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Concentration and Mean Degree of Polymerization of *Rubus* Ellagitannins Evaluated by Optimized Acid Methanolysis

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Ellagitannins are a major class of phenolics largely responsible for the astringent and antioxidant properties of raspberries and blackberries. The *Rubus* ellagitannins constitute a complex mixture of monomeric and oligomeric tannins. *Rubus* oligomeric ellagitannins contain, beside the well-known ellagic acid and gallic acid moieties, the sanguisorboyl linking ester group. When exposed to acids or bases, ester bonds are hydrolyzed and the hexahydroxydiphenic acid spontaneously cyclizes into ellagic acid. This study describes a new, rapid procedure for the acid hydrolysis of *Rubus* ellagitannins in methanol, which results in maximal yield and enables the quantification of all the major reaction products. Additionally, the method provides the rationale for estimating the mean degree of polymerization of *Rubus* ellagitannins.

KEYWORDS: *Rubus*; ellagitannins; ellagic acid; sanguisorbic acid; gallic acid; hexahydroxydiphenic acid

INTRODUCTION

In the past few years there has been an increasing interest in the study of plant-derived phenolics, due to their health promoting properties. Polyphenolic antioxidants are widely distributed in the plant kingdom, and soft fruits are a particularly rich source (*1*) containing high amounts of anthocyanins, flavonol conjugates, ellagitannins, and hydroxycinnamic acids. Ellagic acid, a dimeric derivative of gallic acid, exists in higher plants including fruits in free form, glycosylated with various sugars, or as simple or complex ellagitannins (*2*−*4*), which are esters of hexahydroxydiphenic acid (HHDP) and a polyol, usually glucose or quinic acid (*5*). When exposed to acids or bases, ester bonds become hydrolyzed and the hexahydroxydiphenic acid spontaneously cyclizes into the water-insoluble ellagic acid (*5*). This reaction forms the basis of an assay commonly used for their detection and quantification.

Ellagic acid has received much attention for its nutritional and pharmacological potential as an antioxidant (*6*) and antiviral agent (*7*). Moreover it acts protectively against different types of cancer like colon (*8*), lung, and esophagus cancer (*9*). The occurrence of ellagitannin in common foodstuff is limited to a few fruits, e.g., strawberries (*Fragaria* x *ananassa* D.), blackberries (*Rubus* sp.), raspberries (*Rubus idaeus* L.) (*5*), and muscadine grapes (*Vitis rotundifolia*) (*10*). In raspberry fruits free ellagic acid constitutes only a minor part of the total ellagic acid pool (*11*) while the ellagic acid released after acid hydrolysis from ellagitannins can constitute up to 88% of the total phenolic content (*2, 12*). The ellagitannins of *Rubus* berries and leaves are a complex mixture containing both monomeric and oligomeric tannins (**Figures 1** and **2**). The monomers consist of ellagic acid glycosides (**1**) (*11*) and relatively simple ellagitannins such as galloyl-bis-HHDP-glucosides (**2**) which have been isolated both as α- and *β*-D-glucopyranoside derivatives (*13*). The oligomeric ellagitannins so far characterized are the dimers sanguiin H-6 (**3**) named "T1" (*13*−*14*) and lambertianins A−B (**7**−**8**) (*15*), the trimer lambertianin C (**9**), and the tetramer lambertianin D (**10**) with a molecular weight of 3740 (*15*). Recently, *Rubus* berries have been reported to contain also the dimer sanguiin H-10 (*16*), a compound previously found only in *Sanguisorba officinalis* (*17*). Such oligomers are formed as a second phase in the oxidative metabolism, by intermolecular C−O oxidative coupling between the galloyl and the hexahydroxydiphenoyl moieties of two galloyl-bis-HHDP-glucoside units (*13*). In the case of the *Rubus* and *Sanguisorba* spp. such reaction produces the sanguisorboyl linking ester group (**Figure 3**) (*13*, *15*, *18*). The sanguisorboyl group is a relatively uncommon ester between a galloyl group and a (*S*)-hexahydroxydiphenoyl moiety, which is positionally isomeric as compared with the more widespread valoneoyl ester group. The large majority of *Rubus* ellagitannins are oligomeric. As sanguiin H-6 (**3**) constitutes by far the most abundant phenolic metabolite

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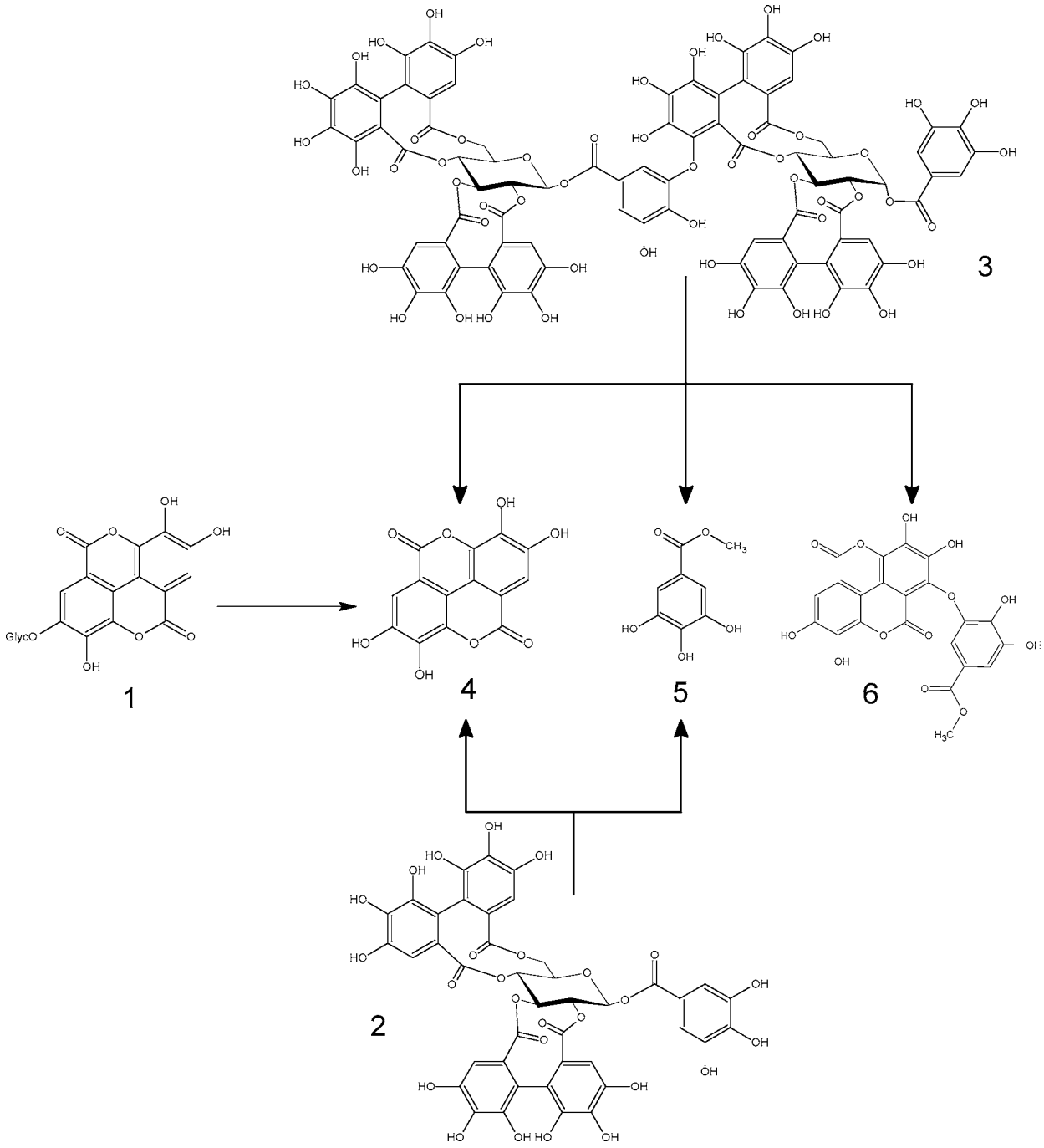

Figure 1. Schematic representation of *Rubus* spp. ellagitannins acid hydrolysis in methanol. The principal components are ellagic acid glycosides (**1**), simple ellagitannins such as β-1-*O*-galloyl-2,3:4,6-bis-HHDP-D-glucose (**2**), and complex oligomeric ellagitannins such as sanguiin H-6 (**3**) and give rise to ellagic acid (**4**), methyl gallate (**5**), and methyl sanguisorboate (**6**). Free HHDP units are converted into the corresponding lactones (**4**, **6**), while benzoic acids moieties are converted into their methyl esters (**5**, **6**) via acid-catalyzed Fisher esterification.

in *Rubus* (*12*−*13*, *15*−*16*) the astringency of raspberry and blackberry and the characteristic properties of the herbal remedies prepared from the fruit and leaves of these plants are normally associated with this compound (*13*).

Procedures for ellagitannins quantification usually involve an hydrolytic treatment followed by HPLC determination of the ellagic acid released. Several methodologies for the acid hydrolysis of ellagitannins are described in the literature. These include treatment of plant extracts with different acid strength 0.6 M (*19*), 1.2 M methanolic HCl (*2*), anhydrous methanolic HCl, prepared by addition of acetyl chloride to a well-stirred cold anhydrous methanolic solution (*20*) or trifluoroacetic acid (*21*, *22*). None of these procedures represent an optimal solution as they either are time-consuming, require the use of dangerous solvents, or result in relatively low yields. Moreover, all the existing procedures rely solely on the quantification of released ellagic acid and do not consider the other phenolics formed during hydrolysis which may provide helpful information on the chemical structure of the *Rubus* ellagitannins.

In this study we investigated a novel hydrolytic procedure which overcomes many of the above-described problems and provides the rationale for estimating the mean degree of polymerization (mDP) of the *Rubus* ellagitannins. The procedure should become a useful base for the development of high throughput methods for the screening of the contents of ellagitannins in plants.

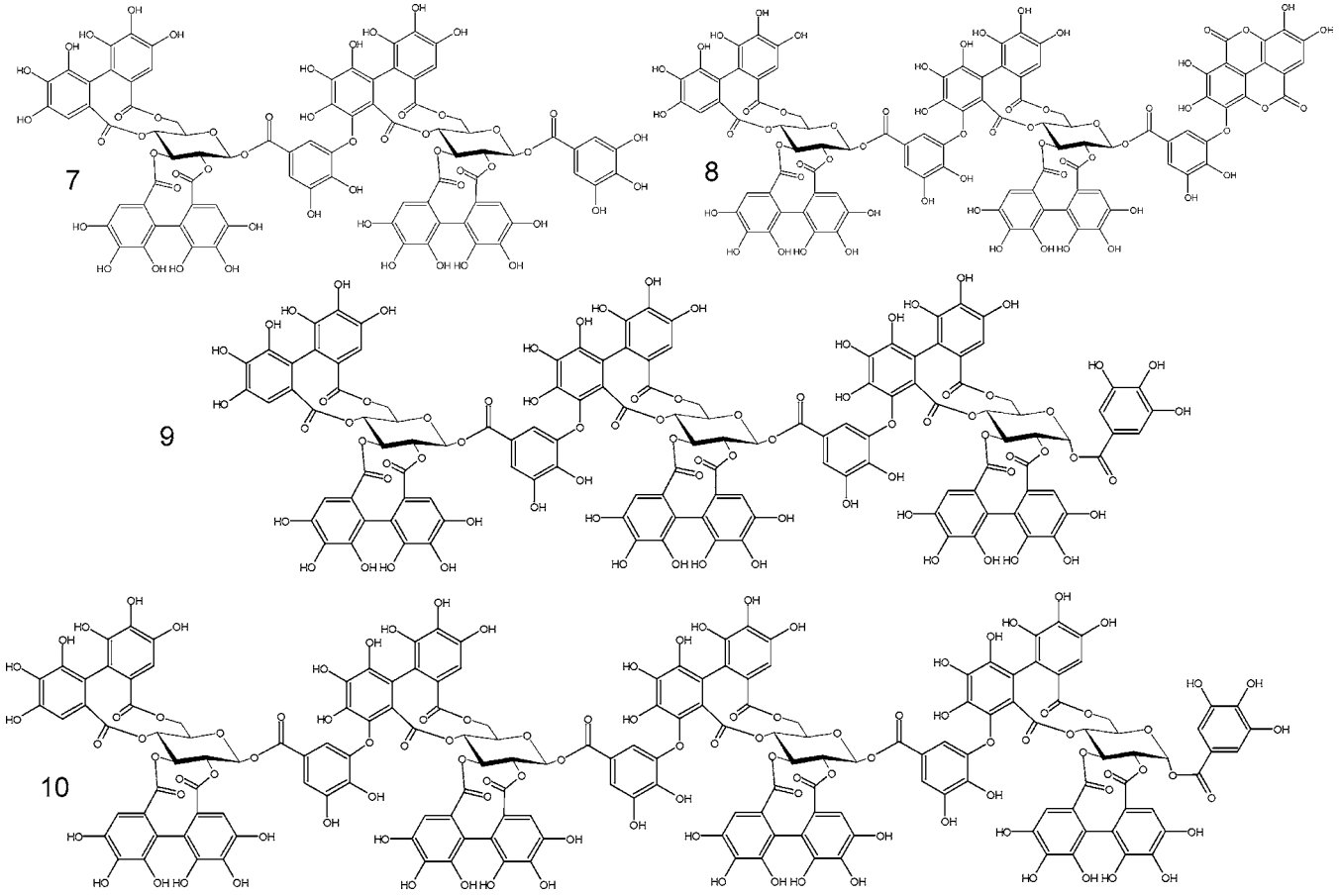

Figure 2. Structure of oligomeric *Rubus* ellagitannins belonging to the family of the lambertianins: lambertianin A (**7**), lambertianin B (**8**), lambertianin C (**9**), and lambertianin D (**10**).

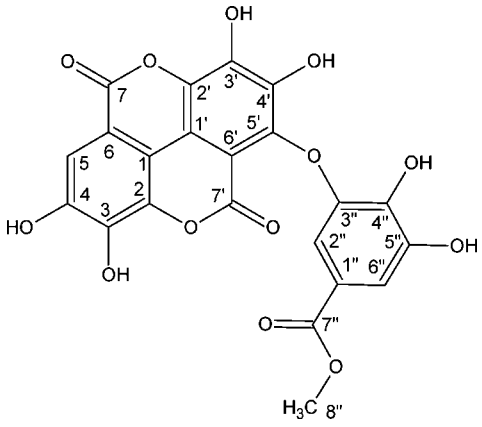

Figure 3. Numbered structure of methyl sanguisorboate (**6**), formed by acid hydrolysis in methanol of the sanguisorboyl ester group of oligomeric ellagitannins such as sanguiin H-10, sanguiin H-6, lambertianin C, and higher oligomers.

MATERIALS AND METHODS

Standards and Solvents. All chromatographic solvents were HPLC grade. Acetonitrile, methanol, and diethyl ether were purchased from VWR International (Milano, Italy). Hexane, formic acid, acetic acid, and hydrochloric acid were purchased from Carlo Erba (Milano, Italy). Ellagic acid standard (purity ≥ 96%) and methyl gallate standard (purity ≥ 98%) were purchased from Fluka (Steinheim, Germany).

Sampling. Freshly collected samples of raspberry (*Rubus idaeus* L.) cv. Tulameen, blackberry (*Rubus fruticosus*) cv. Loch Ness, strawberry (*Fragaria* x *ananassa* D.) cv. Elsanta, and wild strawberry (*Fragaria vesca*) cv. Alpine produced in Trentino in 2005 were provided by the local growers association Sant'Orsola Scarl, Pergine Valsugana, Italy.

Extraction of Polyphenols. Polyphenols were extracted following the method of Mattivi et al. (*23*) in which 60 g of fresh fruit were homogenized in a model 847-86 Osterizer blender at speed one in 250 mL of mixture acetone/water (70/30 v/v) for 1 min. Prior to extraction, fruits and the extraction solution were cooled to 4 °C to limit enzymatic and chemical reactions. The centrifuged extracts were stored at −20 °C until analysis.

For the preparation of aqueous methanolic fruit extracts the extraction solution was methanol/water (60/40, v/v).

Sample Preparation. An aliquot (20 mL) of the extract was evaporated to dryness in a 100 mL pear-shaped flask by rotary evaporation under reduced pressure at 40 °C. The sample was then brought back to 20 mL with methanol immediately prior to processing, due to the limited solubility of phenolics.

Acid Hydrolysis. To optimize the hydrolysis conditions the samples were subjected to hydrolysis at different HCl concentrations (1.2, 2, and 4 M) for different lengths of time (1, 2, 4, 6, and 20 h) at 85 °C. To carry on acid hydrolysis in 1.2, 2, or 4 M HCl, respectively, 5, 8.3, or 16.6 mL of 37% HCl were added to the sample prepared as above and the mixture was diluted to 50 mL with methanol. After hydrolysis the sample was brought to the initial volume (50 mL) with methanol. An aliquot (10 mL) was adjusted to pH 2.5 with 5 N NaOH and diluted to 20 mL with methanol. Subsequently, an aliquot (2 mL) was filtered with a 0.22 μm, 13 mm PTFE syringe-tip filters (Millipore, Bedford, MA) and transferred into LC vials.

HPLC-DAD-ESI-MS Analysis. The HPLC analysis was carried on a Waters 2690 HPLC system equipped with Waters 996 DAD (Waters Corp., Milford, MA) and Empower Software (Waters). The separation was performed using a 250 × 2.1 mm i.d., 5 μm, endcapped reversed-phase Purospher Star column (Merck) and 4 × 4 mm, 5 μm, Purospher

precolumn. The solvents were A (1% formic acid in water) and B (acetonitrile). Gradients were as follows: from 0% to 5% B in 10 min, from 5% to 30% B in 30 min. The column was washed with 100% of B for 2 min and then equilibrated for 5 min prior to each analysis. The flow rate was 0.8 mL/min and oven temperature at 40 °C. The injection volume was 10 μL. Ellagic acid and its derivatives were detected and quantified by UV detection at 260 nm. Ellagic acid and derivative 1 was quantified following calibration with ellagic acid standard (concentration range of 10−200 mg/L, $R^2 = 0.999$). The methyl sanguisorboate (**6**) was quantified following calibration with the pure standard isolated as described below (concentration range of 5−100 mg/L, $R^2 = 0.999$). The methyl gallate (**5**) was quantified following calibration with the corresponding standard compound, under the following conditions: concentration range from 3.3 to 33 mg/L, with the coefficient of $R^2 = 0.998$.

Detailed compound identification was carried out using the Micromass ZQ electrospray ionization-mass spectrometry (ESI-MS) system (Micromass, Manchester, U.K.). The MS detector operated at capillary voltage 3000 V, extractor voltage 6 V, source temperature 105 °C, desolvation temperature 200 °C, cone gas flow (N_2) 30 L/h, and desolvation gas flow (N_2) 450 L/h. The outlet of the HPLC system was split (9:1) to the ESI interface of the mass analyzer. ESI-mass spectra ranging from *m*/*z* 100 to 1500 were taken in negative mode with a dwell time of 0.1 s. The cone voltage (CV) was set in scan mode at the values of 20, 40, and 60 V.

Isolation of Methyl Sanguisorboate. The isolation of ellagic acid derivatives was carried on a preparative HPLC Shimadzu SCL-10 AVP equipped with a Shimadzu SPD-10 AVP UV/vis detector, pumps 8A and a Class VP Software (Shimadzu Corp., Kyoto, Japan). The UV signal was recorded at 260 nm. Flash chromatography was performed using a Toyopearl HW 40 S resin (Tosoh Corp., Tokyo, Japan). The aim of this step was the separation of ellagic acid and its derivatives from nonphenolic compounds and other polyphenols. Aqueous acetone extracts (800 mL) obtained from 202 g of raspberry fruit were evaporated to dryness in a pear-shaped flask by rotary evaporation under reduced pressure at 40 °C. The sample was brought back to 800 mL with methanol containing 2 M HCl, hydrolyzed for 4 h at 85 °C, and brought to pH 2.5 as described above. For flash-chromatography, HW 40 S resin (120 mL) was packed in a single fritted 150 mL reservoir Isolute SPE syringe column, and activated with 100 mL of methanol and 200 mL of 0.5% aqueous acetic acid. One aliquot (200 mL) of hydrolyzed extract was mixed with ca. 40 mL of activated resin and brought to dryness by rotary evaporation under reduced pressure. The resin was resuspended in ca. 50 mL of acidified water, added to the remaining resin packed in the syringe, and washed with 200 mL of acidified water. The column was then inserted in-line in the preparative HPLC system.

The mobile phases used for flash chromatography were A (0.5% acetic acid in water) and B (0.5% acetic acid in methanol). The linear gradient was 50% to 100% B in 20 min. The flow rate was 7 mL/min. The major part of phenolic compounds were eluted with 50% B while ellagic acid and its derivatives were eluted with 100% B. After this first separation the partially purified fraction containing ellagic acid and its derivatives were collected, brought to 30% methanol with distilled water, and passed through a Durapore 0.22 μm filter (Millipore, Vimodrone, Italy). The final separation of the ellagic acid derivatives was performed by preparative HPLC using a 250 × 50 mm, 10 μm, Discovery HS C18 column (Supelco, Bellefonte PA), with a 20 mm Pelliguard LC-18 replacement cartridge precolumn. The column was protected with 2 μm PEEK filter end fittings (Gilson, Milano, Italy). The mobile phase was 0.5% acetic acid in water (A) and 0.5% acetic acid in methanol (B). The column was conditioned with 30% methanol in water. The sample was diluted (1:3) with water, and an aliquot (260 mL) was loaded onto the column. The chromatographic conditions were as follows: isocratic run at 55% B; flow rate 40 mL/min; detection wavelength 260 nm. After the separation, the methyl sanguisorboate was brought to dryness by rotary evaporation under reduced pressure and then dissolved in diethyl ether and crystallized in hexane (40 mg). The pure methyl sanguisorboate was recovered by filtration as a pale-yellow powder which was characterized by MS, NMR, IR, and UV (see below). The derivative 1, which was the last and minor product of the hydrolysis, was isolated in insufficient amount and purity to characterize its structure, which remains unknown.

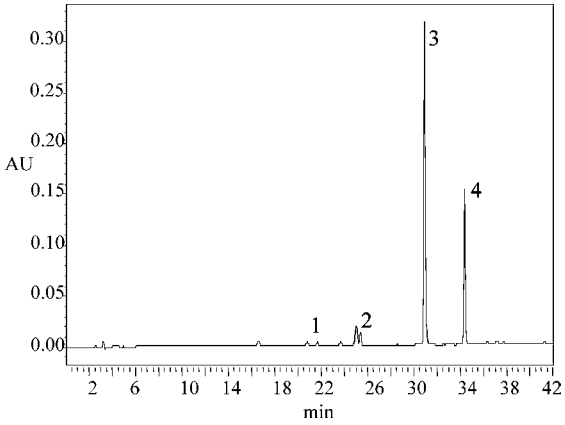

Figure 4. HPLC-DAD chromatogram at 260 nm of raspberry extract after hydrolysis. Compounds: **1**, methyl gallate; **2**, derivative 1; **3**, ellagic acid; **4**, methyl sanguisorboate.

NMR Measurements. ^{1}H and ^{13}C spectra of methyl sanguisorboate (**Figure 3**) were recorded at 500 and 125 MHz, respectively, on a spectrometer Bruker (Rheinstetten, Germany) DRX 500. Measurements were made at 300 K using a 2.5 mm inverse Broad Band probe. Sample (6.5 mg) was dissolved in 100 μL of DMSO-d_6 (99.9% isotope purity, Aldrich) and placed in a 2 mm i.d. capillary for measurement. Chemical shifts are given in δ (ppm) referred to TMS.

^{1}H NMR: 3.65 (3H, s, H8″); 6.50 (1H, d, $J = 1.8$ Hz, H2″); 7.13 (1H, d, $J = 1.83$ Hz, H6″); 7.50 (1H, s, H5); 8−9 (6H, broad signal, OH).

^{13}C NMR: 52.1 (C8″), 101.7 (C6′), 106.2 (C2″), 108.6 (C1), 110.7 (C5), 111.4 (C6″), 112.0 (C1′), 112.4 (C6), 119.2 (C1″), 134.6 (C2′), 137.1 (C2), 139.7 (C3), 140.1 (C3′), 140.4 (C4″), 142.1 (C5′*), 142.3 (C4′*), 146.4 (C5″), 147.1 (C3″), 148.8 (C4), 155.9 (C7′), 159.4 (C7), 166.5 (C7″). (*Positions could be exchanged).

IR Measurements. The IR spectrum of methyl sanguisorboate was obtained at the solid state on the pure sample, on a spectrometer Perkin-Elmer (Boston, MA) Spectrum one FT-IR. Frequency scale is expressed in cm^{-1}: 3195.8, 1709, 1682.8, 1604.3, 1518.9, 1492.3, 1436.1, 1317.8, 1233, 1156.7, 1111.1, 1047.7, 1003.9, 984, 877.8, 826.3, 765.7, 733.1, 700.7.

UV Measurements. The UV spectrum of ellagic acid and methyl sanguisorboate were recorded in methanol on the pure sample, on a Hitachi U-2000 spectrometer (Tokyo, Japan). Molar absorbivities were computed at the maximal absorption. Ellagic acid: $\epsilon_{254nm} = 40704$, $\epsilon_{365nm} = 8066$. Methyl sanguisorboate: $\epsilon_{254nm} = 58543$, $\epsilon_{371nm} = 13022$. Molar absorbivities in the conditions suggested for HPLC analysis with UV detection are as follows: Ellagic acid: $\epsilon_{260nm} = 35822$ (solvent: 21% of acetonitrile in 1% formic acid in water; v/v). Methyl sanguisorboate: $\epsilon_{260nm} = 45114$ (23.9% of acetonitrile in 1% formic acid in water; v/v). The molar absorbivity of methyl gallate in methanol was $\epsilon_{274\ nm} = 11818$.

RESULTS AND DISCUSSION

Identification of Hydrolytic Products. Following acid hydrolysis of red raspberry juice samples with 2 M HCl for 30 min at 100 °C, Rommel and Wrolstad (*24*) reported the presence of ellagic acid and two unidentified compounds with absorbance spectra very similar to that of ellagic acid. Määttä-Riihinen et al. (*25*) observed that after acid hydrolysis with 0.6 M HCl for 2 h the ellagitannins were converted to ellagic acid and an unknown ellagic acid derivative with a UV-DAD spectrum similar to ellagic acid. A similar observation was reported also by Mattila and Kumpulainen (*26*) in strawberry samples. Here we report the presence of ellagic acid and two others compounds with DAD spectra similar to ellagic acid in hydrolyzed *Rubus* extracts (**Figure 4**). Analysis of UV spectra of the hydrolyzed

products suggests different substitutions in the aromatic ring of the ellagic acid nucleus in the derivatives. Identification of hydrolytic products was based on DAD, MS, and NMR spectra.

Ellagic acid and methyl gallate were confirmed by retention time and MS and UV/vis spectra which were identical to the relevant standard. Ellagic acid (RT = 30.8 min, λ_{max} of 254 and 365 nm) was the main peak in the hydrolyzed *Rubus* extract and was characterized by MS from its molecular ion at *m*/*z* 301. Methyl gallate was another hydrolytic product and was characterized by RT = 21.8 min, λ_{max} of 218 and 274 nm, MS molecular ion at *m*/*z* 183.

Derivative 1 (RT = 25.1 min, λ_{max} of 254 and 369 nm) was the less abundant hydrolytic product and was characterized by the following MS data: molecular ion at *m*/*z* 469 which fragmented to *m*/*z* 301 ion. The UV/vis absorption spectra of the ellagic acid derivative 1 (RT = 25.1 min) and of methyl sanguisorboate (RT = 34.6 min) showed a bathochromic shift (shift to longer wavelength, λ = 369 and λ = 371 nm, respectively) in comparison to the spectrum of ellagic acid (RT = 30.8 min, λ_{max} 367 nm).

The methyl sanguisorboate (RT = 34.6 min) was the second main peak in the chromatogram. MS showed a molecular ion at *m*/*z* 483 which fragmented to *m*/*z* 315 and *m*/*z* 301 ions. Its structure was derived by MS, UV/vis, and NMR experiments on the pure standard isolated in our laboratory and is in agreement with the scheme shown in **Figure 1**.

Figure 3 shows the annotated structure of methyl sanguisorboate. Spectra analyses enable assignment of the singlet at δ 7.50 to the ellagic acid moiety, position H5. The two doublets at δ 6.50 and 7.13 are coupled, as confirmed by homonuclear correlation COSY experiment, and can be assigned to the gallic acid moiety, positions H2″ and H6″. The upfield position (δ 6.50) of H2″ with respect to H6″ (δ 7.13) is due to the anisotropic effect of the aromatic ring of the ellagic acid moiety. The position of the OCH_3 (H8″) group was confirmed by NOESY experiment, showing a correlation between H8″ and protons H2″ and H6″. Carbon signals were assigned by 1D $\{^1H\}^{13}C$ and APT (signals of primary, secondary, tertiary, and quaternary carbons were assigned) and 2D HSQC (C8″, C2″, C5, and C6″ signals were assigned), HMBC (C1, C6, C1″, C2, C3, C4″, C5″, C3″, C4, C7, and C7″ signals were assigned) experiments. The other carbon signals were assigned on the basis of their chemical shift and by comparison with ^{13}C spectra of pure ellagic and gallic acid. To our knowledge this is the first time that the isolation and detailed characterization of methyl sanguisorboate has been reported.

Mean Degree of Polymerization. The simultaneous quantification of the three main hydrolytic products of *Rubus* ellagitannins is an important achievement since it provides valuable information on the relative molar abundance of the building units of these phenolic polymers. In particular, an estimate of the mean degree of polymerization (mDP) of *Rubus* ellagitannins can be theoretically derived from the molar ratio between the methyl sanguisorboate and the ellagic acid produced in the reaction, $R_{[MS]/[EA]}$. Taking into consideration the known structure of the major *Rubus* ellagitannins (*11*, *13*−*15*), and assuming a complete hydrolysis, this ratio is expected to increase from 0 for the monomers (ellagic acid glycosides and galloyl-bis-HHDP-glucosides) up to a value of 0.60 for tetrameric lambertianin D, with intermediate values for dimeric sanguiin H-6 and trimeric lambertianin C (0.33 and 0.50, respectively). For oligomeric compounds such as sanguiin H-6 and the lambertianin series, this ratio is expected to grow according to the equation $R_{[MS]/[EA]} = (DP - 1)/(DP + 1)$, shown in **Figure 5A**. In conclusion, the value of $R_{[MS]/[EA]}$ can be obtained experimentally from the HPLC analysis of the hydrolytic products of raw *Rubus* extract and can be used for the computation of the mDP of *Rubus* ellagitannins, which can be derived from the following equation: $mDP = (R_{[MS]/[EA]} + 1)/(1 - R_{[MS]/[EA]})$, as shown in **Figure 5B**. The application of this method to a raspberry extract gives a mDP of ca. 1.9. This suggests that the oligomers with DP > 2 roughly equal the monomers in absolute concentration.

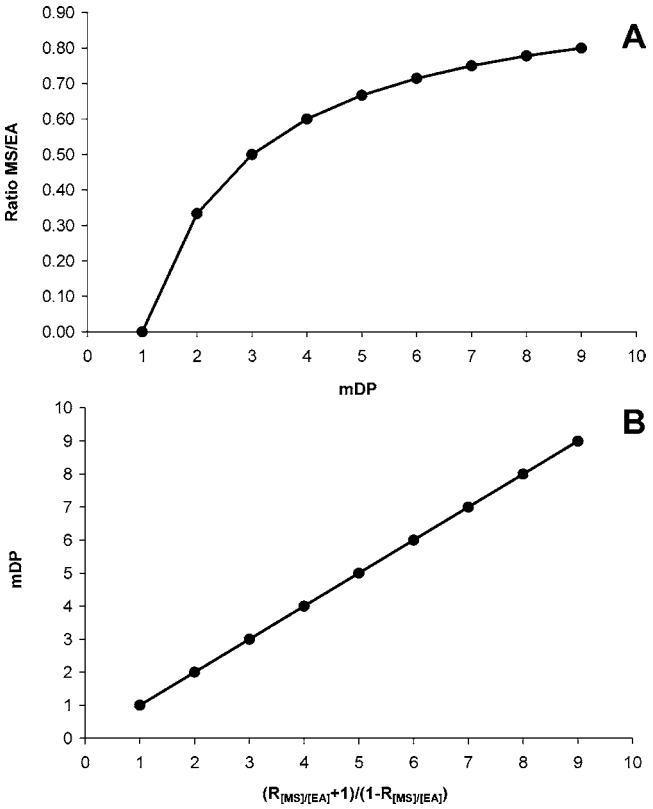

Figure 5. Mean degree of polymerization (mDP) estimates for *Rubus* ellagitannins derived from the molar ratio between methyl sanguisorboate and ellagic acid produced in the reaction, $R_{[MS]/[EA]}$. **Panel A**: Estimates derived from the known structure of the major *Rubus* ellagitannins and assuming a complete hydrolysis [$R_{[MS]/[EA]} = (DP - 1)/(DP + 1)$]. **Panel B**: Estimates of the mDP value of *Rubus* ellagitannins [$mDP = (R_{[MS]/[EA]} + 1)/(1 - R_{[MS]/[EA]})$]. Legend: methyl sanguisorboate (MS) and ellagic acid (EA).

In principle, the mDP of a mixture of ellagitannins could be evaluated also by normal-phase HPLC, since a linear relationship between the molecular weight and the log of the retention time has been reported for different native and partially hydrolyzed ellagitannins (*15*, *17*). The normal-phase HPLC could therefore be used to measure at the same time the concentration and molecular weight of individual ellagitannins, thus providing information also on the mDP of the whole extract. On the other hand, a validation of the normal-phase HPLC method was still not reported. Moreover, it requires a preventive calibration with reference compounds which are not available, which prevents a wider application. On the contrary, the novel method presented here is more simple and can be easily applied by other scientists, since it can be calibrated with commercially available standards.

Kinetics of Acid Hydrolysis. For the optimization of hydrolysis conditions, aqueous methanol extracts were evaporated to dryness, redissolved in methanol, and placed at different HCl concentrations (1.2, 2, and 4 M), for different duration (1, 2, 4, 6, and 20 h). The general scheme of the reaction is shown

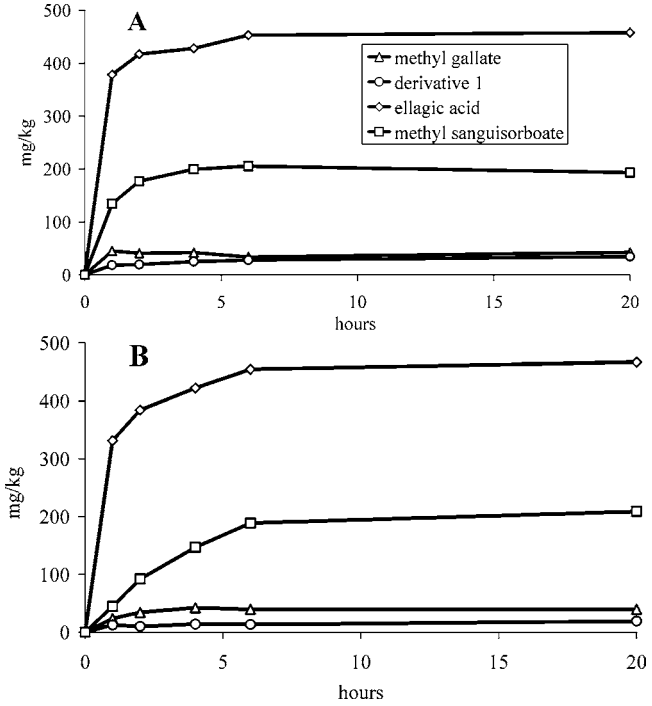

Figure 6. Time course of reaction products appearance following hydrolysis of a raspberry extract with 4 M HCl (**panel A**) or 2 M HCl (**panel B**) in methanol at 85 °C. The data represent the average value of three individual repetitions (two for ellagic acid).

in **Figure 1**. The free HHDP units are converted into the corresponding lactones **4** and **6** (**Figure 1**), while benzoic acid moieties are converted into their methyl esters **5** and **6** (**Figure 1**) via acid-catalyzed Fisher esterification.

When extracts were treated with 2 M trifluoroacetic acid at 85 °C, the ellagic acid rate of release was only 4.2% and 12.3%, after 4 and 20 h, respectively, of that obtained after 20 h of hydrolysis with 2 M HCl. Methyl sanguisorboate and derivative 1 were not detected following hydrolysis with trifluoroacetic acid, suggesting incomplete hydrolysis of the substrate. This finding is in agreement with Häkkinen et al. (*27*) who found the yield of trifluoroacetic acid hydrolysis to be around 15% compared with HCl. The use of trifluoroacetic acid for the hydrolysis of *Rubus* ellagitannins should be avoided.

Additionally, treatments with 1.2 M HCl at 85 °C also resulted in incomplete hydrolysis even after 20 h, resulting in yields of 83% ellagic acid, 90% methyl sanguisorboate, 61% methyl gallate, and 36% derivative 1, compared with hydrolysis in 4 M HCl for 6 h.

Figure 6 shows the average kinetics of hydrolysis of a methanolic raspberry extract at different acid strengths. With 4 M HCl, the release of ellagic acid becomes constant after 6 h of hydrolysis (ca. 453 mg/kg fresh weight) while the concentration of the methyl sanguisorboate becomes constant after 4 h of hydrolysis (ca. 200 mg/kg fresh weight). The amount of derivative 1 released, expressed as equivalents of ellagic acid, is much lower, increasing from 19 mg/kg after 1 h to 34 mg/kg after 20 h of hydrolysis. After 6 h of hydrolysis with 4 M HCl its value was 28 mg/kg. The amount of methyl gallate released reached a maximum after only 1 h of hydrolysis (45 mg/kg) and then remained quite constant up to 20 h. Such kinetics confirms the expected facile hydrolysis of the 1-*O*-gallate ester group. The hydrolysis of the 2,3-HHDP ester group releasing ellagic acid is relatively slower than the hydrolysis of both the 4,6-HHDP-group and the 1-*O*-gallate ester groups, which is required for the release of the sanguisorboyl unit. This is in agreement with the more rapid partial hydrolysis of the free 4,6-HHDP in water compared with the 2,3-HHDP group (*13*). In conclusion, a 6 h hydrolysis with 4 M HCl appears as the most rapid treatment to obtain the maximal yield for the three main hydrolysis products of raspberry ellagitannins. Similar results were also obtained with blackberry samples (data not shown), to which the present method can be also applied.

Table 1. Influence of Extraction Solvent on the Extraction Yield and Degree of Polymerization of Raspberry Ellagitannins, Evaluated for Different Duration of the Hydrolysis (1, 2, 4, and 6 h)[a]

time (h)	methyl gallate	deriv 1	ellagic acid	methyl sanguisorboate	mDP
solvent: aq methanol (60%)					
0	0.00	0.00	0.32	0.00	-
1	4.53	1.86	37.85	13.45	1.57
2	4.09	1.97	41.71	17.70	1.72
4	4.21	2.52	42.80	19.96	1.82
6	3.41	2.82	45.31	20.54	1.79
solvent: aq acetone (70%)					
0	0.00	0.00	0.54	0.00	–
1	7.28	4.38	80.99	33.31	1.69
2	6.82	5.46	88.37	42.55	1.86
4	5.27	5.58	75.98	38.56	1.93
6	5.18	5.61	97.22	48.90	1.91

[a] Data expressed in mg/100 g fresh weight. Hydrolysis condition: 4 M HCl, 85 °C.

Method Validation. Validation of the optimized method was performed on six samples of the same aqueous methanol extract of raspberry samples. Each sample was hydrolyzed separately and analyzed by HPLC-DAD. The coefficients of variation (CV) for ellagic acid, derivative 1, methyl sanguisorboate, and methyl gallate were 2.3, 3.1, 2.8, and 4.5%, respectively. The average value and CV for the mDP were 1.75 and 0.57%, respectively. Recovery experiment was carried out by spiking a red raspberry aqueous methanolic extract with pure ellagic acid (100 mg/kg) and methyl gallate (10 mg/kg). The spiked extract was afterward subjected to acid hydrolysis. The recovery trial was done in triplicate. Recoveries of spiked ellagic acid and methyl gallate were 100% and 96%, respectively.

Extraction Solvents. Large differences were observed in the yield and mDP of extracted ellagitannins when aqueous methanol and aqueous acetone were compared as extraction solvents (**Table 1**). The substantially lower yield obtained with aqueous methanol may be ascribed to the lower ellagitannin solubility in this solvent. On the basis of these results, aqueous acetone should be considered to be the extraction solvent of choice for ellagitannins analyses.

UV Quantification. To encourage the application of this method in other laboratories, overcoming the lack of standard of methyl sanguisorboate, the molar absorbivities of pure standards of methyl sanguisorboate and of ellagic acid were measured also at the optimal wavelength for UV detection in the HPLC analysis. The ratio of the molar absorbivities at $\lambda =$ 260 nm of methyl sanguisorboate vs ellagic acid is 1.259. This value is in agreement with the presence of three and two galloyl units, respectively, in methyl sanguisorboate and ellagic acid, and it has been found to be consistent with the experimental response of the two compounds in our conditions of HPLC analysis.

Strawberry Ellagitannins. There is substantial interest in the development of methodologies for the identification and characterization of ellagitannins in cultivated and wild strawberry. Analyses of aqueous acetone extracts from these fruits after hydrolysis with the above-described method revealed the

presence of ellagic acid and methyl gallate. Methyl sanguisorboate was identified as a minor reaction product together with methyl ellagic acid, and at least four unknown ellagic acid derivatives with different MS spectra and retention time than methyl sanguisorboate were also detected (data not shown). These preliminary results confirm the presence of a more complex mixture of oligomeric ellagitannins in strawberry compared with raspberry and blackberry. Further research is required to isolate and identify these compounds.

ABBREVIATIONS USED

EA, ellagic acid; MS, methyl sanguisorboate; HHDP, 6,6-dicarbonyl-2,2′,3,3′,4,4′-hexahydroxydiphenic acid.

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