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Identification and Quantification of Phenolic Compounds in Berries of *Fragaria* and *Rubus* Species (Family Rosaceae)

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High-performance liquid chromatography combined with diode array and electrospray ionization mass spectrometric detection was used to study soluble and insoluble forms of phenolic compounds in strawberries, raspberries (red and yellow cultivated and red wild), arctic bramble, and cloudberries. Hydroxycinnamic acids were present as free forms in cloudberries and mainly as sugar esters in the other berries. Quercetin 3-glucuronide was the typical flavonol glycoside in all of the berries studied. The composition of the predominant anthocyanins can be used to distinguish the studied red *Rubus* species from each other since cyanidin was glycosylated typically with 3-sophorose (56%) in cultivated red raspberry, with 3-sophorose (30%) and 3-glucose (27%) in wild red raspberry, and with 3-rutinose (80%) in arctic bramble. Ellagic acid was present as free and glycosylated forms and as ellagitannins of varying degrees of polymerization. Comparable levels of ellagitannins were obtained by the analysis of soluble ellagitannins as gallic acid equivalents and by the analysis of ellagic acid equivalents released by acid hydrolysis of the extracts.

KEYWORDS: Food analysis; berries; strawberry; raspberry; arctic bramble; cloudberry; phenolic compounds; HPLC

INTRODUCTION

Berries of the Rosaceae family, namely, raspberry (*Rubus idaeus* L.), arctic bramble (*Rubus arcticum* L.), cloudberry (*Rubus chamaemorus* L.), and strawberry (*Fragaria* × *ananassa* Duch.), provide delicious fruits that can be consumed fresh or in the form of products such as jams, juices, and liquors. Phenolic compounds in the extracts of these berries were shown to have a strong capacity to scavenge oxygen radical species and to inhibit oxidation (*1*−*4*) and the growth of pathogenic bacteria (*5*, *6*). Moreover, strawberry and raspberry were shown to inhibit the growth of certain cancer cell lines in vitro (*3*, *7*, *8*). The evaluation of berries as a source of bioactive phenolic antioxidants involves questions about their absorption, distribution, metabolism, and excretion, which can be affected by the number, position, and nature of conjugated sugars (*9*−*11*). Hence, studies on the quantification of phenolic compounds need to take into account the variable structures of their conjugated forms. An additional aspect of interest for food scientists is to study the characteristic phenolic composition of berries as a marker for the authenticity of the fruit-based products.

Phenolic compounds represent structurally diverse classes of compounds with different kinds of conjugates (*12*). The major class of phenolic compounds in strawberry, red raspberry, arctic bramble, and cloudberry is represented by hydrolyzable tannins (gallo- and ellagitannins) with anthocyanins being the second most abundant class in pigmented berries and hydroxycinnamic acids, flavonols, flavan-3-ols, and proanthocyanidins being the minor ones (**Figure 1**) (*4*, *13*−*15*). Previously, studies on the composition of anthocyanins have been performed on strawberry and red raspberry cultivars (*16*−*18*), on the composition of proanthocyanidins in strawberry and raspberry (*19*), on the composition of ellagic acid glycosides in raspberry jams (*20*), and on the composition of ellagitannins in raspberry (*21*, *22*).

Hydrolyzable tannins consist of a polyol core (e.g., glucose or quinic acid) esterified with either gallic acid(s) (gallotannins) and/or hexahydroxydiphenic acid(s) (HHDP) (ellagitannins) (**Figure 1**). Additional galloyl residues may be attached to glucose via the so-called *meta*-depside bonds (*23*). Mono- and digalloyl esters of glucose are not classified as gallotannins, since they lack the typical property of tannins to complex strongly with carbohydrates and proteins (*24*). Upon acid hydrolysis of ellagitannins, HHPD is released and spontaneously lactonized to ellagic acid (*25*). Therefore, in some studies, the contents of diverse and polymerized ellagitannins are analyzed as ellagic acid equivalents after acid hydrolysis (*14*, *26*). Ellagic acid is found free, in methoxylated and glycosylated forms, and

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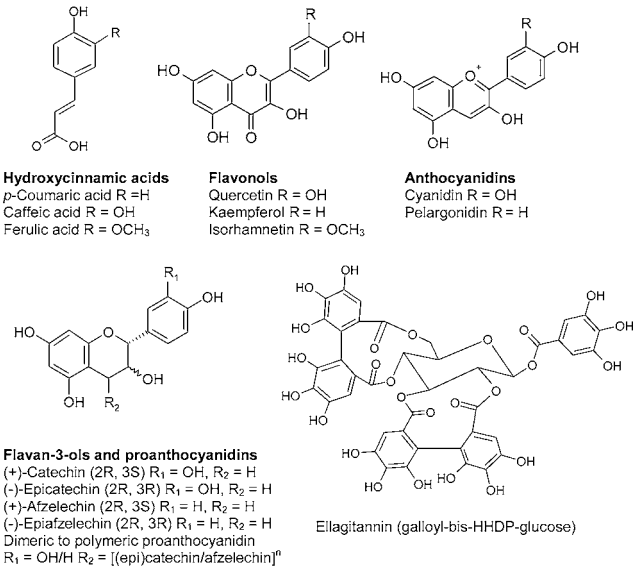

Figure 1. Structures of phenolic compounds.

as part of unknown polymers (*20*, *22*). Anthocyanins and flavonol glycosides consist of phenolic aglycons and sugars. Soluble hydroxycinnamic acids are found as glycosides and as esters with sugars or quinic acid. Flavan-3-ols are found as monomers as well as structural units in proanthocyanidin chains ranging from dimers to long polymers, which are also known as condensed tannins (*12*).

We have previously studied individual conjugated forms of phenolic compounds in the berries of *Ribes* species (*27*). The aim of this paper was to tentatively identify and quantify soluble free and conjugated forms of hydroxycinnamic acids, gallic acid, ellagic acid, and flavonols as well as ellagitannins, flavan-3-ols, and anthocyanins in berry extracts of four species of the Rosaceae family, namely, strawberry; red, yellow, and wild (red) raspberry; cloudberry; and arctic bramble. Moreover, insoluble flavonol glycosides (possibly cell wall bound), polymeric proanthocyanidins, and ellagitannins were quantified in extraction residues after conversion by acid hydrolysis to flavonols, anthocyanidins, and ellagic acid, respectively. In the present study, insoluble means nonextractable in methanol. Attention was paid to the quantification of ellagitannins in their native forms as either gallic acid equivalents or ellagic acid equivalents after acid hydrolysis of the extract.

MATERIALS AND METHODS

Samples and Standards. Finnish berries from the Rosaceae family (strawberries, raspberries, arctic bramble, and cloudberries) were collected in years 2001 and 2002. Strawberry and raspberry are widely cultivated but also grow wild in temperate climate areas. Arctic bramble and cloudberry are confined to arctic regions of the North Temperate Zone. The strawberry cultivars Honeoye, Jonsok, and Polka were purchased from the local market in Kuopio (eastern Finland). The raspberry cultivar Maurin makea, both red and yellow phenotypes, was obtained from the Research Garden of the University of Kuopio; the cultivar Muskoka was obtained from the local market, and wild raspberry was obtained from the open woodland in Kuopio. Arctic bramble, as a mixture of cultivars Mespi and Pima, was obtained from the Berry Know-How Centre of Inner Savo (Suonenjoki, eastern Finland) in 2001 and from a local farmer from Kuopio in 2002. The wild cloudberry was obtained from the swampy woodlands of Kuusamo (northeastern Finland). The berries were frozen at -24 ± 2 °C until analyzed within 2 months. An aliquot of homogenized berries was freeze-dried overnight for the analysis of the moisture content and centrifuged for the separation of juice in the analysis of the soluble solids as °Brix. The collected berries were at maturity according to the following physicochemical characteristics: strawberries (°Brix 9.4−9.6, moisture 90%), raspberries (°Brix 8.3−12.8, moisture 79−86%), arctic brambles (°Brix 11.3−12.1, moisture 80−82%), and cloudberries (°Brix 8.0−8.4, moisture 83−84%).

p-Coumaric acid, caffeic acid, ferulic acid, sinapic acid, gallic acid, ellagic acid, (+)-catechin, (−)-epicatechin, rutin (quercetin 3-rutinoside), quercetin, morin, and kaempferol were purchased from Sigma Chemical Co. (St. Louis, MO). Benzoic acid was purchased from Merck (Darmstadt, Germany), and cyanidin chloride was purchased from Extrasynthese (Geney Cedex, France). These standards were dissolved in methanol to a concentration of ~1 mg/mL and stored at -24 ± 2 °C as stock solutions. Cyanidin 3-glucoside and pelargonidin 3-glucoside (both 20 μmol) were obtained from Polyphenols AS (Sandnes, Norway) and dissolved in 10 mL of methanol for a stock solution.

Extraction of Phenolic Compounds. The extraction procedure for soluble and insoluble phenolic compounds presented in our previous study (*28*) was modified for the analysis of flavan-3-ols and low molecular weight proanthocyanidins. The scheme of the two-phase solvent extraction method is shown in **Figure 2**. Samples of the edible part of the berry (5 g) were weighed in centrifuge tubes, and 0.1 mL of morin (concentrated 1 mg/mL in methanol) was added as an internal standard for the quantification of flavan-3-ols and proanthocyanidins. Morin was a suitable standard to compensate for the losses of flavan-3-ols in the purification step, since it showed a similar solubility in the extraction solvents. The extractions were performed by repeated vigorous vortexing of samples with ethyl acetate (4×10 mL) and intermittent centrifugation. The combined ethyl acetate extracts were divided into two portions (20 mL each) containing soluble free and conjugated forms of hydroxycinnamic acids, gallic acid, ellagic acid, and flavonols as well as flavan-3-ols, low molecular weight proanthocyanidins, and some ellagitannins. One 20 mL portion was evaporated to dryness with a rotary evaporator, dissolved in 1 mL of methanol, and analyzed with LC-DAD (high-performance liquid chromatography combined with diode array detection) and LC-MS (high-performance liquid chromatography combined with electrospray ionization mass spectrometric detection). Another 20 mL portion was extracted with 2×10 mL of sodium acetate buffer (0.1 M, adjusted to pH 7.0) and then with 10 mL of water to remove ionizable phenolic acids into the water phase. After this purification step, the extract was evaporated to dryness with a rotary evaporator and dissolved in 1 mL of methanol for analysis of flavan-3-ols and dimeric proanthocyanidin with LC-DAD. After the ethyl acetate extraction, the berry residue was acidified with HCl (2 M, 2 mL), and anthocyanins were extracted as flavylium cations with methanol four times to a total extraction volume of ~50 mL. An aliquot of the methanol extract (10 mL) was evaporated to dryness in a rotary evaporator, dissolved in 1 mL of methanol, and analyzed with LC-DAD and LC-MS.

Acid Hydrolyses of Methanol Extract and Extraction Residue. The extraction residue of the berry was suspended in 10 mL of methanol, acidified to 0.6 M with concentrated HCl, and refluxed for the first 2 h (60 °C) for analysis of insoluble proanthocyanidins and phenolic glycosides (*28*, *29*). Subsequently, acid hydrolysis was continued until 20 h for analysis of insoluble ellagitannins, since these conditions were found optimal for the conversion ellagitannins to ellagic acid (*26*, *30*). Moreover, an aliquot of the methanol extract was acidified (0.6 M, HCl) and refluxed for 20 h for analysis of the soluble ellagitannins. Acid hydrolysates were analyzed with LC-DAD.

Chromatographic Analyses. All samples were filtered in vials prior to autoinjection and separation in chromatographic systems as described previously (*27*, *28*). Shortly, a 20 min linear gradient of acetonitrile in 1% formic acid was used to separate the free and conjugated forms of all other phenolic compounds but anthocyanins in the extracts and acid hydrolysates. A partly isocratic gradient of acetonitrile in 5% formic acid was used to separate anthocyanins in the extracts.

Identification (LC-DAD and LC-MS) and Quantification (LC-DAD). The analyses with LC-DAD and LC-MS were performed using the same equipment and methods as previously (*27*, *29*, *31*). The basis of identification and quantification of phenolic compounds other than free and conjugated forms of gallic and ellagic acid is described in detail in our previous studies (*27*, *28*). As additional information,

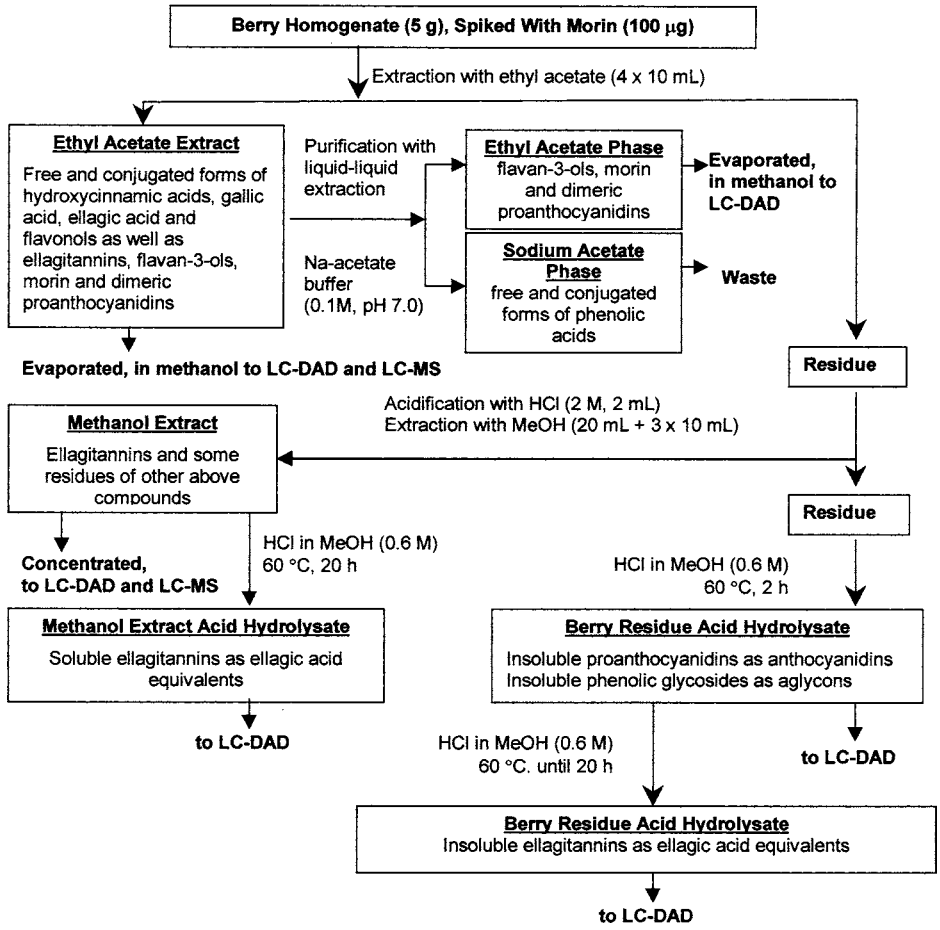

Figure 2. Scheme for the analysis of soluble and insoluble phenolic compounds in berries of the Rosaceae family.

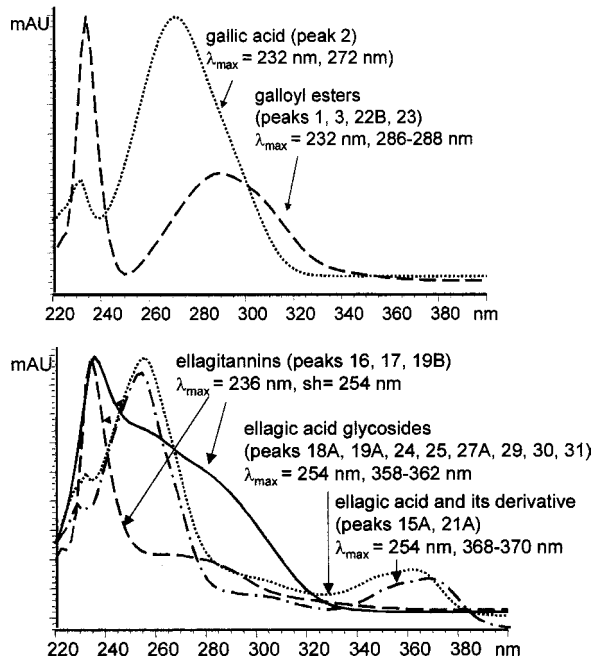

Figure 3. On-line UV–visible spectra of gallic acid, galloyl esters (upper panel), ellagic acid, ellagitannins, and ellagic acid glycosides (lower panel).

pelargonidin released in acid hydrolysis of the extraction residue was quantified using the response factor of cyanidin. Classification of peaks in the DAD chromatograms to free and conjugated forms of gallic and ellagic acid as well as ellagitannins was based on spectral characteristics shown in **Figure 3**. The representative standards for quantification in LC-DAD were gallic acid (detection at 280 nm) for free and conjugated forms of gallic acid and ellagitannins (as gallic acid equivalents) in the extracts and ellagic acid (detection at 360 nm) for free and conjugated forms of ellagic acid in the extracts and acid hydrolysates (ellagic acid equivalents). Ellagitannins were converted to ellagic acid (R_t = 16.1 min) and an unknown ellagic acid derivative (R_t = 18.0 min) in the 20 h acid hydrolysis of the methanol extract and the extraction residue. Because ellagic acid glycosides deconjugate similarly to ellagic acid in acid hydrolysis, the contents of free and glycosylated forms of ellagic acid were subtracted from the contents of ellagitannins in the acid hydrolysates.

RESULTS AND DISCUSSION

Identification of Chromatographic Peaks. The LC-DAD chromatograms of the ethyl acetate extracts in **Figure 4** include all of the compounds discussed hereafter except the anthocyanins and some ellagitannins, which were extracted subsequently in methanol after acidification and were analyzed separately (**Figure 5**). The peaks in the LC-DAD chromatograms in **Figures 4** and **5** were classified into free and conjugated forms of hydroxycinnamic acids, gallic acid, ellagic acid, and flavonols as well as ellagitannins, flavan-3-ols, and anthocyanins by comparison of their UV−visible spectra with those of the available standard aglycons [*p*-coumaric acid, caffeic acid, ferulic acid, gallic acid, ellagic acid, quercetin, kaempferol, cyanidin, (+)-catechin, and (−)-epicatechin] and the glycosides (quercetin 3-rutinoside, pelargonidin 3-glucoside, and cyanidin 3-glucoside). The minor peaks without the typical spectral characteristics of standards remained unidentified. Esterification of aglycons with sugars causes bathochromic shifts (shifts to longer wavelength) while glycosylation caused hypsochromic shifts (shifts to shorter wavelengths) in the UV−visible light absorption spectra of the conjugated forms of phenolic com-

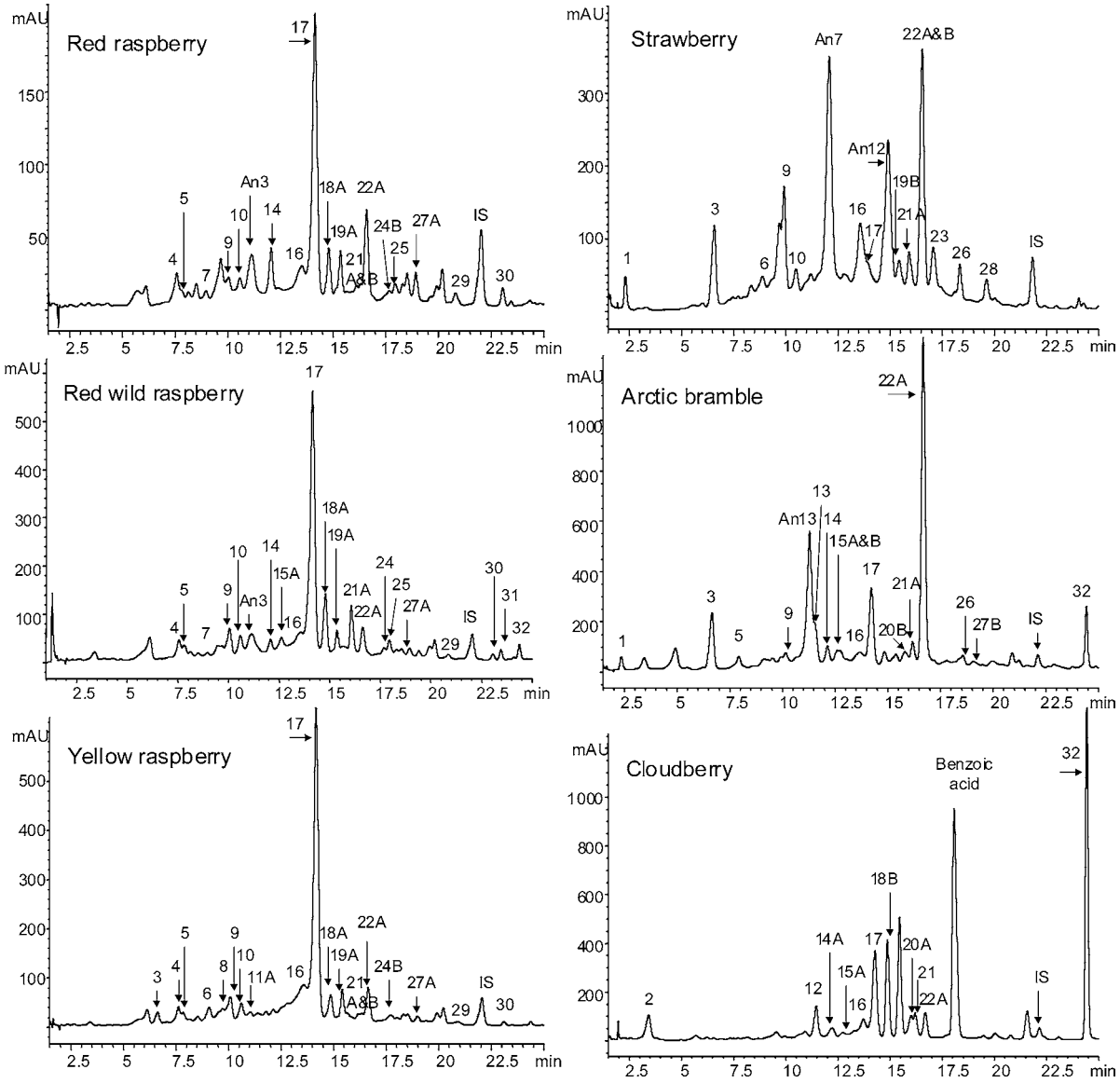

Figure 4. Chromatographic patterns at 280 nm of ethyl acetate soluble phenolic compounds in red, yellow, and wild raspberry, strawberry, arctic bramble, and cloudberry. Peak numbers refer to **Table 1**. IS stands for the internal standard, morin, which is added to the samples prior to extractions.

pounds as compared to the respective aglycons (*27*). LC-MS and the subsequent fragmentation of the predominant positive and negative ions in MS-MS and further the positive fragments ions in MS-MS-MS were used to obtain more information about the molecular masses of conjugates, masses of the sugars bound to the aglycons, and the structures of aglycons. Whenever possible, chromatographic retention and literature were used to support the identification of the peaks. The identified peaks are signed with numbers (1−32 for phenolic compounds other than anthocyanins An1−An12) and letters for overlapping peaks (e.g., 19A and 19B) following the elution order in LC-DAD and LC-MS chromatograms of the ethyl acetate and methanol extracts of strawberry; red cultivated, yellow, and wild raspberry; arctic bramble; and cloudberry (**Figures 4** and **5**). Isolation and characterization of the predominant peak 32 are ongoing in our laboratories since the LC-DAD and LC-MS data obtained in the present study (**Table 1**) could not provide any indication as to the possible identity of this compound. The characterization of the individual peaks belonging to each phenolic class was performed according to data presented in **Table 1**, and quantitative data for the identified compounds are provided in **Table 2**. More berry samples were analyzed for the content of soluble and insoluble phenolic compounds, and these quantitative data are given in **Table 3**. Hereafter, we will discuss the basis of identifications in more detail and compare the quantitative data to literature.

Free and Conjugated Forms of Hydroxycinnamic Acids. The retention times of standards confirmed the identifications of free caffeic (peak 12), *p*-coumaric (peak 18B), and ferulic acid (peak 20A) (**Table 1**). Identification of *p*-coumaric acid 4-glucoside (peak 4), *p*-coumaroyl sugar esters (peaks 7, 9, 10), caffeoylglucose (peak 5), and feruloylglucose (peak 11A) was based on our previous study on the berries of *Ribes* species (**Table 1**) (*27*). Classification of peaks 8 and 15B as esters of caffeic or ferulic acid and peak 13 as an ester of *p*-coumaric acid was based on the bathochromic shifts in their UV−visible spectral maxima due to esterification. The respective signals for these esters were not clearly detected in LC-MS, which hampered further identification of these conjugated forms of hydroxycinnamic acids. Peaks with the UV−visible spectral characteristics (maximum at 288 nm and shoulder at 316 nm) of caffeic and ferulic acid glycosides (*27*, *32*) were not detected in the berries studied. Sinapic acid was not detected in free (236 and 326 nm), esterified, or glycosylated form.

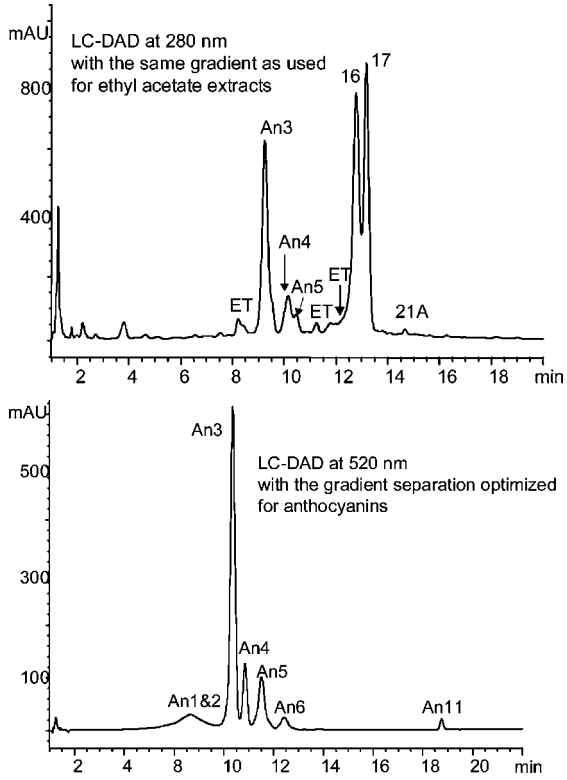

Figure 5. Chromatographic patterns at 280 (upper panel) and 520 nm (lower panel) of methanol soluble phenolic compounds of red cultivated raspberry (extracted after ethyl acetate). Peak numbers refer to **Table 1**. ET stands for minor, not numbered ellagitannin.

Hydroxycinnamic acids in strawberry, raspberry, and cloudberry were extracted in ethyl acetate and were not detected in the subsequent methanol extract, but in the case of arctic bramble, 17% of the total content was quantified in methanol. Soluble hydroxycinnamic acids occurred mainly as esters in the berries studied, except in cloudberry (**Table 2** and **Figure 4**). Free hydroxycinnamic acids are infrequently reported in fruits (*12*). However, wild berries are less studied and it is possible that free hydroxycinnamic acids in cloudberry are released at the late stages of ripening due to the environmental stress factors or that the unbound acids are typical for this wild berry. The composition and contents of conjugated forms of hydroxycinnamic acids in strawberry and raspberry were consistent with those reported in previous studies (*2*, *33*) (**Table 2**). *p*-Coumaric acid was the most abundant aglycon in strawberries, raspberries, and cloudberries but was present at equal level as caffeic and/or ferulic acids in arctic bramble in agreement with previous results on strawberries, cloudberries, and arctic bramble (*34*). In the case of raspberries in the literature, *p*-coumaric acid was found to predominate in some but not in all cultivars (*13*, *33*).

Free and Conjugated Forms of Gallic Acid, Ellagic Acid, and HHDP. The retention time of standard gallic acid (peak 2) confirmed its identification in cloudberry (**Table 1**). The spectral features used to differentiate soluble galloyl esters (peaks 1, 3, 22B, 23) and ellagitannins (peaks 16, 17, 19B) in the extracts are shown in **Figure 3**. Upon esterification to a polyol core, the absorption maximum of gallic acid experiences the bathochromic shift of 10−15 nm without a change in the shape of the spectrum (*35*). In the biosynthetic pathways of plants, gallotannins are transformed to ellagitannins by oxidative C−C coupling between two spatially adjacent galloyl groups and HHDP groups are formed. Therefore, ellagitannins exhibit shorter wavelengths for absorption maxima and weaker absorption intensities in the region of 275−285 nm than galloyl esters (including gallotannins), depending on the number of HHDP and galloyl groups present in the molecules (*35*). No LC-MS data were obtained for the estimation of the molecular size and polyol core of the galloyl esters (peaks 1, 3, 22B, 23) in strawberry, raspberry, and arctic bramble (**Table 1**). These berry extracts were supposed to show the strong background noise in LC-MS, since previously galloyl esters were successfully identified in oak samples using the same ionization parameters in the negative mode (*31*). However, the presence of mono- and digalloyl esters and gallotannins containing a glucose core was established in the literature as typical for the family Rosaceae (*23*). Screening of the negative ions from *m*/*z* 150 to 1000 in LC-MS showed a molecular ion at *m*/*z* 935 described as typical for the galloyl-bis-HHDP-glucose (**Figure 1**) (*35*). This molecular ion fragmented to ions at *m*/*z* 301, 451, 633, and 898 in MS-MS, of which the masses 301 and 633 are known to originate from the HHDP group and galloyl-HHDP-glucose, respectively (**Figure 6**) (*36*). On these bases, peaks 16, 17, and 19B in the ethyl acetate extracts were tentatively identified as ellagitannins including galloyl-bis-HHDP-glucose(s) in their structure. Additional minor ellagitannin peaks were identified and quantified in the methanol extracts using LC-DAD but were not further identified by LC-MS (**Figure 5**). Previously, ellagitannins named sanguiin H-6 and lambertianin C having two and three 1-galloyl-2,3:4,6-bis-HHDP-glucose units, respectively, were identified in raspberry (*22*, *37*).

HHDP is a biogenetic precursor of ellagic acid, which was previously found in free, methoxylated, and glycosylated forms as well as a part of an unknown structure in raspberry (*20*, *22*). The presence of free ellagic acid (peak 21A) in samples was confirmed by its retention time. Peak 15A was distinguished as an ellagic acid derivative on the basis of almost a similar UV−visible spectrum to that of ellagic acid (**Table 1** and **Figure 3**), but no further data were obtained for its tentative identification from the LC-MS analysis. The hypsochromic shifts (8−10 nm) in UV−visible spectra of the peaks 18A, 19A, 24, 25, 27A, 29, 30, and 31 suggested glycosylated forms of ellagic acid (**Figure 3**) (*20*). On the basis of LC-MS and MS-MS data, peaks 18A and 19A were identified as ellagic acid pentosides (arabinoside and xyloside) as described by Mullen et al. (*22*). In the previous study, ellagic acid 4-acetylxyloside and 4-acetylarabinoside were detected on raspberry (*20*). Similarly, one of these two acetylpentosides was detected according to our LC-MS data (**Table 1**), but the exact position of the peak in LC-DAD chromatograms remained obscured by other close-eluting ellagic acid glycosides (peaks 24, 25, 27A, 29, 30, 31).

Conjugated forms of gallic and ellagic acid as well as ellagitannins were present both in the ethyl acetate and in the methanol extracts (**Figures 4** and **5**). Ellagic acid was mainly quantified in methanol (65−100%), which may be partly due to conversion of ellagitannins or deconjugation of glycosides in acidified conditions. There was variation and similarities in the composition galloyl esters, ellagitannins, and ellagic acid glycosides in berries of *Fragaria* and *Rubus* species (**Table 2** and **Figure 4**). Typical was the predominant occurrence of galloyl esters (peaks 1, 3, 22B, 23) in strawberry. Two major ellagitannins (peaks 16, 17) were detected in all of the berries analyzed. Ellagic acid glycosides were detected only in raspberries, of which the wild one showed the most diverse composition and the highest contents.

Flavan-3-ols and Dimeric Proanthocyanidin. (+)-Catechin (peak 6), (−)-epicatechin (peak 14), and B type proanthocyanidin dimer (peak 11B) were identified by their typical LC-

Table 1. Identification of Free and Conjugated Forms of Phenolic Compounds in Ethyl Acetate Extracts of Berries of the Rosaceae Family by Using Their Spectral Characteristics in LC-DAD, Positive or Negative Ions in LC-MS and MS-MS, Respective Standards, and Previous Identification Data[a]

peak no.	LC-DAD R_t (min)[b]	LC-DAD data (nm)	MW	LC-MS data[c] MS (*m*/*z*)	LC-MS data[c] MS-MS (*m*/*z*)	tentative identification
hydroxycinnamic acids				negative ions		
4	7.6	264, 296	326	371 (164 + 162 + 45)	163, **325**	*p*-coumaric acid 4-glucoside (*27*)
5	7.9	246, 300sh, 330	342	341 (180 + 162 − 1)	161, **179**	caffeoylglucose (*27*)
7, 9, 10	9.6, 10.1, 10.6	236, 300sh, 314	326	371 (164 + 162 + 45)	**325**	*p*-coumaroyl sugar esters (*27*)
8, 15B	9.9, 12.6	244, 300sh, 326–328	ND	ND	ND	caffeic/ferulic acid esters
11A	11.1	246, 296sh, 330	ND	ND	ND	feruloylglucose (*27*)
12	11.4	240, 300sh, 324	ND	ND	ND	caffeic acid (std)
13	11.7	228, 300sh, 314	ND	ND	ND	*p*-coumaric acid ester
18B	14.7	236, 300sh, 310	ND	ND	ND	*p*-coumaric acid (std)
20A	16.0	238, 300sh, 326	ND	ND	ND	ferulic acid (std)
gallic acid, ellagic acid, and HHDP				negative ions		
1, 3	2.4, 6.7	232, 288	ND	ND	ND	galloyl esters
2	3.4	232, 272	ND	ND	ND	gallic acid (std)
15A	12.7	256, 370	ND	ND	ND	ellagic acid derivative
16, 17, 19B	13.9, 14.2, 15.4	236, 254sh	[936]^n	**935** (774 + 162 − 1), 934	301, 451, **633**, 898	[galloyl-bis-HHDP-glucose]^n
18A, 19A	14.8, 15.3	252, 362	435	434 (302 + 132 − 1)	**301**	ellagic acid 4-pentosides
21A	16.1	254, 368	302	**301** (302 − 1), 603	**255**, 271	ellagic acid (std)
22B, 23	16.6, 17.1	236, 286	ND	ND	ND	galloyl esters
24, 25, 27A	17.7, 18.0, 18.9	254, 358	476	475 (302 + 132 + 42 − 1) (among other unidentified)	**301**, 415	ellagic acid acetylpentoside (among other ellagic acid glycosides)
29, 30, 31	20.9, 23.2, 23.5	254, 358	ND	ND	ND	ellagic acid glycosides
flavan-3-ols and proanthocyanidin				positive ions		
6, 14	9.2, 12.0	278	290	291 (290 + 1)	**139**,165, 273	(+)-catechin, (–)-epicatechin (std)
11B	11.2	278	578	579 (289 + 289 + 1)	409, **427**, 291	dimer B2 [EC-(4,8)-EC] (*39*)
flavonols				positive ions		
20B	15.8	254, 300sh, 354	624	625 (302 + 176 + 146 + 1)	479, **303**	quercetin 3-glucurone-deoxyhexoside
21B	16.5	254, 300sh, 354	464	465 (302 + 162 + 1)	**303**	quercetin 3-glucoside
22A	16.7	254, 300sh, 354	478	479 (302 + 176 + 1)	**303**	quercetin 3-glucuronide
26	18.5	254, 300sh, 348	462	463 (286 + 176 + 1)	**287**	kaempferol 3-glucuronide
27B	18.9	254, 300sh, 354	492	493 (316 + 176 + 1)	**303**, 317	isorhamnetin 3-glucuronide
28	19.6	254, 300sh, 348	ND	ND	ND	kaempferol glycoside
dominant unknown compound				negative ions		
32	24.2	278	ND	695	**487**, 649	unknown compound
anthocyanidins				positive ions		
An1	8.8	280, 516	449	449 (287 + 162)	**287**	cyanidin hexoside
An2	8.9	280, 516	595	595 (287 + 162 + 146)	**287**	cyanidin hexose-deoxyhexoside
An3	10.0	280, 516	611	611 (287 + 162 + 162)	**287**	cyanidin 3-sophoroside
An4	10.6	280, 516	757	757 (287 + 162 + 162 + 146)	**287**, 611	cyanidin 3-(2^G-glucosylrutinoside)
An5	12.1	280, 516	449	449 (287 + 162)	**287**	cyanidin 3-glucoside
An6	13.9	280, 516	595	595 (287 + 162 + 146)	**287**	cyanidin 3-rutinoside
An7	15.0	276, 504	433	433 (271 + 162)	**271**	pelargonidin 3-glucoside
An8	15.8	276, 504	579	579 (271 + 162 + 146)	**271**, 433	pelargonidin 3-rutinoside
An9	16.7	280, 516	ND	ND	ND	cyanidin glycoside
An10	18.1	276, 504	519	519 (271 + 248)	**271**	pelargonidin 3-malonylglucoside
An11	18.7	274, 524	ND	ND	ND	cyanidin (std)
An12	19.7	276, 504	533	533 (271 + 262)	**271**	pelargonidin 3-succinylglucoside

[a] Abbreviations used in the table: ND, not detected; sh, maximum of the shoulder in the spectrum; std, standard; EC, (–)-epicatechin. [b] HPLC separation was achieved on a LiChroCART Purospher RP-18e column (125 mm × 3 mm i.d., 5 μm, Merck, Darmstadt, Germany) using a gradient of acetonitrile in 1% formic acid for other phenolic compounds than anthocyanins and acetonitrile in 5% formic acid for anthocyanins. [c] 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 bolded. Masses in parentheses refer to supposed structural units: the identified phenolic residues, conjugates (162 to hexose, 132 to pentose, 146 to deoxyhexose, 175 to glucurone, 248 to hexose-malonate, and 262 hexose-succinate), adducts (one to hydrogen, 45 to formate).

DAD, LC-MS, and MS-MS data (**Table 1)**, which are described in previous studies (*27*, *38*). In addition, the B type dimer was assumed to be B2 [(−)-epicatechin-(4,8)-(−)-epicatechin)] since it eluted closely prior to (−)-epicatechin as expected in the reversed phased system (*39*). In the present study, the highest amounts of flavan-3-ols and dimer B2 were quantified in arctic bramble as compared to other berries of the *Rubus* species (**Table 2**).

Flavonol Glycosides. The classification of the peaks of flavonol glycosides was based on the typical shape of the spectrum and absorption maximum at 354 (peaks 20B, 21B, 22A, 27B) and at 348 nm (peaks 26, 28) in LC-DAD (*27*). LC-MS in the positive ionization mode and the subsequent MS-MS were used for further identification of quercetin 3-glucoside (peak 21B), quercetin 3-glucuronide (peak 22A), and kaempferol 3-glucuronide (peak 26) with reference to similar data previously reported for raspberries (*22*). Peak 20B showed a positive molecular ion at *m*/*z* 625 in LC-MS, which fragmented to ions at *m*/*z* 479 (loss of a deoxyhexose) and 303 (loss of a glucurone-deoxyhexose) in MS-MS. Peak 27B showed a positive molecular ion at *m*/*z* 493 in LC-MS, which fragmented to ions at *m*/*z* 303 (loss of a methyl and a glucurone) and 317 (loss of a glucurone)

Table 2. Contents[a] of Phenolic Compounds in Strawberry, Raspberries, Arctic Bramble, and Cloudberries[b]

		raspberry				
	strawberry	red cultivated	yellow	red	arctic bramble	cloudberry
phenolic compounds (peak no.)[c]	Jonsok	Muskoka	cultivated	wild	Mespi and Pima	wild
free and conjugated forms of hydroxycinnamic acids						
p-coumaric acid 4-glucoside (4)	ND	1	2	2	ND	ND
p-coumaroyl esters (7, 9, 10, 13)	23	7	12	13	16	ND
p-coumaric acid (18B)	ND	ND	ND	ND	ND	43
caffeoyl/feruloyl esters (5, 8, 11A, 15B)	ND	1	6	3	16	ND
caffeic acid (12)	ND	ND	ND	ND	ND	10
ferulic acid (20A)	ND	ND	ND	ND	ND	10
free and conjugated forms of gallic acid, ellagic acid and HHDP						
galloyl esters (1, 3, 22B, 23)	115	ND	5	ND	47	ND
gallic acid (2)	ND	ND	ND	ND	ND	42
ellagitannins (16, 17, 19B, minor peaks in methanol)	177	977	1262	1560	2434	1605
ellagic acid pentosides (18A, 19A)	ND	8	14	25	ND	ND
other ellagic acid glycosides (24, 25, 27A, 29, 30, 31)	ND	12	8	36	ND	ND
ellagic acid derivative (15A)	ND	ND	ND	12	26	26
ellagic acid (21A)	41	15	12	112	40	153
flavan-3-ols and proanthocyanidin						
(+)-catechin (6)	24	ND	2	ND	23	5
(−)-epicatechin (14)	ND	11	3	9	18	8
dimer B2 (11B)	ND	3	ND	ND	26	4
flavonol glycosides						
quercetin 3-glucurone-deoxyhexoside (20B)	ND	ND	ND	ND	3	ND
quercetin 3-glucoside (21B)	ND	2	2	ND	ND	ND
quercetin 3-glucuronide (22A)	11	5	6	1	148	5
isorhamnetin 3-glucuronide (27B)	ND	ND	ND	ND	14	ND
kaempferol 3-glucuronide (26) glycoside (28)	6	ND	ND	ND	3	ND
anthocyanidin and anthocyanins						
cyanidin 3-hexoside and hexose-deoxyhexoside (An1, An2)	ND	105	ND	43	ND	ND
cyanidin 3-sophoroside (An3)	ND	499	ND	216	ND	2
cyanidin 3-(2^G-glucosylrutinoside) (An4)	ND	106	ND	117	ND	2
cyanidin 3-glucoside (An5)	22	125	ND	195	160	4
cyanidin 3-rutinoside (An6)	ND	45	ND	137	713	9
cyanidin glycoside (An9)	7	ND	ND	ND	8	ND
pelargonidin 3-glucoside (An7)	248	4	ND	5	2	ND
pelargonidin 3-rutinoside (An8)	12	2	ND	4	5	ND
cyanidin (An11)	5	3	ND	13	2	ND
pelargonidin 3-malonylglucoside (An10)	25	ND	ND	ND	ND	ND
pelargonidin 3-succinylglucoside (An12)	45	ND	ND	ND	ND	ND

[a] The contents of ethyl acetate and methanol extractable phenolic compounds together are expressed in milligrams per kilogram of fresh weight for the weight of aglycon. The mean values are for duplicate assays of the selected berries harvested in year 2002. The contents of flavan-3-ols and dimer B2 were quantified after a purification step (**Figure 2**) using morin as an internal standard to compensate losses in this step. [b] Abbreviation used in the table: ND, not detected. [c] Peak numbers refer to the identified phenolic compounds in ethyl acetate and methanol extracts (**Table 1**).

in MS-MS. On the basis of these fragmentation patterns, the rare quercetin glycosides in arctic bramble were identified as quercetin 3-glucurone-deoxyhexoside (peak 20B) and isorhamnetin 3-glucuronide (peak 27B). The further fragmentation of aglycons in MS-MS-MS to the typical fragment ions of quercetin and isorhamnetin at *m*/*z* 229, 257, and 285 (collision energy was set at 30%) confirmed these LC-MS identifications (*29*). The late-eluting kaempferol glycoside (peak 28) in strawberry remains without further identification.

Flavonol glycosides were extracted in ethyl acetate and were not detected in methanol, except in the case of arctic bramble where 14% of the total content was extracted in methanol. Quercetin 3-glucuronide was typical to berries of *Fragaria* and *Rubus* species. The contents and composition of quercetin 3-glucuronide and kaempferol glycosides in strawberry were consistent with the results reported by Wang and Zheng (*2*). The contents of quercetin and kaempferol in strawberries; red, yellow, and wild raspberries; and cloudberries were in good accordance with the respective results published previously, but the content of these flavonols in arctic bramble was 10 times higher in the present study (*13*).

Anthocyanins. Anthocyanins (anthocyanidin glycosides) are responsible for the black and red pigments in berries. For identification and quantification, they were extracted with methanol after ethyl acetate extraction and acidification of the berry matrix (**Figure 2**). The UV−visible spectral characteristics showed a difference of 12 nm in the absorption maxima between the glycosides of pelargonidin (504 nm) and the glycosides of cyanidin (516 nm). The identification of cyanidin 3-sophoroside (peak An3), 3-(2^G-glucosylrutinoside) (peak An4), 3-glucoside (peak An5), 3-rutinoside (peak An6), pelargonidin 3-glucoside (peak An7), and 3-rutinoside (peak An8) was based on LC-MS data and the identity of anthocyanins in strawberry and red raspberry comparable to those published in earlier studies (*16*−*18*, *40*). The identification of anthocyanins in arctic bramble and cloudberry was then made by reference to the respective anthocyanins in raspberry. Two nonpolar pelargonidin glycosides (An10 and An12) in strawberry showed molecular ions at *m*/*z* 519 and 533, of which mass units 248 (hexoside-malonate) and 262 (hexoside-succinate) were, respectively, lost in the subsequent fragmentation in MS-MS. Peaks An10 and An12 were, thus, identified as pelargonidin 3-malonylglucoside and 3-succinylglucoside in agreement with previous studies (*41*,

Table 3. Contents[a] of Selected Phenolic Classes in Strawberries, Raspberries, Arctic Bramble, and Cloudberries[b]

			ellagitannins GA equivalents (EA equivalents)[c]					
berry, cultivars, harvest year	HCA soluble	GA/GE soluble	soluble in EtOAc	soluble in MeOH	insoluble	EA/EAC soluble	flavonol soluble	anthocyanin soluble
strawberry								
Honeoye, 2002	37 ± 2	162 ± 6	56 ± 2	115 ± 5 (524 ± 28)	(224 ± 20)	40 ± 0	29 ± 1	361 ± 6
Jonsok, 2002	23 ± 0	115 ± 1	69 ± 1	108 ± 8 (455 ± 10)	(151 ± 6)	41 ± 2	17 ± 2	365 ± 37
Polka, 2002	17 ± 1	94 ± 7	46 ± 8	79 ± 9 (416 ± 54)	(150 ± 7)	35 ± 9	15 ± 6	314 ± 4
raspberry								
red, Maurin makea, 2001	11 ± 1	ND	55 ± 6	968 ± 81	NA	45 ± 5	2 ± 0	536 ± 27
red, Muskoka, 2002	9 ± 0	ND	52 ± 1	924 ± 15 (1030 ± 33)	(745 ± 6)	35 ± 3	6 ± 1	888 ± 12
yellow, 2001	12 ± 1	6 ± 0	68 ± 9	966 ± 74	NA	30 ± 2	3 ± 0	ND
yellow, 2002	19 ± 0	5 ± 0	168 ± 5	1094 ± 34 (1130 ± 69)	(605 ± 29)	33 ± 2	8 ± 2	ND
red, wild, 2001	12 ± 2	ND	100 ± 16	1460 ± 23	NA	90 ± 16	1 ± 0	761 ± 15
red, wild, 2002	18 ± 1	ND	158 ± 5	1402 ± 31 (1199 ± 8)	(1159 ± 135)	185 ± 9	1 ± 0	730 ± 3
arctic bramble								
Mespi and Pima, 2001	25 ± 2	97 ± 2	60 ± 2	2017 ± 127	NA	62 ± 4	91 ± 9	702 ± 55
Mespi and Pima, 2002	32 ± 2	47 ± 4	81 ± 1	2352 ± 91 (2425 ± 16)	(1286 ± 23)	66 ± 9	168 ± 13	890 ± 30
cloudberry								
wild, 2001	52 ± 3	70 ± 5	25 ± 5	682 ± 68	NA	28 ± 8	5 ± 1	20 ± 4
wild, 2002	63 ± 7	42 ± 4	101 ± 4	1504 ± 72 (1646 ± 94)	(1647 ± 9)	179 ± 17	5 ± 0	18 ± 0

[a] Contents are expressed as means ± SD in milligrams per kilogram of fresh weight ($n = 4$ in year 2001 and $n = 2$ in year 2002). Soluble means ethyl acetate and subsequently methanol extractable compounds, and insoluble means their extraction residues in the berry matrix. [b] Abbreviations used in the table: HCA, hydroxycinnamic acids; GA, gallic acid; GE, galloyl esters; EA, ellagic acid; EAC, ellagic acid conjugates; EtOAc, ethyl acetate; MeOH, methanol; NA, not analyzed; ND, not detected. [c] Ellagitannins were quantified as gallic acid equivalents in soluble form in the extracts and as ellagic acid equivalents after 20 h of acid hydrolysis in the methanol extract and extraction residue (results shown in parentheses).

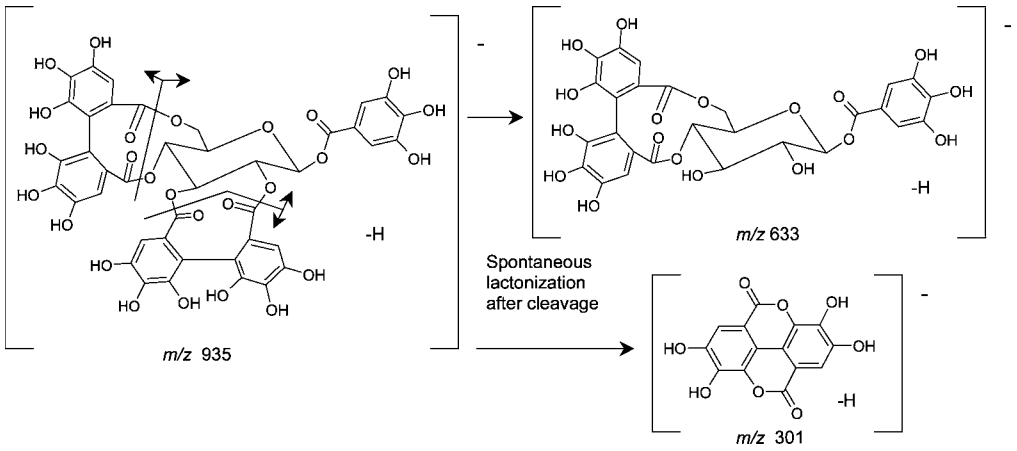

Figure 6. Proposed diagnostic MS-MS fragmentation of galloyl-bis-HHDP-glucose (*m/z* 935) in negative ion LC-MS. The arrows indicate the sides of cleavages.

42). Cyanidin 3-hexoside and cyanidin 3-hexose-deoxyhexoside (peaks An1 and An2) eluted at early retention times in raspberry as part of a polar "hump" of anthocyanins (**Figure 5**).

Anthocyanins were extracted mainly in methanol, but negligible amounts (1−2%) were pre-extracted in ethyl acetate (**Figures 4** and **5**). However, in the case of strawberry, the amounts of anthocyanins in the ethyl acetate extract were higher (21−24%), due to the less polar pelargonidin glycosides. Pelargonidin 3-glucoside (68%) was the major anthocyanin in the strawberry (cultivar Jonsok) in accordance with previously published chromatographic profiles (*18*, *43*) and the quantified amounts (recalculated for the weight of aglycon) (*2*, *44*). The contents and composition of anthocyanins in red cultivated raspberry Muskoka were comparable to those of Ceva, one of the four Spanish raspberry cultivars reported by de Ancos et al. (*16*), although another study showed a different anthocyanin composition for the same Spanish cultivar (*45*). Cyanidin 3-sophoroside was the major anthocyanin (56%) in cultivated red raspberry in the present study and is known to be typical for European cultivars (*16*). However, a distinguishable anthocyanin profile was found in wild raspberry with equal amounts of cyanidin 3-sophoroside (30%) and cyanidin 3-glucoside (27%) (**Table 2**). The characteristic anthocyanin in arctic bramble was cyanidin 3-rutinoside (80%) (**Table 2**).

Contents of Phenolic Classes. The contents of selected phenolic classes in three cultivars of strawberry and two cultivars of red raspberry, yellow raspberry, wild rasberry, arctic bramble, and cloudberry analyzed for two seasons' harvest are shown in **Table 3**. The contents of insoluble hydroxycinnamic acids, flavonols, and proanthocyanidins are summarized later on in this chapter, since they were not detected in all of the samples and the methods are considered semiquantitative.

In agreement with the previous study (*4*), ellagitannins are the major phenolic class that characterizes berries of *Fragaria* and *Rubus* species (**Table 3**). The presence of ellagitannins in both the ethyl acetate and the methanol extracts as well as in their extraction residues suggests variable molecular sizes and structures. Ellagitannins were analyzed with two different methods: in the soluble form (gallic acid equivalents in ethyl acetate and methanol extracts) and in the form of conversion

products after acid hydrolysis (ellagic acid equivalents in methanol extracts and extraction residue). Ellagitannins were converted by acid hydrolysis to ellagic acid and a less polar derivative, 20−26% of the content, which was assumed to be a methoxylated ellagic acid formed by the electrophilic attack of methanol to unsubstituted carbons in an intermediate structure (*25*). It should be noted that in previous studies ellagic acid was quantified as one conversion product (*14*, *26*, *30*). The contents of ellagitannins as gallic acid equivalents and ellagic acid equivalents in the methanol extracts were comparable in the berries of *Rubus* species but not in strawberries (**Table 3**). The possible explanation is that the fibrous berry matrix of the strawberries, which was partly solubilized in methanol, may contain insoluble or extensive, unresolved ellagitannins. Our results on the total contents of ellagitannins were similar to those where soluble forms were extracted with 70% acetone and quantified using ellagic acid as a standard (*4*). The estimated intake of ellagic acid should sum up free and conjugated forms of ellagic acid as well as soluble and insoluble ellagitannins. According to our results, these contents (mg/kg in fresh weight) would be approximately 650−850 in strawberries, 1900 in cultivated raspberries, 2700 in wild raspberries, 3900 in arctic bramble, and 3600 in cloudberries. In addition, soluble galloyl esters were a noteworthy phenolic class in strawberries, arctic bramble, and cloudberries but not in raspberries.

The second major phenolic class was anthocyanins in berries of *Fragaria* and *Rubus* species in consistency with the literature (*4*). The content of anthocyanins (mg/kg in fresh weight) varied from 18 to 20 in cloudberries to 314−361 in strawberries and 536−890 in raspberries and arctic bramble (**Table 3**). Hydroxycinnamic acids and flavonols were minor phenolic classes. The content of hydroxycinnamic acids (mg/kg in fresh weight) varied from 9 to 19 in raspberries to 52−63 in cloudberries in accordance with previously published studies (*4*, *14*). The total content of soluble flavonols was clearly highest in arctic bramble.

The levels of insoluble phenolic compounds were quantified after acid hydrolysis of the extraction residue (*26*, *28*, *29*). Ellagic acid equivalents and anthocyanidins were released from the extraction residues of all of the berries (**Table 3**), but flavonols were only released from strawberries (Honeoye and Polka 12 mg/kg and Jonsok 5 mg/kg) and hydroxycinnamic acids from none of the berries. Insoluble flavonols (kaempferol) in strawberry are supposed to represent the cell wall bound phenolic compounds. The levels of insoluble (polymeric or cell wall bound) condensed tannins were around 10−20 mg/kg in the berries studied. According to the spectral characteristics, the released anthocyanidins were cyanidin and pelargonidin, which represent the constituent units (epi)catechin (procyanidin) and (epi)afzelechin (propelargonidin), respectively (**Figure 1**). In strawberries, cloudberries, and arctic bramble, (epi)catechin units dominated (approximately 80%), but in raspberries, both units were detected in approximately equal amounts. In the study of Gu et al. (*19*), the molar proportion of epiafzelechin was 6% in strawberry and 14% in raspberry for those proanthocyanidins, which were soluble in 70% acetone.

The quantification data enable comparison of three strawberry cultivars Honeoye, Jonsok, and Polka and the related raspberries (two red cultivars, a yellow cultivar, and a wild one) (**Table 3**). The highest contents of flavonols and hydroxycinnamic acids were detected in Honeoye, consistent with the respective previous comparisons (*46*). The lowest contents of all phenolic classes were in the cultivar Polka. Unpigmented, yellow raspberries lack anthocyanins, but the contents of other phenolic classes were comparable to red cultivated raspberries. The wild red raspberry showed a pronounced content of ellagitannins as well as free and conjugated forms of ellagic acid as compared to cultivated red raspberries in both years 2001 and 2002.

Phenolic Compounds in *Fragaria* and *Rubus* Berries. The identification and quantification of individual conjugated forms of phenolic compounds revealed the similarities and variation in the composition of *Fragaria* and *Rubus* species. *p*-Coumaric acid was the predominant hydroxycinnamic acid as sugar esters in strawberries and raspberries and as free form in cloudberries, but arctic bramble showed its own typical composition of conjugated forms of hydroxycinnamic acids. Quercetin 3-glucuronide was the predominant flavonol glycoside in all of the berries studied, although strawberry and arctic bramble showed other flavonol glycosides, which distinguished them from the other berries studied. The anthocyanin composition was distinguishable from others among the species and between cultivated and wild raspberries. Galloyl esters were typical to strawberries. The same ellagitannins were found in strawberries as in the berries of the *Rubus* species, although the contents were lower. As a new methodological note, the content of ellagitannins evaluated as conversion products after acid hydrolysis was consistent with the values achieved for soluble, tentatively identified ellagitannins.

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