sambubioside. The other four anthocyanins detected in the *Ribes rubrum* L. cultivars were glycosides of cyanidin (λ_{max} at 512 nm). Peak 3 was identified as cyanidin 3- sophoroside. Peak 4 produced a mass spectrum, with a M⁻ at *m/z* 579, which yielded a fragment ion at *m/z* 285 (M – 294) and was identified as cyanidin 3- sambubioside. Peak 5 was recognized as cyanidin 3-xylosylrutinoside (Cy 3-xylrut), presenting a molecular weight of 725 M⁻ and a fragment ion at *m/z* 285.
The last anthocyanin (peak 6) was identified as cyanidin 3-rutinoside. Two anthocyanin compounds were detected in cv. Rokula, the only *Ribes grossularia* L. cultivar: delphinidin 3-rutinoside and cyanidin 3-rutinoside.
Three anthocyanin compounds were detected in the two analyzed hybrids: delphinidin 3-glucoside, delphinidin 3-rutinoside and cyanidin 3-rutinoside. The anthocyanin content in the red currants, gooseberries and hybrids ranged from 5.74 (gooseberry, cv Rokula) to 85.74 mg/100 g (red currant, cv Jonker van Tets), with a mean

value of 33.83 mg/100 g, and showed statistically significant differences (*P*<0.05). The anthocyanins in the *Ribes rubrum* L. cultivars, ranged from 14.09 (cv Red win) to 85.74 mg/100 g (cv Jonker van Tets), with a mean value of 38.29 mg/100 g.
The most abundant anthocyanin found in these cultivars of *Ribes rubrum* L. was cyanidin 3-xylosylrutinoside, with an average value of 21.80 mg/100 g. The Jogranda cultivar showed a higher anthocyanin value (32.98 mg/100 g) among the hybrids.

Analysis of proanthocyanidins
The proanthocyanidins in the 10 analyzed cultivars of *Ribes* spp. were procyanidins and prodelphinidin; (epi)catechin and (epi)gallocatechin were present as subunits.

Analysis of vitamin C
Strawberries
Statistically significant differences were found for the singular AA and DHAA contents for the five analyzed strawberry selections. The AA content ranged from 25.74 (150M61) to 30.36 (29K55) mg/100 g. the DHAA content varied from 3.03 (29K55) to 7.46 (160M77) mg/100 g.
The total vitamin C content (AA+DHAA) varied from 31.74 (150M61) to 35.87 (160M77) mg/100 g, with a mean value of 33.32, but no statistically significant differences were observed (Tab. 3).

Tab. 3: Ascorbic acid (AA) and dehydroascorbic acid (DHAA) contents. Results are expressed as mg per 100 g_{FW} (mean ± SD, n=3). Values with different letters showed statistically significant differences.

	Vitamin C		
	AA	DHAA	**Total**
Fragaria x ananassa Duch.			
163M88	26.72 ± 1.10 b	6.74 ± 1.11 a	**33.46 ± 2.21 a**
148M6	26.38 ± 0.55 b	5.78 ± 0.59 a	**32.16 ± 1.14 a**
150M61	25.74 ± 1.63 b	6.00 ± 0.64 a	**31.74 ± 2.27 a**
29K55	30.36 ± 0.91 a	3.03 ± 0.30 b	**33.39 ± 1.21 a**
160M77	28.41 ± 1.69 ab	7.46 ± 0.98 a	**35.87 ± 2.67 a**

Discussion
Phenolic compounds
Strawberries
The phenolic compounds found in the five strawberry selections have already been mentioned in literature, but different concentrations have been found (AABY et al., 2005). The selections that showed the highest phenolic compounds were 160M77, 29K55 and 150M61. The mean value of the phenolic compound concentration was 63.46 mg/100 g.
Ellagitannins were detected at 280 nm. The ellagitannins, together with anthocyanins were the most abundant phenolic compounds in the strawberries. In this study, ellagitannins were the main phenolic class in the strawberries, representing 49 % of the analyzed phenolic compounds; this result is in agreement with a previous study performed by KÄHKÖNEN et al., (2001). The mean content of ellagitannins found in this research (30.77 mg/100 g) was within the range found in literature (25-59 mg/100 g) (HÄKKINEN and TÖRRÖNEN, 2000; SKUPIEN et al., 2004). Ellagitannins and ellagic acid have been reported to show *in vitro* and in *vivo* antitumori-

genic and antipromoting activities (LARROSA et al., 2006); for this reason, there is great interest in evaluating the concentrations of ellagitannins and ellagic acid.

Ellagic acid is a dimeric derivative of gallic acid and is generally recognized as a hydrolytic byproduct of the release of a hexahydroxydiphenoyl (HHDP) ester group from ellagitannins, which spontaneously converts to its characteristic bislactone structure. Ellagic acid was mostly present as ellagitannins, and, in this research, the relative amount of ellagic acid and its glycosides was <2 % of the total phenolics. The mean content of ellagic acid found in this study was 1.18 mg/100 g, a similar value to those previously reported for strawberries by AABY et al. (2005), but lower than the values obtained by KOPONEN et al. (2007). This is feasible because the present extraction used a medium containing acetone, as in AABY et al. (2005), whereas KOPONEN et al. (2007) applied acid hydrolysis, which favours the release of ellagic acid from ellagitannins esterified with glucose.

The second phenolic class in the strawberries was anthocyanins, a result that is in agreement with the literature (KÄHKÖNEN et al., 2001). Anthocyanins are a group of flavonoids with exceptionally good antioxidant properties, which have been attributed to the phenolic hydroxyl groups attached to the ring structures (WANG et al., 2002). The mean value of anthocyanins detected in this work was 26.56 mg/100 g; this value is similar, but slightly lower than that found by MÄÄTTÄ et al. (2004) (34.66 mg/100 g). Two anthocyanin glycosides, pelargonidin 3-glucoside and cyanidin 3-glucoside, are almost exclusively responsible for the red color of strawberries. In this study, pelargonidin 3- glucoside was found to be the predominant pigment of the strawberries, and on average represented 75 % of the total anthocyanin content, in agreement with previous studies (MÄÄTTÄ et al., 2004). The fruit color is affected by many ecological factors, such as light and temperature. For this reason, it is possible to find differences in the anthocyanin concentration among studies carried out under different climatic conditions.

The average flavonol content found in this study in the five strawberry selections was 1.24 mg/100 g; a similar value of 2.03 mg/100 g was found in the strawberries by MÄÄTTÄ et al. (2004). It is important to remember that the flavonoid content in strawberries (anthocyanins, flavonols, catechins and flavanones), as in any other fruit, depends on a series of factors, such as the stage of maturity, cultivar, storage conditions and analytical methods, which can readily be inferred from a comparison of different studies on this topic (MIKKONEN et al., 2001).

Quercetin 3-glucuronide has been reported to be the main flavonol in strawberry (AABY et al., 2007), and, again in this work, it has been the most abundant detected flavonol. Flavonols are usually present in food as their glycosides, but are found in biological fluids also as their glucuronidated derivatives (MANACH and DONOVAN, 2004). P-coumaric acid 4-glucoside was the only hydroxycinnamic acid detected in the analysed strawberry selections, with a mean value of 3.30 mg/100 g, a comparable, but slightly higher value, than formerly found by MAATTA et al. (2004) (2.57 mg/100 g) in *Fragaria* x *ananassa* cultivars. The literature data confirm that p-coumaric acid is the most abundant aglycon in strawberries. Soluble hydroxycinnamic acids mainly occur as esters in berry fruit, except in cloudberries (MAATTA et al., 2004); free hydroxycinnamic acids have infrequently been reported in fruit, but it is possible that free hydroxycinnamic acids are released at the late stages of ripening in cloudberry, due to environmental stress factors, or that the unbound acids are typical for these wild berries (KUMPULAINEN and SALONEN, 1996).

Currants and gooseberries

Anthocyanins are mportant polyphenolic components in *Ribes* spp. (MÄÄTTÄ et al., 2001). The mean content of anthocyanins found in

this work for the black currant cultivars was 313.37 mg/100 g; this value was lower than the content of 476.17 mg/100 g found by WU et al. (2004). The most abundant anthocyanin found in these black currant cultivars was delphinidin 3- glucoside, as demonstrated by WU et al. (2004).

The mean amount of anthocyanins detected in the red currant cultivars was 38.29 mg/100 g; this value was higher than that found by WU et al. (2004) (12.09 mg/100 g). The differences can be explained by the fact that the average value in this work is a mean value obtained from 13 different *Ribes rubrum* L. cultivars, while WU et al. (2004) analyzed just one cultivar. Cyanidin 3- xylosylrutinoside was the most abundant anthocyanin detected in the analyzed red currant cultivars, as already found by WU et al. (2004).

Rokula, the only gooseberry cultivar considered in this research, showed a mean anthocyanin value of 5.74 mg/100 g, a similar value to that detected by WU et al. (2004) (5.77 mg/100 g).

Finally, the two hybrids showed a mean anthocyanin value of 25.55 mg/100 g, a lower value than that found by JORDHEIM et al. (2007). It is interesting to note that the individual anthocyanins, identified in the two hybrids, reflected that these cultivars contained more anthocyanins than both parents. This result confirms what has already been observed by JORDHEIM et al. (2007).

Phloroglucinol: identification of proanthocyanidins

Proanthocyanidins are polymeric flavonoid compounds. In this study it was possible to identify the different proanthocyanidins present in 11 cultivars of *Ribes* spp. and the mDP (apparent mean degree of polymerization) was also found. The mDP value was 19.60, although the black currant cultivars showed a mDP of 22.42, a lower value than that found by WU et al. (2004) (47.90). This result can be considered positive, because some studies have suggested that low proanthocyanidin oligomers (DP<4) could be absorbed in the gastrointestinal tract (SANTOS-BUELGA et al., 2000). Dimers have been detected in human blood, after a proanthocyanidins-rich diet has been consumed, while trimers have been shown to be absorbed through the human intestinal cell line Caco-2 (DEPREZ et al., 2001). For this reason, identification of heterogenous proanthocyanidins, especially the low oligomers, are emphasized. (HOLT et al., 2002).

Analysis of vitamin C

The mean value of total vitamin C (ascorbic acid + dehydroascorbic acid) contents in strawberry cultivars analyzed in this study was 33.32 mg/100 g; this value falls within the range of 23-47 mg/100 g detected in strawberry genotypes by TULIPANI et al. (2008). It has been demonstrated that strawberries are rich in vitamin C [a handful of strawberries is sufficient to cover the recommended daily allowance (RDA) of vitamin C] (TULIPANI et al., 2008).

In this study, the strawberries showed higher vitamin C values compared to the other analyzed fruit, but lower than average values of other fruit species, such as grapefruit (52 mg/100 g) or oranges (46 mg/100 g), which are well known for their high vitamin C content (PROTEGGENTE et al., 2002).

Conclusions

The aim of this work was to contribute to the knowledge of the nutraceutical features of some berry fruit species that so far have been studied less than other berry species, such as blueberry and raspberry.

As far as the *Ribes* spp. cultivars are concerned, the presence of a high phenolic compound content, especially in *Ribes nigrum* L., has been confirmed through qualitative analysis. *Ribes* spp. cultivars can therefore be considered as an important source of

antioxidant compounds. Moreover, the results have shown that the tested strawberry cultivars and selections are good sources of bioactive compounds, especially phenolics. In particular, selections 150M61, 160M77 and 29K55 have shown to possess the highest values in polyphenol contents, among the analyzed strawberry fruit selections.

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