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High-Performance Liquid Chromatography (HPLC) Analysis of Phenolic Compounds in Berries with Diode Array and Electrospray Ionization Mass Spectrometric (MS) Detection: *Ribes* Species

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High-performance liquid chromatography combined with diode array and electrospray ionization mass spectrometric (MS) detection was used to study phenolic compounds in berries of black, green, red, and white currants (*Ribes* spp.). UV−visible spectrometry was a valuable tool for the identification of the class of the phenolic compound, whereas MS and MS-MS fragmentation data were useful for further structural characterization. Distinct similarities were found in the relative distribution of conjugated forms of phenolic compounds among the four currants. Phenolic acids were found mainly as hexose esters. Flavonol glycosides and anthocyanin pigments were mainly found as 3-*O*-rutinosides and second as 3-*O*-glucosides. However, cyanidin 3-*O*-sambubioside and quercetin hexoside−malonate were notable phenolic compounds in red currant. Flavonol hexoside−malonates were identified and quantified in the berries of currants for the first time.

KEYWORDS: Food analysis; berries; currants; phenolic compounds; HPLC; mass spectrometry; diode array detection

INTRODUCTION

Dietary phenolic compounds have received much attention during recent years due to their antioxidant and other biological properties imparting possible benefits to human health (*1*−*4*). Berries and fruits are rich sources of phenolic compounds (*5*) including anthocyanins, flavonols, ellagitannins, and hydroxybenzoic and hydroxycinnamic acid derivatives as well as flavan-3-ols and their polymeric forms proanthocyanidins (*6*−*9*). The evaluation of berries as a source of dietary phenolic antioxidants involves questions about their absorption, distribution, metabolism, and excretion. It was suggested that the number, position, and nature of conjugated sugars have an influence on the absorption and bioavailability of flavonols (*10*−*12*). Hence, studies on the quantification of phenolic compounds should take into account the variable structures of their conjugated forms.

The most frequently used analytical technique for the separation of phenolic compounds is reversed-phase high-performance liquid chromatography (RP-HPLC) (*13*). Detection techniques for HPLC methods are various, but diode array detection (DAD) is currently the most widely available and commonly used technique for routine qualitative and quantitative analysis of phenolic compounds (*6*, *7*, *9*, *13*−*15*). The UV−visible absorption spectra of phenolic compounds enable identification and classification of chromatographic peaks into classes, but the combination of these data with mass spectra (MS) data and information from the respective literature can be used for tentative identification of the conjugated forms (*15*−*18*).

Soft ionization electrospray mass spectrometry provides the molecular masses of chromatographically separated molecules, and tandem mass spectroscopy (MS-MS) provides extra structural details of thermally labile, nonvolatile polar phenolic compounds. Ionization may be performed in the positive and/or negative ion mode. In the negative ionization mode, acidic hydroxybenzoic and hydroxycinnamic acids deprotonate easily (*18*−*21*), and in the positive ionization mode, they form adducts with the cations in the sample or mobile phase, for example, sodium ions (*17*, *22*, *23*). Depending on the chromatographic conditions, monomeric flavan-3-ols and dimeric and trimeric proanthocyanidins favor both protonation to positive ions (*24*) and deprotonation to negative ions (*20*, *25*−*27*); the latter takes place more easily with the longer proanthocyanidin chains (*28*). Flavonol glycosides also show response in both positive and negative ionization modes (*16*, *24*, *29*, *30*), and anthocyanins are mainly identified in positive ion mode in their native forms, that is, positive flavylium cations (*31*−*33*).

We have previously reported the contents of phenolic compounds as classes in currant berries (*Ribes* spp.; family,

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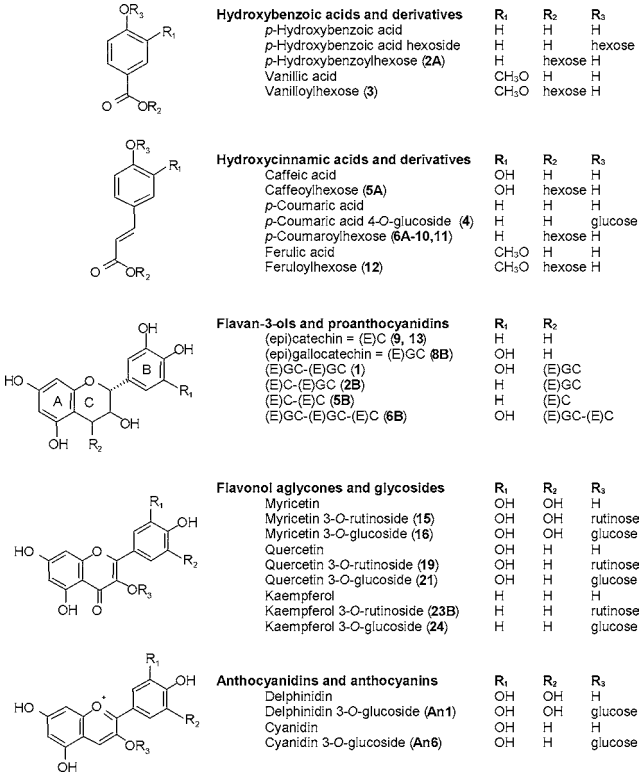

Figure 1. Structures of aglycons and conjugates of phenolic compounds found in *Ribes* spp. (peak numbers in parentheses).

Grossulariaceae; syn. Saxifragaceae) (*9*). Black (*Ribes nigrum*) and red (*Ribes* × *pallidum*) currants are widely cultivated in North America and Europe, but their unpigmented variants, green (*Ribes nigrum*) and white (*Ribes* × *pallidum*) currants, are less common. The aim of this paper is tentatively to identify the structures of the conjugated forms of phenolic compounds in currants by combining data obtained by DAD and electrospray ionization MS after separation by RP-HPLC. The sample preparation is based on sequential extraction, which means prefractionation of anthocyanins from the other phenolic compounds (*9*). After identification, individual derivatives of hydroxycinnamic acids, flavonol glycosides, and anthocyanins (**Figure 1**) were quantified using LC-DAD.

MATERIALS AND METHODS

Samples and Standards. Berries of black (*R. nigrum* cv. Öjebyn), green (*R. nigrum* cv. Vertti), red (*R.* × *pallidum* cv. Red Dutch), and white (*R.* × *pallidum* cv. White Dutch) currants obtained from a local market in 1999 were used for the identification of phenolic compounds with LC-DAD and LC-MS. For the quantification, fresh berries were harvested at maturity in 2000 and analyzed within 2 days (*9*).

The phenolic compounds *p*-hydroxybenzoic acid (H5376), vanillic acid (V2250), chlorogenic acid (C3878), *p*-coumaric acid (C9008), (+)-catechin (C1251), (−)-epicatechin (E1753), rutin (quercetin 3-*O*-rutinoside or quercetin 3-*O*-glucose-rhamnoside) (R5143), quercetin (Q0125), and kaempferol (K0133) were purchased from Sigma Chemical Co. (St. Louis, MO), and myricetin was obtained from Fluka (Buchs, Switzerland). Delphinidin and cyanidin 3-*O*-β-glucosides were obtained from Polyphenols AS (Sandnes, Norway), and delphinidin (0904S) and cyanidin (0909S) chlorides were purchased from Extrasynthese (Geney Cedex, France). These commercial standards were dissolved in methanol to a concentration of ∼1 mg/mL and stored at −20 °C as stock solutions. A base hydrolysate of flaxseed extract containing 4-*O*-glucosides of *p*-coumaric acid and ferulic acid was obtained as described by Johnsson et al. (*22*).

Sample Preparation. The extraction of berries in a two-step procedure with ethyl acetate and methanol has previously been described by Määttä et al. (*9*). Frozen berries were homogenized, and samples were weighed in centrifuge tubes for extraction. Hydroxycinnamic acid derivatives, flavan-3-ols, dimeric and trimeric proanthocyanidins, and flavonol glycosides were first extracted using ethyl acetate with intermittent mixing and centrifugation. The residue of the berry matrix was acidified with hydrochloric acid (2 M, 2 mL), and anthocyanins and some residues of flavonol glycosides and hydroxycinnamic acid derivatives were extracted into methanol. The ethyl acetate extract and an aliquot of the methanol extract were separately evaporated to dryness and reconstituted into methanol for analysis by HPLC. Anthocyanins of black currant were analyzed directly from the methanol extract. The occurrence of quercetin hexoside−malonate in the red currant was confirmed according to the modified method of Lin et al. (*34*), in which an ethyl acetate extract reconstituted in methanol was heated in a sealed vial at 70 °C for 30 h and cooled to room temperature prior to analysis.

LC-DAD and LC-MS Analyses. The HPLC apparatus consisted of a Hewlett-Packard instrument with a 1100 series quaternary pump, an autosampler, and a diode array detector linked to an HP-ChemStation data handling system (Waldbronn Analytical Division, Germany). The separation of the phenolic compounds was achieved on a LiChroCART Purospher RP-18e column (125 × 3 mm i.d., 5 μm, Merck, Darmstadt, Germany) protected with a guard column of the same material (4 × 4 mm). The system used for LC-MS analysis was a Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA) equipped with a Rheos 400 HPLC pump (Danderyd, Sweden). Conditions for the initial ionization in the positive and negative ionization modes included capillary voltages at +4.5 and −3 kV and a temperature at 225 °C. The MS data were acquired as full scan mass spectra at *m*/*z* 150−1500 by using 200 ms for collection of the ions in the trap. Tandem MS (MS-MS) was performed using helium as the collision gas, and the collision energy was set at 30%. MS revealed the positive or negative molecular ions; MS-MS broke down the most abundant ones with dependent collision-induced dissociation.

For the analysis of hydroxycinnamic acid derivatives, flavan-3-ols, dimeric and trimeric proanthocyanidins, and flavonol glycosides in LC-DAD and all phenolic compounds in LC-MS, a 20 min linear gradient from 5 to 30% acetonitrile in 1% formic acid in water was used. The gradient for LC-DAD was achieved using acetonitrile and 1% formic acid in the separate bottles, but for the accurate performance of the pump in LC-MS, 10% of organic solvent was premixed in the water phase. For the quantification of anthocyanins in LC-DAD, the separation was achieved using 5% formic acid in water and acetonitrile according to the following gradient: 5−10% acetonitrile (0−5 min), 10% acetonitrile (5−10 min), 10−40% acetonitrile (10−25 min), and finally 40−90% acetonitrile (25−35 min). Both gradients were at a flow rate of 0.5 mL/min. Because different gradients were used for anthocyanins and the other phenolic compounds, the retention times for these classes are not comparable.

LC-DAD was used for spectral and chromatographic analysis and quantification. Identification and quantification of the peaks in DAD chromatograms were the same as in the previous study (*9*) and in accordance with common practice (*13*). Peak assignment of the conjugated forms of phenolic compounds in the chromatograms was based on the comparison of their spectral characteristics with those of the representative standards of the phenolic classes (*9*). UV−visible spectral maxima were approximated from the isoplot of the peak within a variation of ±2 nm. The conjugated forms of phenolic compounds were quantified using the response factors of their representative standards near their characteristic wavelengths of maximum absorption (hydroxycinnamic acids at 320 nm, flavonol glycosides at 360 nm, and anthocyanins at 520 nm). Response factors of anthocyanins were determined in acidified methanol (0.6 M HCl). The contents of hydroxycinnamic acid derivatives and flavonol glycosides in the ethyl acetate extract and their residues in the methanol extracts were combined. The minimum for the shown quantified values (0.4 mg of aglycon/kg of fresh weight) was determined by the lowest concentration of standards in the calibration curves.

The conjugated forms of phenolic compounds were further identified by HPLC with electrospray ionization mass spectrometric (ESI) detection. The parameters for positive ionization were adapted from Häkkinen et al. (*29*) and those for negative ionization from Mämmelä

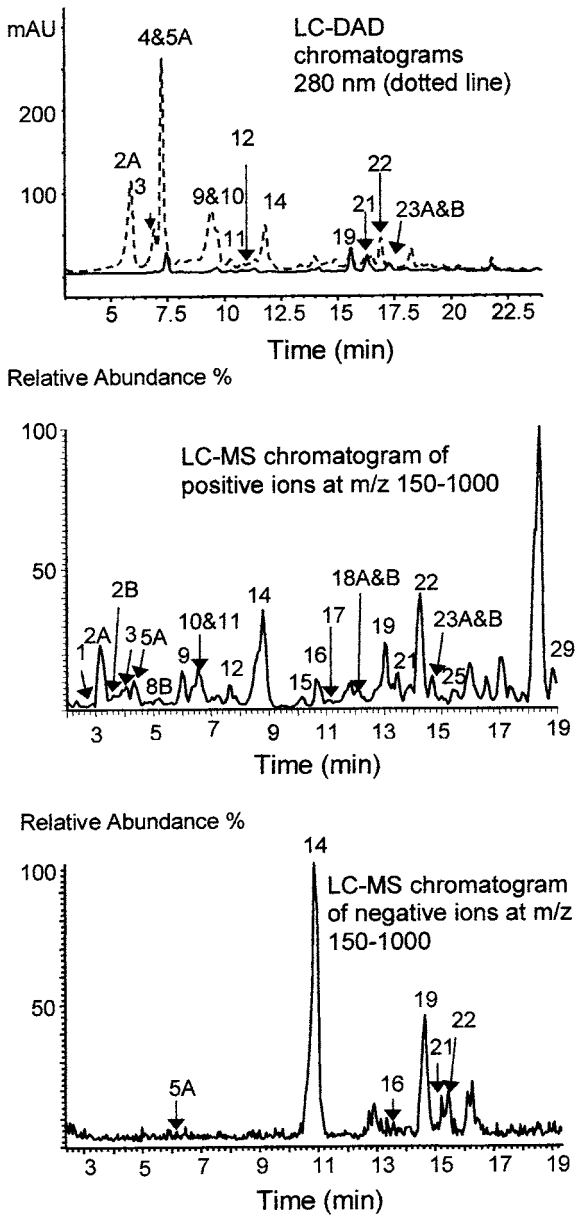

Figure 2. Chromatograms of LC-DAD and LC-MS in positive and negative ionization modes for ethyl acetate extract of white currant. Peak numbers refer to **Table 1**.

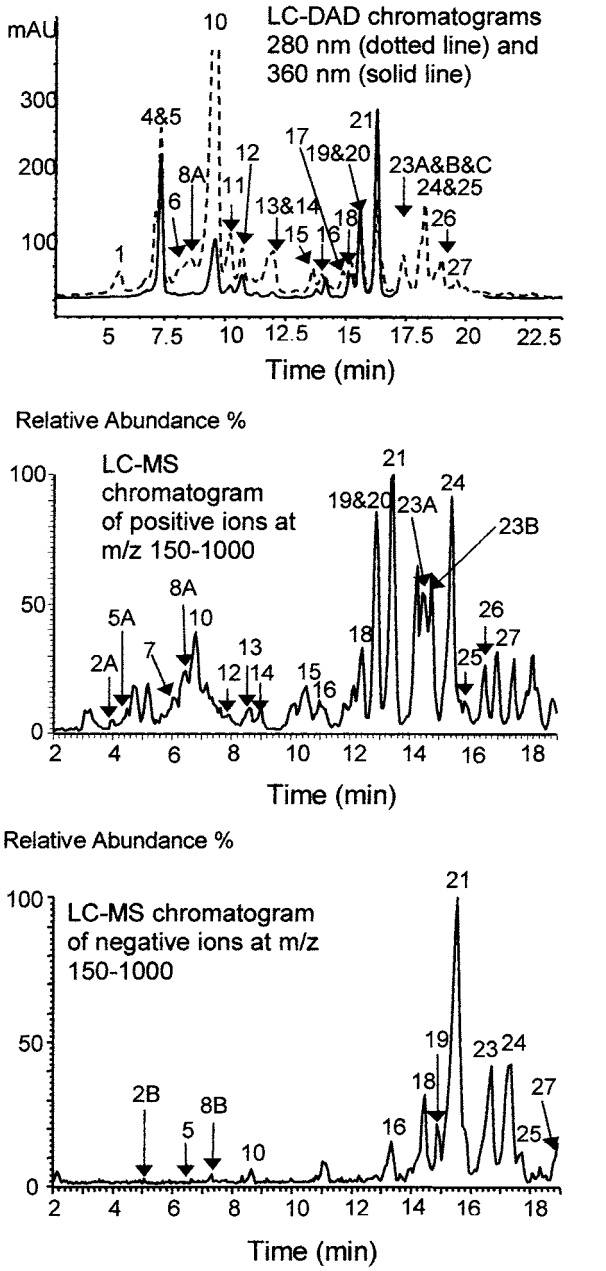

Figure 3. Chromatograms of LC-DAD and LC-MS in positive and negative ionization modes for ethyl acetate extract of green currant. Peak numbers refer to **Table 1**.

et al. (*35*). The ionization conditions were optimal in the positive ionization mode for (+)-catechin, (−)-epicatechin, rutin, flavonols (quercetin, myricetin, and kaempferol), and anthocyanins (delphinidin and cyanidin 3-*O*-*β*-glucosides) and in the negative ionization mode for chlorogenic acid, rutin, and flavonols.

RESULTS AND DISCUSSION

Identification of the Chromatographic Peaks. In the previous paper (*9*), we examined the contents of phenolic compound classes in black, green, red, and white currants using LC-DAD. Peaks in the chromatograms were classified into hydroxybenzoic and hydroxycinnamic acid derivatives, flavonol glycosides, and anthocyanins by comparison of their UV−visible spectra with those of the available standards. In this study, we used LC-MS and MS-MS in both positive and negative ionization modes in order to obtain more information on the structural features of the conjugated forms of phenolic compounds. The masses of the sugars bound to the aglycons and the specific fragmentation patterns of the compounds were used for identification of the chromatographic peaks. The bound sugar moieties consist of hexoses with a mass unit of 162 (glucose or galactose), deoxyhexoses with a mass unit of 146 (rhamnose), and pentoses with a mass unit of 132 (xylose or arabinose). When the conjugated phenolic forms presented bathochromic shifts in the UV−visible absorption spectra compared to their respective aglycons (shifts to longer wavelength), this indicated esterification of the aglycons with sugars, and when they presented hypsochromic shifts to shorter wavelengths, this indicated glycosidation. Whenever possible, chromatographic retention was used to support the tentative identification of some peaks. However, this was sometimes complicated by the fact that retention is governed not only by the polarity of the molecules but also by their size.

Figures 2 and **3** show the LC-DAD chromatograms recorded at 280 and 360 nm and the LC-MS ion chromatograms obtained in the positive and negative ionization modes for the ethyl

Table 1. Identification of Phenolic Compounds in Ethyl Acetate Extracts of Black, Red, Green, and White Currants by Using Their Spectral Characteristics in LC-DAD, Positive and Negative Ions in LC-MS and MS-MS, and Respective Standards

peak(s)	t_R (min)	spectral characteristics[b] (nm)	MW	positive ions[a] MS (*m*/*z*)	positive ions[a] MS-MS (*m*/*z*)	negative ions MS (*m*/*z*)	negative ions MS-MS (*m*/*z*)	tentative identification[c]
				Hydroxybenzoic Acid Derivatives				
2A	5.3	262	300	323 (138 + 162 + 23)	**185**, 226, 249	ND	ND	*p*-hydroxybenzoylhexose
3	6.3	264, 296	330	353 (168 + 162 + 23)	**185**, 226, 231	ND	ND	vanilloylhexose
				Hydroxycinnamic Acid Derivatives				
4	6.5	234, 296	326	ND	ND	ND	ND	*p*-coumaric acid 4-*O*-glucoside (std)
5A	6.9	244, 300sh, 330	342	365 (180 + 162 + 23)	**185**, 226, 243	341 (180 + 162 − 1)	161, **179**	caffeoylhexose
6A, **7**, **8A**, **10**	7.3–9.1	236, 300sh, 314	326	349 (164 + 162 + 23)	**185**, 226	325 (164 + 162 − 1)	145, **163**	*p*-coumaroylhexose
11	9.7	236, 300sh, 314	326	349 (164 + 162 + 23)	**187**, 228	325 (164 + 162 − 1)	**145**, 163, 265	*p*-coumaroylhexose
12	10.1	244, 296sh, 330	356	379 (194 + 162 + 23)	**185**, 226, 257	ND	ND	feruloylhexose
20	14.9	ND	535	536 (180 + 96 + 162 + 98)	**276**, 438	ND	ND	caffeic acid hexose derivative
25	17.5	236, 300sh, 314	520	520 (164 + 96 + 162 + 98)	**260**, 358, 422	421 (164 + 96 + 162 − 1)	163	*p*-coumaric acid hexose derivative
26	17.8	234, 296, 330	452	452 (194 + 96 + 162)	**290**, 177	ND	ND	ferulic acid hexose derivative
				Flavan-3-ols and Proanthocyanidins				
1	5.1	270	610	611 (305 + 305 + 1)	305, 317, 425, **443**	609 (305 + 305 − 1)	441	(E)GC–(E)GC
2B	5.5	ND	594	595 (289 + 305 + 1)	287, 427, **443**	593 (289 + 305 − 1)	425	(E)C–(E)GC
5B	7.0	ND	578	579 (289 + 289 + 1)	409, **427**	ND	ND	(E)C–(E)C
6B	7.8	ND	898	899 (304 + 305 + 289 + 1)	**595**, 609, 611	897 (304 + 305 + 289 − 1)	607, **718**	(E)GC–(E)GC–(E)C
8B	8.4	ND	306	307 (306 + 1)	139, **289**	305 (306 − 1)	137, **179**, 261	EGC
9	8.6	236, 278	290	291 (290 + 1)	**139**, 165, 273	ND	ND	(+)-catechin (std)
13	11.3	236, 278	290	291 (290 + 1)	**139**, 165, 273	ND	ND	(−)-epicatechin (std)
				Flavonol Glycosides				
15	13.0	254, 300sh, 354	626	627 (318 + 162 + 146 + 1)	**319**, 481	625 (319 + 162 + 146 − 1)	316	myricetin hexose–deoxyhexoside
16	13.3	254, 300sh, 354	480	481 (318 + 162 + 1)	319	479 (319 + 162 − 1)	316	myricetin hexoside
18A	14.2	254, 300sh, 354	566	567 (318 + 248 + 1)	319	521 (319 + 248 − 45)	316	myricetin hexoside–malonate
19	14.7	254, 262sh, 300sh, 354	610	611 (302 + 162 + 146 + 1)	**303**, 465	609 (303 + 162 + 146 − 1)	301	quercetin 3-*O*-rutinoside (std, syn. rutin)
21	15.4	254, 262sh, 300sh, 354	464	465 (302 + 162 + 1)	303	463 (303 + 162 − 1)	301	quercetin hexoside
23A	16.4	254, 300sh, 354	550	551 (302 + 248 + 1)	303	505 (303 + 248 − 45)	301	quercetin hexoside–malonate
23B	16.6	ND	594	595 (286 + 162 + 146 + 1)	**287**, 449	ND	ND	kaempferol hexose–deoxyhexoside 1
24	17.1	264, 290, 348	448	449 (286 + 162 + 1)	**327**, 287	447 (287 + 162 − 1)	285	kaempferol hexoside
27	18.2	264, 348	594	595 (286 + 162 + 146 + 1)	**287**, 449	593 (286 + 162 + 146 − 1)	285	kaempferol hexose–deoxyhexoside 2
28	18.8	264, 348	534	535 (286 + 248 + 1)	287	489 (287 + 248 − 45)	285	kaempferol hexoside–malonate
29	23.0	ND	756	757 (302 + 162 + 146 + 146 + 1)	611	ND	ND	quercetin hexose–deoxyhexose–deoxyhexoside
				Group of Unidentified Compounds				
14	11.4	270, 278sh, 286sh	337	338 (164 + 173 + 1)	176, **218**, 320	673 (337 + 337 − 1)	336	
17	13.8	234, 266	304	ND	ND	ND	ND	
18B	14.4	340sh, 400	448	449 (286 + 162 + 1)	287	447	ND	
22	16.3	234, 274	ND	ND	ND	ND	ND	

[a] In MS-MS, the most abundant parent ion of LC-MS is fragmented; in the case of several ions, the most abundant one is shown in boldface. Masses in parentheses refer to supposed structural units: the identified phenolic residues, sugars (162 to hexose, 146 to deoxyhexose, 248 to hexose-malonate), adducts (1 to hydrogen, 23 to sodium), and lost functional groups (45 to carboxyl). [b] ND, not detected; sh, maximun of the shoulder in the spectrum. Spectral characteristics were obtained from the samples where the signals of the respective compounds were most intense and pure. [c] Abbreviations for flavan-3-ols: (E)GC, (epi)gallocatechin; (E)C, (epi)catechin.

acetate extracts of green and white currants. Peak numbers (1−29 and An1−An8) in this paper are in the same order of retention times as before (*9*), whereas additional overlapping peaks are indicated with letters A and B. The differences in the absolute retention times between different chromatograms were caused by the different HPLC devices used for LC-DAD and LC-MS analyses and the time period between the runs.

Table 1 shows the tentative identification of the chromatographic peaks on the basis of the data obtained for the ethyl acetate extracts of black, red, green, and white currants in LC-DAD, LC-MS, and MS-MS analyses. **Table 2** shows the respective detection data of anthocyanins for the acidified methanol extracts of black and red currants. In the following, we discuss the characterization of the peaks with regard to each phenolic compound class.

Hydroxybenzoic Acid Derivatives (Peaks 2A and 3). Bathochromic shifts were observed for hydroxybenzoic acid derivatives compared to their respective aglycons: from 256 to 262 nm in the case of a *p*-hydroxybenzoic acid derivative (peak 2A) and from 262 to 264 nm and from 292 to 296 nm in the case of a vanillic acid derivative (peak 3). Previously published UV−visible spectral maxima were at 249 nm for a *p*-hydroxybenzoic acid 4-*O*-glucoside, at 254 and 293 nm for a vanillic acid 4-*O*-glucoside (*19*), and at 265 and 294 nm for vanilloylglucose (*23*). These data reveal that sugar esters cause bathochromic shifts, whereas *O*-glycosidic bonds cause hypsochromic shifts compared to their aglycons. On this basis, peaks 2A and 3 are assumed to be sugar esters of hydroxybenzoic acids, despite the general notion that these acids exist mainly as glycosides (*5*).

The positive ion mass spectra of peaks 2A and 3 showed that these hexose esters of phenolic acids form sodium adduct ions at *m*/*z* 323 and 353, respectively (**Table 1**). In MS-MS, both hydroxybenzoic acid derivatives fragmented to the sodium adduct ions of the hexose moiety (a route is suggested in **Figure 4A**). To our knowledge, this assumed fragmentation pattern of

Table 2. Identification of Anthocyanins in Methanol Extracts of Red and Black Currants by Using Their Spectral Characteristics in LC-DAD, Positive Ions in LC-MS and MS-MS, Respective Standards, and the Literature

peak(s)	t_R (min)	spectral characteristics (nm)	MW	positive ions[a] MS (*m*/*z*)	positive ions[a] MS-MS (*m*/*z*)	tentative identification	literature data[b]
An1	9.5	278, 524	465	465 (303 + 162)	303	delphinidin 3-*O*-glucoside (std)	delphinidin 3-*O*-glucoside
An2	10.4	278, 524	611	611 (303 + 162 + 146)	303, 465	delphinidin hexose–deoxyhexoside	delphinidin 3-*O*-rutinoside
An3	9.9	280, 516	611	611 (287 + 162 + 162)	287	cyanidin hexose–hexoside	cyanidin 3-*O*-sophoroside
An4	10.6	280, 516	757	757 (287 + 162 + 162 + 146), 611	287	cyanidin hexose–hexose–deoxyhexoside	cyanidin 3-*O*-(2^G-glucosylrutinoside)
An5	11.5	280, 516	581	581 (287 + 162 + 132)	287	cyanidin hexose–pentoside	cyanidin 3-*O*-sambubioside
An6	12.0	280, 516	449	449 (287 + 162)	287	cyanidin 3-*O*-glucoside (std)	cyanidin 3-*O*-glucoside
An7	12.2	280, 516	727	727 (287 + 162 + 132 + 146)	287, 581	cyanidin (hexose + pentose)–deoxyhexoside	cyanidin 3-*O*-(2^G-xylosylrutinoside)
An8	13.8	280, 516	595	595 (287 + 162 + 146)	287, 449	cyanidin hexose–deoxyhexoside	cyanidin 3-*O*-rutinoside

[a] See **Table 1**. Substituent 132 refers to pentose. [b] Identification of the sugars of anthocyanins is based on literature data obtained by partial acid hydrolysis of anthocyanins and identification of sugar moieties by thin-layer chromatography (*47*).

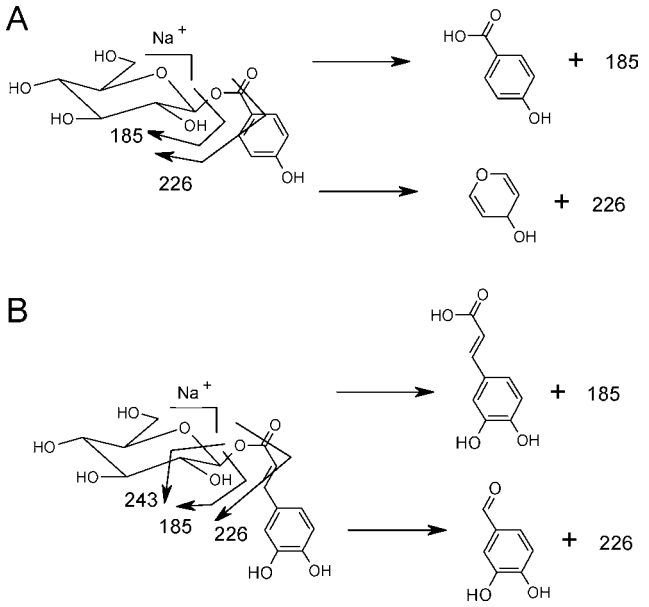

Figure 4. Proposed diagnostic MS-MS fragmentation of sodium adducts of *p*-hydroxybenzoylhexose (**A**) and caffeoylhexose (**B**).

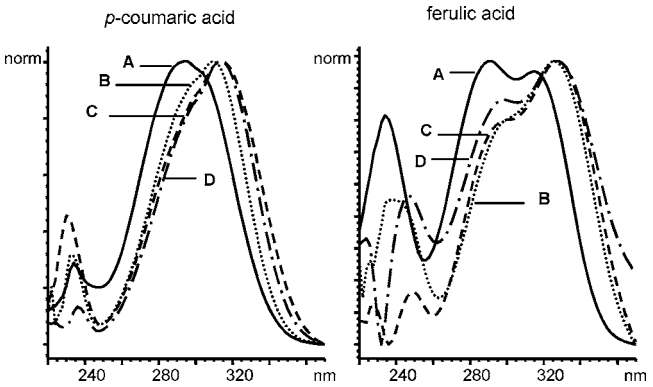

Figure 5. On-line UV–visible spectra of 4-*O*-glucoside (**A**), aglycon (**B**), hexose ester (**C**), and less polar hexose derivative (**D**) of *p*-coumaric and ferulic acids in HPLC-DAD.

sodium adducts of phenolic−hexose structures has not so far been described in the literature. In this study (**Table 1**), phenolic acids did not deprotonate in the negative ionization mode as expected from the literature (*18*, *20*, *21*). According to one previous study, the detector sensitivity of LC-MS is lower for phenolic acids than for other phenolic compounds (*26*).

Hydroxycinnamic Acid Derivatives (Peaks 4, 5A, 6A, 7, 8A, 10−12, 20, 25, and 26). Because hydroxycinnamic acid derivatives are not commercially available, 4-*O*-glucosides of *p*-coumaric and ferulic acids isolated from flaxseed and identified using LC-MS and NMR (*22*) were used as authentic standards. The UV−visible spectrum and the retention time of peak 4 matched that of *p*-coumaric acid 4-*O*-glucoside. The glycosylation of the hydroxyl group in *p*-coumaric acid caused a hypsochromic shift (from 310 to 296 nm) and the disappearance of the typical spectral feature of the aglycon (**Figure 5**).

UV−visible spectral identification allows the assignment of peaks 6A, 7, 8A, 10, 11, and 25 as *p*-coumaric acid derivatives and peaks 5A, 12, 20, and 26 as caffeic and/or ferulic acid derivatives (**Table 1**; **Figures 2** and **3**). These peaks have experienced a shift of the major absorption maximum from 310 to 314 nm (*p*-coumaric acid) and from 326 to 330 nm (caffeic and ferulic acid) compared to the respective free standards (**Figure 5**). These bathochromic shifts suggest that the substituents are esterified to the carboxylic functions of the hydroxycinnamic acids as found in a previous study (*23*).

The LC-MS data were used to obtain the molecular weights of *p*-coumaroyl-, caffeoyl- and feruloylhexoses (**Table 1**). The positive MS-MS fragmentation spectra of the hydroxycinnamic acid esters consisted of the sodium adducts of hexose, as did those of the hydroxybenzoic acid esters (**Table 1**; **Figure 4B**). In the negative ion LC-MS, caffeoylhexose (peak 5A) and *p*-coumaroylhexose (peak 10) exhibited deprotonated ions with very low responses at *m*/*z* 341 and 325, respectively (**Figures 2** and **3**). In the MS-MS spectra, these two compounds displayed ions of *p*-coumaric and caffeic acids at *m*/*z* 163 and 179, after elimination of a hexose moiety, and at *m*/*z* 161 and 145, after the subsequent elimination of water (**Table 1**). Previous identification of caffeoylglucose, *p*-coumaroylglucose, and feruloylglucose in black currant by NMR and different chemical properties (*36*) supports our identification. Other derivatives such as quinic acid esters and glucosides of hydroxycinnamic acids reported before (*37*) were not found in this study.

The UV spectra of the less polar derivatives of caffeic, *p*-coumaric, and ferulic acids (peaks 20, 25, and 26) have the same spectral characteristics as the corresponding hexose esters (peaks 5A, 6A, 7, 8A, 10, 11, and 12) (**Table 1**; **Figure 5**). The structures responsible for the positive molecular ions at *m*/*z* 536, 520, and 452 (peaks 20, 25, and 26) were difficult to interpret. The eliminated structural units in MS-MS included an unknown mass unit 98, a hexose, a hexose + 98, and a hexose + 96 (**Table 1**). According to our knowledge, the masses 96 and 98 do not correspond with any of the common acylation moieties known to form conjugates with phenolic acids in fruits (*5*). It is not possible, on the basis of LC-DAD, LC-MS, and MS-MS data alone, to identify the exact structures of these less polar hydroxycinnamic acid derivatives. For this purpose, additional NMR data will be required.

Flavan-3-ols and Dimeric and Trimeric Proanthocyanidins (Peaks 1, 2B, 5B, 6B, 8B, 9, and 13). In the previous study using LC-DAD (*9*), (+)-catechin (C, peak 9) was present in red and white currants and (−)-epicatechin (EC, peak 13) was present in black and green currants, but no other flavan-3-ols or dimeric and trimeric proanthocyanidins were detected. LC-MS in the positive ionization mode confirmed the presence of (+)-catechin and (−)-epicatechin and showed that (epi)gallocatechin (peak 8B) is present in all of the currants. The close retention time of peak 8B to that of (+)-catechin supports the assumption that this peak is epigallocatechin (EGC) (*20*, *38*, *39*). The respective MS-MS fragmentation of flavan-3-ols was described by Lin et al. (*24*).

Positive and negative ion LC-MS also revealed the presence of dimeric and trimeric proanthocyanidins (peaks 1, 2B, 5B, and 6B) that were not detected in LC-DAD. The MS-MS fragmentation pathways led to the tentative identification of the proanthocyanidins (E)GC−(E)GC (peak 1), (E)C−(E)GC (peak 2B), (E)C−(E)C (peak 5B), and (E)GC−(E)GC−(E)C (peak 6B**)** (**Table 1**) as described by Friedrich et al. (*27*). The published UV−visible spectral characteristic of GC−(4,8)−GC with the absorption maximum at 271 nm support this identification (*39*).

The existence of (+)-catechin, (−)-epicatechin, EGC, GC−(4,8)−GC, C−(4,8)−GC and C−(4,8)−C (dimer B3) in red currant was consistent with previous results (*8*) and provided more precise identification data for the dimers found in our study. In another previous study, (+)-catechin was detected in black, red, and white currants, but (−)-epicatechin was detected only in black currant and GC only in red currant (*40*).

Flavonol Glycosides (Peaks 15, 16, 18A, 19, 21, 23A/B, 24, and 27−29). Peaks 15, 16, 18A, 19, 21, and 23A displayed UV spectra similar to that of rutin (quercetin 3-*O*-glucose-rhamnoside) with two absorption maxima at 254 and 354 nm. Peaks 24, 27, and 28 showed a shift of the UV maximum from 354 to 348 nm compared to rutin. The lack of additional hydroxyl groups attached to ring B in kaempferol, compared to quercetin and myricetin, is responsible for this shift (**Figure 1**). Systematic identification of flavonol glycosides was feasible because they have intense peaks of positive protonated molecular and negative deprotonated pseudomolecular ions in LC-MS (**Figures 2** and **3**). The tentative identification of flavonol *O*-glycosides on the basis LC-MS and MS-MS data is previously described by Häkkinen and Auriola (*29*). In agreement with our results, and as additional identification of the nature of the sugar moiety of peaks 15, 16, 19, 21, 23B, and 24, myricetin, quercetin, and kaempferol have previously been identified as 3-*O*-glucosides and 3-*O*-rutinosides in black currant (*41*).

The sensitive LC-MS detection revealed the occurrence of myricetin glycosides (peaks 15, 16, and 18A) and kaempferol glycosides (peaks 23B, 24, 27, and 28) in the ethyl acetate extracts of red and white currant (**Figure 2**). The peak intensity for these compounds in LC-DAD was too weak for any identification on the basis of UV−visible spectral characteristic (*9*). LC-MS revealed the late-eluting peak as quercetin hexose−deoxyhexose−deoxyhexoside (peak 29) in white and red currants. Although triglycosides of flavonols are quite rare in fruits, the previous identification of quercetin 3-dirhamnosyl−glucoside in red currants supports the identification of peak 29 (*41*).

Peaks 18A, 23A, and 28 of flavonol glycosides showed a loss of 248 mass units from the parent compound in the positive ionization mode of MS-MS (**Table 1**). The loss of this mass unit and a UV−visible spectrum similar to those of other

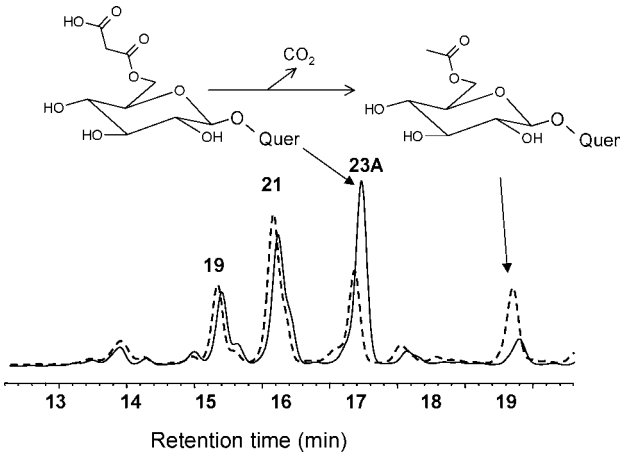

Figure 6. Assumed decarboxylation of quercetin hexoside–malonate (23A) to quercetin hexoside–acetate in the heating process. The partial LC-DAD chromatogram at 360 nm shows the ethyl acetate extract of red currant before (solid line) and after (dotted line) heating.

flavonol glycosides indicate the presence of a malonyl group ($OCCH_2COOH$) attached to the glycosyl part of the molecule (*34*). In the negative ionization mode, the carboxylic function (mass unit 45) of the malonyl group was lost from the pseudomolecular ion. Acyl functions in glycosides were reported in red clover, lettuce, and endive (*34*, *42*) but were considered to be a rare phenomenon in berries and fruits (*5*). However, 6′-*O*-malonylated glycoconjugates have been found in raspberry and strawberry, allowing the authors to conclude that malonylation of glycoconjugates is a common pathway in plant secondary metabolism (*43*). When the ethyl acetate extract of red currant was heated, quercetin hexoside−malonate (peak 23A) decomposed easily, probably by decarboxylation to an artifactual hexoside−acetate as described by Horowitz and Asen (*44*), whereas the other flavonol glycosides (peaks 19 and 21) did not change (**Figure 6**). The labile structure of glycoside−malonates is known to easily decompose by heat, light, and other environmental stress factors, producing respective glycosides, glycoside−acetates, or other derivatives (*34*, *42*). It is possible that this instability makes some sample preparation conditions unfavorable, which might explain why glycoside−malonates were not detected in berries in previous studies (*29*, *41*).

Unidentified Peaks in the Ethyl Acetate Extracts (Peaks 14, 17, 18B, and 22). Peaks 14, 17, and 22 remained without identification according to LC-DAD and LC-MS data (**Table 1**). One coeluting peak, 18B, could be assumed to be kaempferol hexoside, according to the LC-MS and MS-MS spectra (**Table 1**). However, the UV spectrum of this peak reached the longer wavelengths and had a shorter retention time than another kaempferol hexoside (peak 24) (**Table 1**). There is a wide range of phenolic compounds in nature, but this UV−visible spectrum refers to aurone, and the MS-MS data could be from tetrahydroxyaurone glucoside (*45*, *46*). Isolation and NMR identification are needed for an unequivocal identification of all unidentified peaks.

Anthocyanins (Peaks An1−An8). Anthocyanins (anthocyanidin glycosides) are responsible for the black and red pigments in currants. The identity of anthocyanins in black and red currants is well-known and documented (*32*, *48*, *49*). Anthocyanins showed very intense peaks in the positive ionization mode of LC-MS because of acidic flavylium cations, their natural and most stable forms. The identification on the basis of the MS and MS-MS data of anthocyanins in black and red currants (**Table 2**) is in good agreement with the literature (*32*,

Table 3. Contents[a] of Individual Hydroxycinnamic Acid Derivatives, Flavonol Glycosides, and Anthocyanins in Black, Green, Red, and White Currants

compound (peak)	currant			
	black	green	red	white
Hydroxycinnamic Acid Derivatives				
caffeoylglucose (5A)	22	17	NA[b]	NA
caffeic acid hexose derivative (20)	3.2	3.8	ND	3.0
total	**26**	**21**		**3**
p-coumaric acid 4-*O*-glucoside (4)	2.8	1.6	1.9	3.4
p-coumaroylglucose and hexoses (6A–10, 11)	34	67	2.9	9
p-coumaric acid hexose derivative (25)	11	13	ND	NA
total	**48**	**82**	**5**	**12**
feruloylhexose (12)	6	15	0.6	5.6
ferulic acid hexose derivative (26)	3.1	4.0	2.5	1.5
total	**10**	**19**	**3**	**7**
Flavonol Glycosides				
myricetin 3-*O*-rutinoside (15)	16	4.0	NA	NA
myricetin 3-*O*-glucoside (16)	18	1.3	0.4	NA
myricetin hexoside-malonate (18A)	5.4	2.1	0.4	NA
total	**40**	**7**	**0.8**	**0.4**
rutin (19)	23	30	2.2	3.5
quercetin 3-*O*-glucoside (21)	17	23	1.8	1.1
quercetin hexoside-malonate (23A)	6	10	2.4	0.6
total	**47**	**63**	**7**	**5**
kaempferol 3-*O*-rutinoside 1 (23B)	NA	NA	NA	ND
kaempferol 3-*O*-glucoside (24)	3.7	3.3	NA	ND
kaempferol hexoside–deoxyhexoside 2 (27)	0.8	NA	ND	ND
kaempferol hexoside–malonate (28)	10.4	3.7	0.4	NA
total	**15**	**7**	**0.4**	
Anthocyanins				
delphinidin 3-*O*-glucoside (An1)	538	ND	ND	ND
delphinidin 3-*O*-rutinoside (An2)	979	ND	ND	ND
total	**1518**			
cyanidin 3-*O*-sophoroside (An3)	ND	ND	24	ND
cyanidin 3-*O*-(2^G-glucosylrutinoside) (An4)	ND	ND	29	ND
cyanidin 3-*O*-sambubioside (An5)	ND	ND	72	ND
cyanidin 3-*O*-glucoside (An6)	331	ND	42	ND
cyanidin 3-*O*-(2^G-xylosylrutinoside) (An7)	ND	ND	*c*	ND
cyanidin 3-*O*-rutinoside (An8)	1163	ND	10	ND
total	**1494**		**177**	

[a] Contents are expressed in milligrams per kilogram of fresh weight, for the weight of the aglycon. Our previously published standard errors for contents of the phenolic classes in berries of *Ribes* spp. were low (SE < 4 mg/kg) (*9*) and therefore are not shown for individual derivatives in this study. [b] ND, not detected; NA, not analyzed in LC-DAD because of an overlapping peak (23B) or the peak area was below the minimum of the calibration curve (0.4 mg/kg). [c] Cyanidin 3-*O*-(2^G-xylosylrutinoside) coeluted with cyanidin 3-*O*-glucoside.

48, *49*). Recent research has revealed that black currant contains 15 anthocyanins, of which the 4 major ones (An-1, -2, -6, and -8) analyzed in this study represent >97% of the total content (*32*).

Contents and Relative Distributions of the Individual Phenolic Compounds. The contents of phenolic classes in black, green, red, and white currants have previously been published (*9*). In the present study, we have requantified the amounts of different conjugated forms of phenolic compounds (**Table 3**). Because the method presented in this paper was not validated, the quantitative results presented here are considered as the best estimations assuming complete recovery. The standards (*p*-coumaric, caffeic, and ferulic acids, rutin, and cyanidin and delphinidin 3-*O*-glucosides) were used to quantify the respective conjugates in LC-DAD. Because the weight of the sugar varies in the conjugates and interest is focused on the phenolic residue, the quantified results are expressed for the weight of aglycon.

In our previous study, we quantified the hexose esters of *p*-hydroxybenzoic and vanillic acids in red and white currants (*9*). However, it should be noted that our quantification did not include hydroxybenzoic acid glycosides, which were previously quantified after methanolic acid hydrolysis in black, green, red, and white currants by Häkkinen et al. (*7*). Flavan-3-ols and dimeric and trimeric proanthocyanidins were overlapped by the major hydroxycinnamic acid glucose esters in LC-DAD and were, therefore, not quantified in the present study.

The same hydroxycinnamic acid derivatives were found in black, green, red, and white currants. The most abundant derivatives of hydroxycinnamic acids were glucose esters (60−87% of all the derivatives) (**Table 3**). Previous knowledge reveals that 3′-caffeoylquinic acid and caffeoylglucose were the dominant acids in black currant, and *p*-coumaric acid 4-*O*-glucoside was dominant in red currant (*37*). As an aglycon, *p*-coumaric acid dominated in black (57%), green (67%), red (41%), and white (54%) currants in agreement with the literature (*7*, *50*).

Flavonols and anthocyanidins were found as glycosides. In black, green, and white currants, quercetin was found mainly as 3-*O*-rutinoside (47−68%) and 3-*O*-glucoside (21−36%) (**Table 3**). Exceptionally, quercetin hexoside−malonate (38%) dominated in red currant. Delphinidin and cyanidin were found as 3-*O*-rutinosides (65 and 78%) and 3-*O*-glucosides (35 and 22%) in black currant, similar to flavonols and consistent with the literature (*32*). Cyanidin 3-*O*-sambubioside (40%) was the major anthocyanin in red currant in agreement with the literature (*48*).

In the case of aglycons, the amounts of myricetin compared to quercetin as well as delphinidin compared to cyanidin were approximately at the same level in black currant. The higher content of myricetin for the same cultivar of black currant in a previous study might be explained by a different maturity level and the sample extraction techniques (*51*).

Conclusions. LC-DAD and LC-MS with MS-MS provided a valuable tool for studying the conjugated forms of phenolic compounds of black, green, red, and white currants. LC-MS revealed overlapping signals of flavan-3-ols and some proanthocyanidins, which could possibly be detected with LC-DAD after removal of phenolic acid derivatives. Ethyl acetate extraction was found to be useful in the analysis of easily destructable structures of hexoside−malonates and minor flavonol glycosides in white and red currants. Hexose esters of hydroxybenzoic acids, acylated derivatives of hydroxycinnamic acids, and malonylated flavonol glycosides were detected for the first time in *Ribes* spp. Distinct similarities were found in the relative distributions of the conjugated forms of phenolic compounds in black, green, red, and white currants, even though the contents of phenolic compounds differed significantly.

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