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Separation, Characterization and Quantification of Phenolic Compounds in Blueberries and Red and Black Currants by HPLC−DAD−ESI-MS^n

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ABSTRACT: The phenolic profile of four blueberry varieties (*Vaccinium corymbosum* L., cv. *Toro*, *Legacy*, *Duke* and *Bluecrop*) and two varieties (*Rosenthal* and *Rovada*) of red currants (*Ribes rubrum* L.) and black currants (*Ribes nigrum* L.) cultivated in Macedonia have been analyzed using HPLC coupled to diode-array detection and tandem mass spectrometry with electrospray ionization. A complex profile of anthocyanins, flavonols, flavan-3-ols and hydroxycinnamic acid derivatives has been assayed in acetone−acetic acid (99:1, v/v) extracts. Anthocyanins comprised the highest content of total phenolic compounds in currants (>85%) and lower and variety dependent in blueberries (35−74%). Hydroxycinnamic acid derivatives comprised 23−56% of total phenolics in blueberries and 1−6% in currants. Chlorogenic acid was the major hydroxycinnamic acid in blueberries, only in the *Legacy* variety, two malonyl-caffeoylquinic acid isomers were major components. Flavonols, mainly quercetin and myricetin glycosides, were a minor group, but glucosides of laricitrin and syringetin were also detected in the blueberry varieties counting for 10−34% of total flavonols. From flavan-3-ols, catechin was detected in most samples; the dimer B2 was specific for blueberries whereas epigallocatechin was detected in currants.

KEYWORDS: blueberries, red and black currants, phenolic compounds, identification, quantification, HPLC−DAD−ESI-MS^n

INTRODUCTION

A wide variety of phytonutrients are produced by plants, many of which have antioxidant properties and are extensively studied for their beneficial effects on the health of animals and humans, based on scavenging of free radicals generated by environmental and metabolic factors. The interest in the role of phenolic antioxidants in human health has prompted research into the separation and characterization of active phenolic components in various plant-derived foods.[1] Most of these compounds are an integral part of the human diet, and they are also taken as medicinal preparations.[1] Studies have shown that polyphenols, besides vitamins C, E and A, are the components in fruits and vegetables that contribute significantly to their total antioxidant capacity.[2] Berry fruits have been proven as a rich source of various classes of phenolic compounds and their characterization has been of interest for many research groups.[2−12] Kähkönen et al.[3] have done a thorough study of anthocyanins and their antioxidant activities in berries (black currants, bilberries, cowberries), whereas Wu and Prior[4] have studied the anthocyanins in a group of 25 fruits including berries (blueberries) using HPLC coupled to mass spectrometry as well as total phenolics and anthocyanins content, maturity and variety influence on the antioxidant capacity.[2] The composition and stability of black currant anthocyanins have been studied by Rubinskiene et al.,[5] whereas a survey of anthocyanins in wild blueberries has been published by Nicoué et al.[6] Anthocyanins and procyanidins in *Vaccinuim* species (blueberries and cranberries) using normal-phase HPLC coupled to MS have been studied by Prior et al.,[7] whereas catechins and procyanidins in the same species and its correlation to antioxidant activity has been assayed by Määttä-Riihinen et al.[8] Häkkinen et al.[9] have developed a method for detection of flavonoids and phenolic acids in berries (black currants and strawberries) using HPLC−DAD, whereas a study focused on flavonol aglycons and glycosides in berries using HPLC coupled to DAD and electrospray MS has been published by Häkkinen and Auriola.[10] Määttä et al.[11] have studied the phenolic profile of four varieties of the *Ribes* species (black, red, green and white currants) giving very useful spectral data (UV and MS) for identification as well as quantitative data for the contents and relative distributions of the individual phenolic compounds in currants. A similar study has been published by Määttä-Riihinen et al.[12] on the phenolic composition of *Fragaria* and *Rubus* species.

High-performance liquid chromatography (HPLC) has been proved as the most convenient technique for separation and characterization of phenolic compounds in plant materials, coupled to diode array detection (DAD) for routine analysis and mass spectrometry as a more sophisticated one especially for identification of phenolic compounds. UV−vis absorption spectra of phenolic compounds enable their tentative identification and classification into corresponding groups, but combining these data with mass spectral data and information from literature can be used for identification of the many different forms of

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phenolic compounds.[13−15] Electrospray is a commonly used ionization technique that provides the molecular masses, and tandem mass spectrometry (MS^n) provides structural details of the phenolic compounds. Ionization in the positive ion mode is used mainly for anthocyanins detected in their native forms (positive flavylium cations[16]) whereas hydroxybenzoic and hydroxycinnamic acids, flavonol glycosides and condensed tannins show good response in the negative ionization mode.[17,18]

Blueberries, red and black currants are widely cultivated in Macedonia, especially in the east part of the country, but also there are a lot of wild growing varieties. The aim of this study was to develop methodology and characterize the phenolic profiles of blueberries, red and black currants cultivated in Macedonia by combining data obtained by HPLC−DAD and mass spectrometry (MS^n) with electrospray ionization (ESI). All individual derivatives of phenolic acids, flavonols, flavan-3-ols and anthocyanins were identified using UV and MS spectra and quantified using LC−DAD. The obtained data give an insight in the variety and quantity of phenolic compounds in these fruits and can be used for further studies aimed at introducing promising varieties for cultivation as well as for optimizing agricultural practices for production of berry fruits with higher phenolic content.

MATERIALS AND METHODS

Reagents and Standards. Formic acid of analytical grade, methanol and water of gradient grade for liquid chromatography were purchased from Merck KGaA (Darmstadt, Germany). Acetone and acetic acid of analytical grade were purchased from Alkaloid (Skopje, R. Macedonia).

Standards of cyanidin-3-rutinoside, malvidin-3-glucoside, and proanthocyanidin B2 were purchased from Phytolab (Germany); (+)-catechin, rutin and quercetin were purchased from Sigma (Germany); *p*-coumaric acid, ferulic acid and caffeic acid were purchased from Genay (France).

The commercial standards were dissolved as follows: cyanidin-3-rutinoside and malvidin-3-glucoside in mixture of H_2O:MeOH:HCl (88/10/2 v/v/v); quercetin, proanthocyanidin dimer B2, catechin and rutin in water; *p*-coumaric, caffeic and ferulic acid in 70% MeOH in water. The concentration of the stock standard solutions was 1 mg/mL, and they were stored at −20 °C. Calibration curves were prepared for quantitative analysis of phenolic compounds in the studied samples by diluting the stock solutions in the following concentration ranges: cyanidin-3-rutinoside in the range of 10−500 μmol/L; malvidin-3-glucoside in range of 100−500 μmol/L; quercetin, proanthocyanidin B2, catechin, rutin, *p*-comaric acid and ferulic acid all in range 10−50 μmol/L, whereas caffeic acid in two concentration ranges 100−500 μmol/L and 10−50 μmol/L.

Berry Samples. Fresh, ripe samples of cultivated highbush blueberries of the *Vaccinium corymbosum* L. species represented by four varieties (*Toro*, *Legacy*, *Duke* and *Bluecrop*), cultivated red currants of the *Ribes rubrum* L. species represented by two varieties (*Rosenthal* and *Rovada*), and black currants of the *Ribes nigrum* L. species represented by two varieties (*Rosenthal* and *Rovada*) were analyzed for the polyphenols composition. They have been cultivated in different regions in Republic of Macedonia: blueberries were harvested near the city Strumica, and samples of red and black currants were harvest near the cities Pehcevo and Skopje. The berry fruits were harvested at the usual state of maturity, and they were frozen and stored at −80 °C until analysis.

Extraction. For extraction, 5 g of frozen material was extracted with 10 mL of extraction solvent mixture containing acetone and acetic acid (99:1, v/v), as optimized in a previous study[19] and used on strawberry samples.[20] The extracts were sonicated for 15 min in ultrasonic bath (Branson model 3510, USA) and centrifuged for 15 min at 3000 rpm, and the supernatants were concentrated in a rotary evaporator at low temperature (37 °C). The volume of the extract (aqueous residue) was made up to 10 mL with 20% methanol in water, and it was filtered through 0.45 μm pore-size polyethersulfone filter (Econofilter, 25/0.45 μm NL, Agilent Technologies, Germany) before analysis. All extracts were analyzed by HPLC−DAD−ESI-MS.

HPLC/DAD/ESI-MS^n Analysis. The HPLC system was equipped with an Agilent 1100 series diode array and ion trap mass detector in series (Agilent Technologies, Waldbronn, Germany). It consisted of a G1312A binary pump, a G1329A autosampler, a G1379B degasser, G1316A thermostat column and G1315B photodiode array detector, controlled by ChemStation software (Agilent, v.01.03).

Chromatographic separations were carried out on 150 mm × 4.6 mm, 5 μm Zorbax SB-C18 column (Agilent Technologies, Germany). The mobile phase consisted of two solvents: formic acid (5%, v/v) in water (A) and methanol (B). A linear gradient starting with 5% B, 5% B at 5 min, was set to reach 50% B at 45 min, 75% B at 55 min, 100% B at 65 min. The flow rate was 0.4 mL/min and the injection volume was 10 μL for extracts of blueberries and black currants and 20 μL for red currants, because prior experimental results with 10 μL showed very low peak intensity.

Spectral data from all peaks were accumulated in the range 190−600 nm, and chromatograms were recorded at 280 nm for flavan-3-ols, at 320 nm for conjugated forms of hydroxycinnamic acids, at 360 nm for flavonols and at 520 nm for anthocyanins.

The mass detector was an Agilent G2449A ion-trap mass spectrometer equipped with an electrospray ionization (ESI) system and controlled by LCMSD software (Agilent, v.6.2). Nitrogen was used as nebulizing gas at pressure of 50 psi, and the flow rate was adjusted to 12 L/min. The temperature and the voltage of the capillary were maintained at 325 °C and 3.1 kV, respectively. MS data were acquired in positive and negative ionization mode. The full scan covered the mass range from m/z 100 to 1000. Collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas with voltage ramping cycle from 0.3 up to 2 V. Maximum accumulation times of the ion trap and the number of MS repetitions to obtain the MS average spectra were set at 400 ms and 3, respectively.

RESULTS AND DISSCUSSION

LC−DAD was used for spectral and chromatographic analysis and quantitation. Peak assignment of the various forms of phenolic compounds in the chromatograms was based on the comparison of their UV−vis absorption spectra to those of the authentic samples. The conjugated forms of phenolic compounds were further characterized by electrospray ionization mass spectrometric detection in the positive ionization mode for anthocyanins and in negative ionization mode for flavonols, phenolic acids and flavan-3-ols. The HPLC−MS conditions were optimized to obtain an acceptable compromise between separation efficiency and limits of detection.

Qualitative Analysis. The LC−DAD chromatograms obtained for the acetone−acetic acid (99:1, v/v) extract of blueberries (*Legacy* variety) at the four wavelengths are presented in Figure 1 (the identified peaks are numbered An1−An17 for anthocyanins and 1−14 for the other phenolic compounds, which corresponds to the peak data in Table 1). The corresponding chromatograms obtained for red currants (*Rovada* variety) and black currants (*Rovada* variety) at the four wavelengths are presented in Figure 2 and Figure 3, respectively (peak assignments as in Table 2 and Table 3). The peaks i.e. phenolic compounds detected in all studied samples were classified into four groups: conjugated forms of hydroxycinnamic acids,

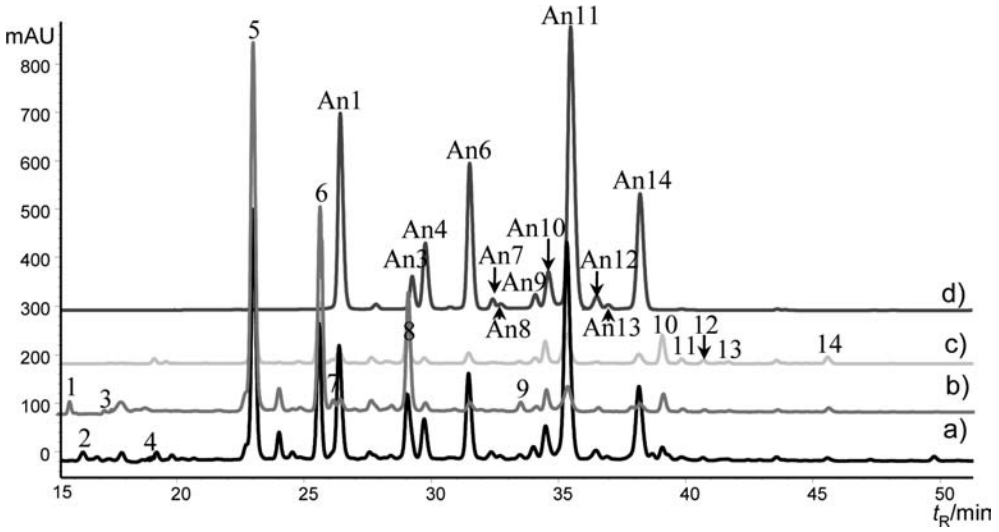

Figure 1. Chromatograms of extracts of blueberries (*Vaccinium corymbosum* L. variety *Legacy*) monitored at (a) 280 nm, (b) 320 nm, (c) 360 nm, and (d) 520 nm for optimal detection of flavan-3-ols, hydroxycinnamic acid derivatives, flavonols and anthocyanins (peak numbers correspond to those in Table 1).

flavonols, flavan-3-ols and anthocyanins. A summary of all identified phenolic compounds for each studied sample type, that is, blueberries, red currants and black currants, is given in Tables 1, 2 and 3, respectively, with the respective retention time, UV−vis absorption maxima, molecular ion and fragment ions for each component (numbered as in Figures 1, 2 and 3). LC−MS and subsequent fragmentation of the dominant ions in MS^2 and further fragmentation in MS^3 were used to obtain more information about the molecular masses of conjugates, masses of the sugars bonded to the aglycons, and the structures of aglycons. Whenever possible, chromatographic retention and literature were used to support the identification of the peaks.

Identification of Anthocyanins. Anthocyanins are responsible for the black and red pigments in berries. They exhibit specific UV−visible absorption maxima allowing the first step in their identification. The identification of all anthocyanins was based on LC−MS data and the identity of anthocyanins in blueberries and red and black currants comparable to those published in earlier studies.[3−7,11,12] The data used for identification of anthocyanins in the extracts of blueberries, red currants and black currants are given in Tables 1, 2 and 3, respectively.

The most abundant anthocyanin in blueberries was malvidin with six derivatives (two hexosides, two pentosides and two acetylated hexosides). 3-*O*-Galactosides and 3-*O*-arabinosides of all five major anthocyanidins have been identified in all four studied blueberry varieties, malvidin-3-*O*-galactoside being the major anthocyanin in accordance with previously published chromatographic profile.[7] 3-*O*-Glucosides of delphinidin, cyanidin, petunidin and malvidin have also been detected as well as acetyl derivatives malvidin-3-*O*-glacatoside and malvidin-3-*O*-glucoside. The order of elution in most investigated cases is galactoside before glucoside, which is before pentosides (arabinoside before xyloside), and acylation of the sugar moieties of anthocyanins causes decrease in polarity and increasing the retention time, so they elute after the respective glycosides (Table 1).[4,6,7]

Only three cyanidin glycosides have been detected in red currants extracts, out of which cyanidin (hexose + pentose)-deoxyhexoside was identified as cyanidin-3-*O*-(2^G-xylosylrutinoside)[11] as the major one. 3-*O*-Glucosides and 3-*O*-rutinosides of delphinidin and cyanidin have been found as as dominant anthocyanins in black currants together with *p*-coumaroyl derivatives of the glucosides of petunidin and peonidin. These results were in accordance with published data for detected anthocyanins in red and black currants.[3,5,11] Literature data were used for comparison and tentative identification was based on UV−vis absorption maxima and MS^n fragmentation pathways. In that way, cyanidin (hexose + pentose) could be identified as cyanidin 3-*O*-sambubioside, and cyanidin (hexose + pentose)-deoxyhexoside as cyanidin 3-*O*-(2^G-xylosylrutinoside).[21]

Two anthocyanins petunidin-3-(6″-coumaroyl) glucoside and peonidin-3-(6″-coumaroyl) glucoside that have not been found before in currants were detected in the studied black currants. These coumaroyl glucoside derivatives have previosly been reported in concord grape and red grape in an earlier study.[4]

Identification of Flavonols. Flavonols as *O*- and *C*-glycosides of quercetin, myricetin and kaempferol have been reported in berry fruits.[10] Their identification was based on the typical UV−vis spectral appearance with absorption maximum around 360 nm and LC−MS in the negative ionization mode with the subsequent MS^2 and MS^3 for further identification with reference to similar data previously reported for blueberries and currants.

Quercetin derivatives were dominant flavonols in blueberries and red currants, and myricetin derivative was found as major flavonol in black currants.

Three peaks with absorption maximum at 356 nm and characteristic MS^2 fragment at *m*/z 301 in negative mode were identified as quercetin glycosides. The detected quercetin 3-*O*-hexoside was identified as quercetin 3-*O*-glucoside,[12] quercetin-3-*O*-(hexoside + deoxyhexoside) as rutin (compared to standard), and quercetin pentoside was identified as quercetin 3-*O*-arabinoside as previously identified in cranberry.[12,22]

Two other peaks (12 and 14) with absorption maximum at 358 nm also had specific flavonol absorption spectra. Peak 12 with $[M - H]^-$ in negative mode at *m*/*z* 493 and MS^2 fragment ion at 331 was identified as laricitrin-3-*O*-glucoside and peak 14 with $[M - H]^-$ in negative mode at 507 and MS^2 fragment ion at 345 was identified as syringetin-3-*O*-glucoside. These flavonol

Table 1. Retention Times, UV−Vis and Mass Spectral Data of Phenolic Compounds in Extracts of Blueberries (*Vaccinium corymbosum* L.)

peak	t_R (min)	λ_{max} (nm)	MW	MS (m/z)	MS^2 (m/z)	compd[a]	varieties
				Anthocyanins			
An1	26.6	276, 526	465	465	303 (100)	delphinidin-3-*O*-gal	all
An2	27.9	276, 526	465	465	303 (100)	delphinidin-3-*O*-glc	*Bluecrop*
An3	29.3	280, 518	449	449	287 (100)	cyanidin-3-*O*-gal	all
An4	29.9	278, 526	435	435	303 (100)	delphinidin-3-*O*-arab	all
An5	30.8	280, 518	449	449	287 (100)	cyanidin-3-*O*-glc	*Bluecrop*
An6	31.6	276, 528	479	479	317 (100)	petunidin-3-*O*-gal	all
An7	32.5	278, 518	419	419	287 (100)	cyanidin-3-*O*-arab	all
An8	32.8	278, 528	479	479	317 (100)	petunidin-3-*O*-glc	all
An9	34.1	280, 518	463	463	301 (100)	peonidin-3-*O*-gal	all
An10	34.6	278, 530	449	449	317 (100)	petunidin-3-*O*-arab	all
An11	35.5	278, 528	493	493	331 (100)	malvidin-3-*O*-gal	all
An12	36.5	278, 528	493	493	331 (100)	malvidin-3-*O*-glc	all
An13	37.0	280, 518	433	433	301 (100)	peonidin pentose	all
An14	38.3	278, 530	463	463	331 (100)	malvidin-3-*O*-arab	all
An15	42.8	278, 532	463	463	331 (100)	malvidin-3-*O*-xyl	*Bluecrop*
An16	43.5	278, 532	535	535	491 (15), 331 (100)	malvidin-3-(6″-acetyl) gal	*Toro*, *Bluecrop*
An17	46.6	278, 532	535	535	331 (100)	malvidin-3-(6″-acetyl) glc	*Bluecrop*
				Flavonols			
10	39.1	256, 356	464	463	301 (100)	quercetin-3-*O*-glc	all
11	39.8	256, 356	610	609	301 (100)	rutin	*Legacy*, *Duke*, *Bluecrop*
12	40.7	262, 360	494	493	331 (100)	laricitrin-3-*O*-glc[b]	all
13	41.6	256, 356	434	433	301 (100)	quercetin-3-*O*-arab	*Legacy*
14	45.6	254, 358	508	507	345 (100)	syringetin-3-*O*-glc[b]	all
				Flavan-3-ols			
2	15.8	242, 280	578	577	425 (100), 407 (72)	dimer B2	*Toro*, *Legacy*, *Bluecrop*
4	18.5	242, 278	290	289	245 (100), 205 (31)	(+)-catechin	*Toro*, *Legacy*, *Bluecrop*
				Hydroxycinnamic Acid Derivatives			
1	15.1	244, 324	354	353	191 (100)	chlorogenic acid	all
3	17.3	246, 330	342	341	179 (100),161 (39)	caffeoylhexose	all
5	23.0	246, 326	354	353	191 (100)	chlorogenic acid	all
6	25.5	246, 326	396	439	395 (100), 233 (55), 173 (4)	malonyl-caffeoylquinic acid[c]	*Legacy*
7	26.0	244, 330	356	355	193 (100), 175 (46)	feruloylhexose	all
8	29.0	246, 326	396	439	395 (100), 233 (75), 173 (8)	malonyl-caffeoylquinic acid[c]	*Legacy*
9	33.4	246, 326	396	601	439 (100)	malonyl-dicaffeoylquinic acid[c]	*Legacy*

[a] gal, galactoside; glc, glucoside; arab, arabinoside; xyl, xyloside. [b] Identified for the first time in blueberries (*Vaccinium corymbosum*), previously found in red grape skins[22] and in bog bilberries, *Vaccinium uliginosum* L.[24] [c] Identified the first time in blueberries (*Vaccinium corymbosum*), previously found in extracts of *Erigeron breviscapus*[25] and *Helichrysum devium*.[26]

glucosides have been previously identified in red grape skins[23] and only in the latest report for bog bilberries (*Vaccinium uliginosum* L.) from Finland.[24]

Only rutin was identified in red currants whereas rutinosides of myricetin and quercetin were detected in black currant extracts (Tables 2 and 3). Previous results from other studies have demonstrated the presence of rutinosides, glucosides[10] and also hexoside-malonates of myricetin, quercetin and kaempferol,[11] but in this survey only rutinosides of myricetin and quercetin were detected in currants.

Identification of Flavan-3-ols. Condensed tannins or proanthocyanidins are homogeneous or heterogeneous polymers of flavan-3-ols. The monomers can be linked through C4−C8 or C4−C6 to form B type proanthocyanidins, or an additional C2−C7 linkage may be present to form doubly linked A type proanthocyanidins.[8] (+)-Catechin (peak 2) and B type proanthocyanidin dimer (peak 4 in Table 1) were identified in all varieties of blueberries except in *Duke* variety according to the retention times, UV−vis spectra and MS data of standards. The MS of catechin in full scan mode with deprotonated molecule $[M - H]^-$ at m/z 289 (peak 2) and MS^2 ions at m/z 245, 205 and UV maximum at 278 nm was in accordance with reported data.[8]The typical MS−MS fragment ions of the molecular ion of the B-type dimer with m/z 577 were detected at m/z 425 and in MS^3 at m/z 407 in the negative mode due to the neutral loss 152 and 170 m/z.[8]

In red currants, only in the *Rovada* variety, (+)-catechin and epigallocathecin were identified (peaks 2 and 3, Figure 2,

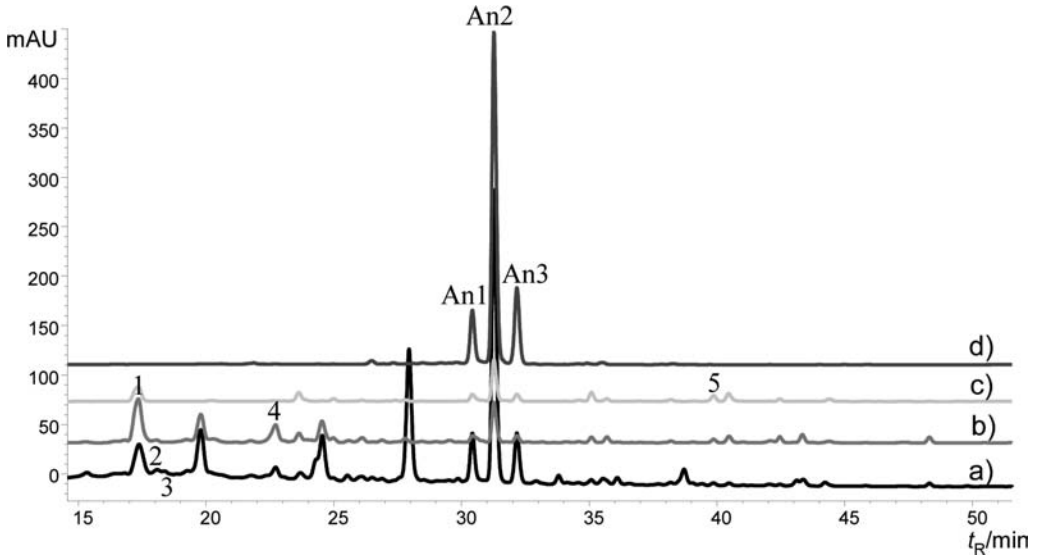

Figure 2. Chromatograms of extracts of red currants (*Ribes rubrum* L. variety *Rovada*) monitored at (a) 280 nm, (b) 320 nm, (c) 360 nm, and (d) 520 nm for optimal detection of flavan-3-ols, hydroxycinnamic acid derivatives, flavonols and anthocyanins (peak numbers correspond to those in Table 2).

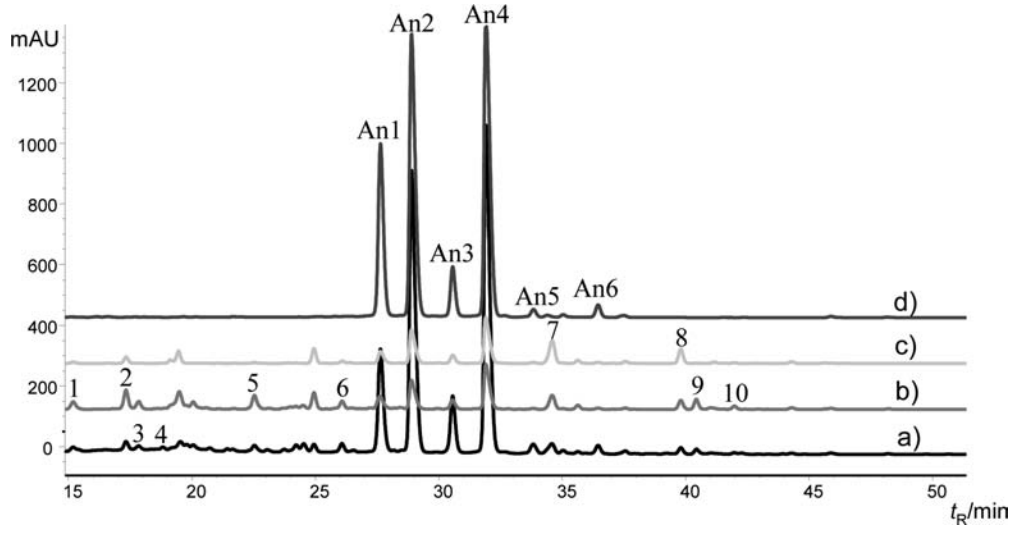

Figure 3. Chromatograms of extracts of black currants (*Ribes nigrum* L. variety *Rovada*) monitored at (a) 280 nm, (b) 320 nm, (c) 360 nm, and (d) 520 nm for optimal detection of flavan-3-ols, hydroxycinnamic acid derivatives, flavonols and anthocyanins (peak numbers correspond to those in Table 3).

Table 2) with absorption maxima at 276 nm and with m/z 289 and 305 and characteristic MS^2 fragments in negative mode with accordance with literature.[11] The same flavan-3-ol monomers were identified in the extracts of both black currant varieties (Figure 3, Table 3).

Identification of Conjugated Forms of Hydroxycinnamic Acids. Phenolic acids are usually found in plants as derivatives of the three hydroxycinnamic acids: caffeic, *p*-coumaric and ferulic acid. In all studied blueberry varieties, ferulic and caffeic acids linked to sugars were detected and chlorogenic acid was the major phenolic acid, which is also an ester of caffeic and quinic acid.[7] Peaks 1 and 5 (Table 1) were due to chlorogenic acid isomers with absorption maximum at 326 nm with m/z 353 and characteristic MS^2 ions at m/z 191, in negative mode due to a loss of caffeoyl moiety giving quinic acid as a main ion. Peak 3 was identified as caffeoylhexose and peak 7 as feruloylhexose giving deprotonated ions with responses at m/z 341 and 355 in MS, and 179 and 193 in MS^2 after elimination of a hexose moiety, respectively.[11]

Only in the extracts of blueberries from the *Legacy* variety, three peaks with m/z 439 (peaks 6 and 8) and m/z 601 (peak 9) with UV spectra implying hydroxycinnamic acid derivatives were detected (Figure 1, Table 1). Peaks 6 and 8 with $[M - H]^-$ at m/z 439 and MS^2 ion at 233 were tentatively identified as malonyl-monocaffeoylquinic acids. Two analogous isomers (malonyl-mono-CQA) with similar fragmentation pattern have been previously described in *Erigeron breviscapus*.[25] Peak 9 with $[M - H]^-$ at m/z 601 and MS^2 ion at 439 was tentatively identified as malonyl-dicaffeoylquinic acid (malonyl-diCQA) according to the fragmentation pattern analogous with the ones found by

Table 2. Retention Times, UV−Vis and Mass Spectral Data of Phenolic Compounds in Extracts of Red Currants (*Ribes rubrum* L.)

peak	t_R (min)	λ_{max} (nm)	MW	MS (m/z)	MS^2 (m/z)	compd	varieties
				Anthocyanins			
An1	30.5	280, 520	581	581	287 (100)	cyanidin 3-*O*-sambubioside	all
An2	31.3	280, 522	727	727	581 (7), 287 (100)	cyanidin 3-*O*-(2^G-xylosylrutinoside)	all
An3	32.2	280, 520	595	595	449 (10), 287 (100)	cyanidin hexose-deoxyhexoside	all
				Flavonols			
5	39.9	256, 356	610	609	301 (100)	rutin	all
				Flavan-3-ols			
2	18.1	244, 276	290	289	245 (100), 205 (31)	(+)-catechin	*Rovada*
3	18.4	244, 276	306	305	261 (42), 179 (100), 137 (14)	EGC	*Rovada*
				Hydroxycinnamc Acid Derivatives			
1	17.2	248, 328	342	341	179 (100), 161 (38)	caffeoylhexose	*Rovada*
4	22.7	242, 316	326	325	163 (100), 119 (13)	*p*-comaroylhexose	all

Table 3. Retention Times, UV−Vis and Mass Spectral Data of Phenolic Compounds in Extracts of Black Currants (*Ribes nigrum* L.)

peak	t_R (min)	λ_{max} (nm)	MW	MS (m/z)	MS^2 (m/z)	compd	varieties
				Anthocyanins			
An1	27.8	276, 526	465	465	303 (100)	delphinidin-3-*O*-glc	all
An2	29.0	276, 528	611	611	465 (7), 303 (100)	delphinidin-3-*O*-rutinoside	all
An3	30.7	280, 518	449	449	287 (100)	cyanidin-3-*O*-glc	all
An4	32.1	280, 520	595	595	449 (9), 287 (100)	cyanidin-3-*O*-rutinoside	all
An5	33.91	276, 530	625	625	317 (100)	petunidin-3-(6″-coumaroyl) glc[a]	all
An6	36.6	280, 520	609	609	301 (100)	peonidin-3-(6″-coumaroyl) glc[a]	all
				Flavonols			
7	34.6	260, 356	626	625	317 (100)	myricetin hexose-deoxyhexoside	all
8	39.8	260, 356	610	609	301 (100)	rutin	all
				Flavan-3-ols			
3	18.5	244, 276	290	289	245 (100), 205 (28)	(+)-catechin	all
4	18.8	244, 276	306	305	261 (35), 179 (100), 137 (14)	EGC	all
				Hydroxycinnamic Acid Derivatives			
1	15.2	244, 324	354	353	191 (100)	chlorogenic acid	all
2	17.3	246, 328	342	341	179 (100), 161 (37)	caffeoylhexose	all
5	22.5	314, 240	326	325	163 (85), 145 (100)	*p*-coumaroylhexose	all
6	26.1	246, 328	356	355	193 (100)	feruloylhexose	all
9	40.4	242, 314	422	421	163 (100), 119 (6)	*p*-coumaric acid hexose deriv	all
10	42.0	244, 330	452	451	193 (100)	ferulic acid hexose deriv	all

[a] Identified for the first time in currants, previously found in concord grape and red grape.[4]

Zhang et al. who described three such isomers in the extracts of *Erigeron breviscapus*,[25] and Gouveia et al. who also found three malonyl-diCQA isomers in the extracts of *Helichrysum devium*.[26] To the best of our knowledge, this the first report on their presence in berry fruits and, which is more interesting, they were detected only in the *Legacy* variety and not in the other three analyzed varieties (*Toro*, *Duke* and *Bluecrop*) of the *Vaccinium corymbosum* species.

p-Coumaric acid derivatives dominated in red and black currant extracts. *p*-Coumaric acid has maximum absorption at 310 nm, but peak 4 (Figure 2, Table 2) and peaks 5 and 9 (Figure 3, Table 3) have experienced a shift of the major absorption maximum from to 316 and 314 nm compared to the respective standard of *p*-coumaric acid due to esterification. *p*-Coumaroylhexose was identified in red currants by its deprotonated ion at m/z 325 in the negative mode MS, and the fragments in MS^2 at m/z 163 after elimination of a hexose moiety and 145 after the subsequent elimination of water.[11]

In black currants, besides *p*-coumaric acid as the main hydrocinnamic acid, derivatives of caffeic and ferulic acids were also detected, which is in accordance with previosly reported data.[10,11] Peaks 9 and 10, (Table 3) with m/z 421 in negative ion mode (163 in MS^2) and m/z 451 (193 in MS^2), respectively, could be just tentatively identified as *p*-coumaric acid hexose derivative and ferulic acid hexose derivative in accordance with literature data.[11]

Table 4. Contents of Phenolic Compounds in Blueberries (*Vaccinium corymbosum* L.) Determined by HPLC−DAD and Expressed in mg per 100 g Fresh Weight ± SD (n = 3)

compd	*Toro*	*Legacy*	*Duke*	*Bluecrop*
Phenolic Compds: Total	**94.60 ± 0.93**	**137.74 ± 1.05**	**113.02 ± 1.28**	**120.14 ± 1.02**
Anthocyanins: Total	**56.35 ± 1.04**	**68.55 ± 2.35**	**83.64 ± 3.16**	**41.99 ± 0.25**
delphinidin-3-*O*-galactoside	7.68 ± 1.42	11.44 ± 3.70	14.99 ± 3.97	2.29 ± 0.21
delphinidin-3-*O*-glucoside	nd[a]	nd	nd	1.21 ± 0.10
cyanidin-3-*O*-galactoside	1.63 ± 0.09	2.29 ± 0.38	2.83 ± 0.60	0.80 ± 0.10
delphinidin-3-*O*-arabinoside	3.99 ± 0.72	4.07 ± 1.15	5.10 ± 1.22	1.66 ± 0.10
cyanidin-3-*O*-glucoside	nd	nd	nd	0.56 ± 0.04
petunidin-3-*O*-galactoside	6.29 ± 1.05	9.42 ± 2.81	11.17 ± 3.78	2.57 ± 0.20
cyanidin-3-*O*-arabinoside	0.84 ± 0.04	0.96 ± 0.12	1.09 ± 0.147	0.42 ± 0.01
petunidin-3-*O*-glucoside	0.70 ± 0.16	0.73 ± 0.10	0.67 ± 0.08	1.29 ± 0.10
peonidin-3-*O*-galactoside	1.16 ± 0.12	1.37 ± 0.32	1.63 ± 0.47	0.77 ± 0.05
petunidin-3-*O*-arabinoside	3.19 ± 0.52	3.34 ± 0.95	4.27 ± 1.27	1.82 ± 0.12
malvidin-3-*O*-galactoside	18.88 ± 4.55	24.79 ± 9.90	30.29 ± 13.76	12.11 ± 0.98
malvidin-3-*O*-glucoside	0.68 ± 0.04	1.37 ± 0.37	1.51 ± 0.35	6.03 ± 0.78
peonidin-3-*O*-pentose	0.68 ± 0.06	0.69 ± 0.15	0.68 ± 0.09	0.52 ± 0.03
malvidin-3-*O*-arabinoside	8.88 ± 1.91	8.08 ± 3.26	9.41 ± 4.09	6.77 ± 0.70
malvidin-3-*O*-xyloside	nd	nd	nd	0.56 ± 0.05
malvidin-3-(6″-acetyl) galactoside	1.74 ± 0.28	nd	nd	0.99 ± 0.08
malvidin-3-(6″-acetyl) glucoside	nd	nd	nd	1.63 ± 0.03
Flavonols: Total	**2.28 ± 0.80**	**5.17 ± 0.03**	**3.41 ± 0.16**	**6.08 ± 0.45**
quercetin-3-*O*-glucoside	0.91 ± 0.19	1.59 ± 0.02	1.15 ± 0.26	0.97 ± 0.25
rutin	nd	1.58 ± 0.07	0.78 ± 0.34	3.52 ± 1.25
laricitrin-3-*O*-glucoside[b]	0.61 ± 0.02	0.63 ± 0.03	0.65 ± 0.05	0.63 ± 0.05
quercetin-3-*O*-arabinoside	nd	0.58 ± 0.01	nd	nd
syringetin-3-*O*-glucoside[b]	0.77 ± 0.05	0.79 ± 0.07	0.83 ± 0.16	0.97 ± 0.12
Flavan-3-ols: Total	**2.85 ± 0.54**	**1.75 ± 0.07**	nd	**4.52 ± 0.43**
dimer B2	0.91 ± 0.42	0.40 ± 0.01	nd	1.51 ± 0.15
(+)-catechin	1.94 ± 0.99	1.35 ± 0.13	nd	3.01 ± 0.84
Hydroxycinnamc Acid Deriv: Total	**33.12 ± 1.78**	**62.27 ± 1.97**	**25.97 ± 3.21**	**67.54 ± 3.03**
chlorogenic acid	0.40 ± 0.04	0.26 ± 0.02	0.29 ± 0.05	0.46 ± 0.07
caffeoylhexose	0.19 ± 0.01	0.22 ± 0.03	0.17 ± 0.01	0.21 ± 0.02
chlorogenic acid	31.39 ± 5.03	31.61 ± 5.81	24.87 ± 9.07	65.24 ± 8.55
malonyl-caffeoylquinic acid[c]	nd	19.20 ± 4.15	nd	nd
feruloylhexose	1.15 ± 0.17	0.91 ± 0.32	0.64 ± 0.26	1.63 ± 0.38
malonyl-caffeoylquinic acid[c]	nd	9.32 ± 1.79	nd	nd
malonyl-dicaffeoylquinic acid[c]	nd	0.76 ± 0.18	nd	nd

[a] Not detected. [b] Identified for the first time in blueberries (*Vaccinium corymbosum*), previously found in red grape skins[22] and in bog bilberries, *Vaccinium uliginosum* L.[24] [c] Identified for the first time in blueberries (*Vaccinium corymbosum*), previously found in extracts of *Erigeron breviscapus*[25] and *Helichrysum devium*.[26]

Quantitative Analysis. The contents of selected phenolic classes of the studied fruits are shown in Table 4 and Table 5 expressed as mg per 100 g of fresh weight of berry fruits. Quantification of all phenolic compounds was made by LC−DAD using the corresponding standards for calibration for each group (malvidin-3-glucoside for anthocyanins in blueberries; cyanidin-3-rutinoside for anthocyanins in black and red currants; rutin for flavonols; catechin and proanthocyanidin dimer B2 for flavan-3-ols; *p*-coumaric, ferulic and caffeic acid for the corresponding hydroxycinnamic acid derivatives) and detection wavelength where each group exhibits absorption maximum. As previously reported,[7,8,11] anthocyanins are the major phenolic class that characterizes berries of *Vaccinium* and *Ribes* species. It was found that from anthocyanins, malvidin-3-galactoside, cyanidin-3-*O*-(2^{G}-xylosylrutinoside) and cyanidin-3-*O*-rutinoside were the most abundant phenolic components in blueberries, red currants and black currants, respectively.

In our study, five of the six most widespread anthocyanindins were found mostly as glycosides in the investigated four blueberry varieties, which is in agreement with those reported in the literature.[4,6,7,11] Maximal anthocyanin content was found in the variety *Duke* (83.65 mg/100 g fresh weight) and minimum content in the variety *Bluecrop* (41.99 mg/100 g fresh weight). The relative content of anthocyanins in the total content of

Table 5. Contents of Phenolic Compounds in Red Currants (*Ribes rubrum* L.) and Black Currants (*Ribes nigrum* L.) Determined by HPLC−DAD and Expressed in mg per 100 g Fresh Weight ± SD (n = 3)

	red currants		black currants	
compd	*Rosenthal*	*Rovada*	*Rosenthal*	*Rovada*
Phenolic Compds: Total	**18.05 ± 0.58**	**17.97 ± 0.31**	**207.77 ± 1.14**	**187.69 ± 1.84**
Anthocyanins: Total	**15.93 ± 0.95**	**14.73 ± 0.29**	**180.44 ± 3.59**	**162.83 ± 2.46**
delphinidin-3-*O*-glucoside	nd[a]	nd	16.86 ± 4.07	13.92 ± 7.33
cyanidin-3-*O*-glucoside	nd	nd	2.89 ± 1.26	13.62 ± 14.58
cyanidin 3-*O*-sambubioside	1.91 ± 0.34	1.93 ± 0.16	nd	nd
cyanidin-3-*O*-(2^G-xylosylrutinoside)	10.57 ± 2.22	10.71 ± 0.84	nd	nd
delphinidin-3-*O*-rutinoside	nd	nd	89.66 ± 8.33	65.27 ± 25.39
cyanidin-3-*O*-rutinoside	3.45 ± 0.59	2.09 ± 0.13	67.42 ± 8.18	66.26 ± 24.21
petunidin-3-(6″-coumaroyl) glucoside	nd	nd	2.34 ± 0.42	2.01 ± 0.74
peonidin-3-(6″-coumaroyl) glucoside	nd	nd	1.27 ± 0.29	1.75 ± 0.52
Flavonols: Total	**1.89 ± 0.08**	**0.48 ± 0.005**	**7.36 ± 0.57**	**6.95 ± 0.92**
rutin	1.89 ± 0.28	0.477 ± 0.07	4.24 ± 0.94	4.58 ± 1.64
myricetin hexose-deoxyhexoside	nd	nd	3.12 ± 0.65	2.37 ± 0.85
Flavan-3-ols: Total		**1.60 ± 0.002**	**13.35 ± 0.90**	**11.02 ± 1.23**
(+)-catechin	nd	0.77 ± 0.01	7.49 ± 1.31	5.07 ± 2.00
EGC	nd	0.84 ± 0.01	5.86 ± 1.77	5.95 ± 2.26
Hydroxycinnamc Acid Deriv: Total	**0.23 ± 0.002**	**1.16 ± 0.10**	**6.62 ± 0.18**	**6.89 ± 0.24**
chlorogenic acid	nd	nd	0.79 ± 0.16	0.97 ± 0.30
caffeoylhexose	nd	0.86 ± 0.19	2.07 ± 0.49	2.17 ± 0.66
feruloylhexose	nd	nd	1.32 ± 0.32	1.18 ± 0.31
p-comaroylhexose	0.23 ± 0.02	0.30 ± 0.02	0.90 ± 0.11	0.89 ± 0.12
p-coumaric acid hexose derivative	nd	nd	0.91 ± 0.05	0.97 ± 0.08
ferulic acid hexose derivative	nd	nd	0.63 ± 0.01	0.71 ± 0.14

[a] Not detected.

phenolics varied in the studied blueberries from around 1/3 in *Bluecrop* (35% contribution of total anthocyanins to total polyphenolics in sample), around 50−60% in *Legacy* (49.76%) and *Toro* (59.55%), to 74% in the variety *Duke*. Prior et al.[7] have found 2.2 mg/g of total anthocyanins in blueberries of the variety *Rubel* of the same *Vaccinium corymbosum* species employing extraction of freeze-dried sample and converting the result to fresh weight based upon dry matter content of samples.

Lower content of anthocyanins was found in red currants (*Rosenthal*, 15.93 mg/100 g; *Rovada*, 14.72 mg/100 g fresh weight) but it comprised over 80% of total phenolics in these berries (83% and 82%, respectively). Ten times higher content of anthocyanins was measured in black currants (*Rosenthal*, 180.44 mg/100 g, *Rovada*: 162.85 mg/100 g fresh weight) with similar relative content of anthocyanins of around 87% of total phenolics. Similar content and also ratio between the anthocyanins content in black and red currants were found by Määttä et al.[11]

Hydroxycinnamic acid derivatives are the second major phenolic class in blueberries. All conjugates of these phenolics are more abundant in blueberries than in currants. The most abundant phenolic acid in blueberries was chlorogenic acid (Figure 1, peak 5 at 320 nm) in a concentration range from 24.87 to 65.24 mg/100 g fresh weight followed by ferulic and other caffeic acid derivatives. The total content of hydroxycinnamic acid derivatives varied from 67.54 mg/100 g fresh weight (56% of total phenolic compounds) in the *Bluecrop* variety and 62.28 mg/100 g (45%) in *Legacy* to the smaller content of 33.12 mg/100 g (35%) in *Toro* and 25.97 mg/100 g (23%) in the *Duke* variety. The interesting finding here was the detection of the two malonyl derivatives of chlorogenic acid in the blueberry variety *Legacy* in significant quantity (19.20 mg/100 g fresh weight, Figure 1, peaks 6 and 8 at 320 nm) together with one malonyldicaffeoylquinic acid isomer in minor quantity (0.756 mg/100 g fresh weight, Figure 1, peak 9 at 320 nm). As pointed out above, they have not been previously reported in other berries.

Smaller amounts of hydroxycinnamic acid derivatives were detected in red currants compared to black currants. The content and distribution related to total phenolic compounds found was as follows: for red currants, 0.23 mg/100 g fresh weight (1%) in *Rosenthal* variety and 1.16 mg/100 g (6%) in *Rovada*, and for black currants, 6.6 mg/100 g (3%) in *Rosenthal* and 6.89 mg/100 g (4%) in *Rovada* variety. These values correspond to the ones found by Määttä et al.,[11] who measured 8 mg/kg fresh weight for red currants and 84 mg/kg fresh weight for black currants for total content of glycosides of caffeic, *p*-coumaric and ferulic acid.

Flavonols were found in all samples. Quercetin was the major flavonol in blueberries and red currants, and a significant content of myricetin was measured in black currants. The content and distribution of flavonols in blueberries found was as follows: in *Toro* 2.28 mg/100 g fresh weight (2%), in *Duke* 3.4 mg/100 g (3%), in *Legacy* 5.17 mg/100 g (3%) and in *Bluecrop* as 6.08 mg/100 g (5%). Also, the 3-*O*-glucosides of the less abundant

flavonols laricitrin and syringetin were measured in all four blueberry varieties in quantities between 0.607 and 0.967 mg/100 g fresh weight comprising 26−60% of total flavonols detected. The recent report for bog bilberries (*Vaccinium uliginosum* L.) from Finland demonstrated the presence of these two flavonols in ranges 47−89 mg/kg dry weight and 28−73 mg/kg dry weight for laricitrin and syringetin, respectively, each comprising around 5% of total flavonols.[24] In currants, slightly higher flavonol content was measured in the variety *Rosenthal* (1.89 mg/100 g fresh weight for red currants; 7.36 mg/100 g for black currants) than in *Rovada* (0.48 mg/100 g for red currants; 6.94 mg/100 g for black currants). This report was in accordance with some literature data[10,11] but not the same with another reported profile[9] where quercetin was the main flavonol in black currants followed by myricetin and kaempferol (30%, 16% and 6% of total phenolic content, respectively). In our study, kaempferol was not found, and the total flavonols content in currants counted for 2−10% in red currants and between 3 and 4% of total phenolics in black currants due to the presence of rutin only in red currants and rutin and myricetin hexose-deoxyhexoside (tentatively myricetin-rutinoside) in black currants.

The condensed tannins i.e. flavan-3-ols were the minor group in blueberries and red currants but second major in black currants. The content of these polyphenols varied in blueberry extracts from 1.75 mg/100 g (1%) in *Legacy* to 4.52 mg/100 g (3%) in *Bluecrop*. Condensed tannins were found only in red currants *Rovada* as 1.6 mg/100 g, but not in *Rosenthal*. In both varieties of black currants similar content of this type of polyphenols was measured: 13.34 mg/100 g for *Rosenthal* and 11.02 mg/100 for *Rovada*, which counted for around 6% of total phenolic compounds. These results are in accordance with the literature.[9,11]

In conclusion, 55 phenolic compounds have been detected and measured in acetone−acetic acid extracts (99:1, v/v) of cultivated blueberries and red and black currants from Macedonia illustrating a complex profile of anthocyanins, flavonols, flavan-3-ols and conjugated forms of hydroxycinnamic acids assayed with HPLC−DAD−MS^n. An interesting finding here was the presence of 3-*O*-glucosides of the less abundant flavonols laricitrin and syringetin in all four blueberry varieties comprising 26−60% of total flavonols detected, which is a first report for their presence in *Vaccinium corymbosum* species. Also found were two malonyl derivatives of chlorogenic acid in the blueberry variety *Legacy* in significant quantity (19.20 mg/100 g fresh weight, Figure 1, peaks 6 and 8 at 320 nm) together with one malonyl-dicaffeoylquinic acid isomer in minor quantity (0.756 mg/100 g fresh weight, Figure 1, peak 9 at 320 nm). As pointed out above, they have not been previously reported in other berries.

The wide variety of the phenolic compounds found in the studied blueberries and red and black currants implies their potential beneficial effects for human health due to their use in the everyday diet. The obtained data can also be used for further studies aimed at introducing promising varieties for cultivation as well as for optimizing agricultural practices for production of berry fruits with higher phenolic content.

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