Evolution of Ellagitannin Content and Profile during Fruit Ripening in *Fragaria* spp.

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Ⓢ Supporting Information

ABSTRACT: Ellagitannins and ellagic acid conjugates are polyphenols present in the human diet, in particular strawberries (*Fragaria* spp.). The first aim of this study was isolation and structural characterization of casuarictin and 3-*O*-methyl ellagic acid 3′-*O*-α-rhamnopyranoside, which were found to be abundant in *Fragaria* spp., along with agrimoniin. The second aim was accurate profiling and quantification of 26 ellagitannins and ellagic acid conjugates in six *Fragaria* x *ananassa* cultivars and two *Fragaria vesca* species. The third aim was to describe the ellagitannins behavior during fruit ripening from the green stage to over-ripeness. It was shown

that there are major qualitative and quantitative differences in the amount and profile of ellagitannins and ellagic acid conjugates between *Fragaria* spp. Genotype is a major factor in defining ellagitannin concentration and patterns between strawberries, and variable behavior of the genotypes was observed, in the context of a significant drop in ellagitannins during ripening.
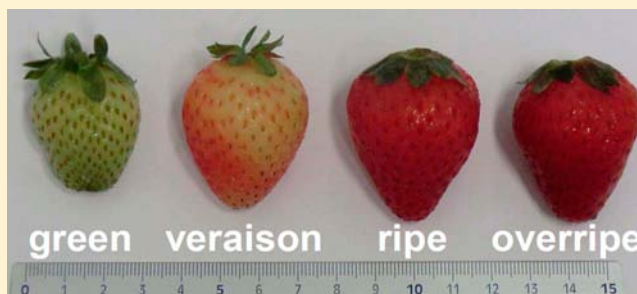
KEYWORDS: *Fragaria, strawberry, fruit ripening, ellagitannins, casuarictin, methyl ellagic acid rhamnoside*

INTRODUCTION

Ellagitannins are a complex family of hydrolyzable tannins which have been found only in dicotyledoneous angiosperms.[1] Ellagitannins are present in the human diet, as they are contained in berries and a number of other sources, such as pomegranates, walnuts, muscadine grapes, and many medicinal plants.[2] This class of natural polyphenols has recently received considerable attention in the light of experimental evidence regarding purported anticancer properties,[3,4] antiproliferative properties,[5,6] antibacterial activity in relation to intestinal pathogens,[7] and very recently anti-inflammatory activity at gastric level.[8] Ellagitannins are present at a relatively high concentration in some berries, such as raspberries, blackberries, strawberries, boysenberries, cloudberries, rose hips, and sea buckthorn.[9−12] Due to the greater awareness of producers and consumers as regards health benefits, the selection of cultivars in the last years has also focused on factors influencing the content of bioactive compounds in fruit,[13] besides the usual agronomical and product characteristics.

There is little knowledge about the native forms of ellagitannins in strawberries and their biosynthetic behavior during ripening,[14−19] although they are probably the most important dietary sources of ellagitannins in the human diet.[15] Due to the sheer complexity, the qualitative and quantitative composition of this class in strawberries has not yet been thoroughly resolved to date.[14] This represents one of the major limitations in the study of health benefits and the human

metabolism at the molecular level. In order to assign the correct health properties to these compounds it is important to have specific knowledge about the chemical structure of the native form of ellagitannins, their concentration, and the ellagitannin profiles present in fruits at different ripening stages and not only in ripe fruit.

It has been shown that there are both qualitative and quantitative differences in the concentrations of secondary metabolites in different strawberry cultivars.[16,19−22] Besides the cultivars, the chemical composition of fruit is also strongly influenced by the ripening stages, since both primary[23,24] and secondary metabolism change[23−25] while the fruit ripens. Specifically, the influence of ripening on polyphenol content, including ellagitannins, was recently investigated by Aaby et al.,[14] who reported that the changes observed in three strawberry cultivars during the stages considered suitable for consumption were doubtful.

From the point of view of plant biochemistry it is important to understand the fate of these compounds throughout ripening of the fruit (from green to red), while on the other hand it could be in the interest of producers and consumers concerned about the healthy properties of food to know how these

compounds change while the fruit is edible. Strawberries are usually harvested prematurely in order to prevent postharvest losses, whereas consumers are likely to get fully ripe fruits. Fruit quality and metabolite synthesis are complex traits that are genetically controlled, but at the same time they depend on the plant's stage of development and of course on agronomical and other environmental factors. To better understand the role played by each factor, the variation in metabolite content is often correlated with data derived from genetics, transcriptomics, and environmental studies.[26] The results can be used to further investigate metabolite biosynthesis, which in the case of ellagitannins is still not well-defined, and to develop new molecular markers useful in marker-assisted selection.[27]

A previous work described the isolation and structural elucidation of agrimoniin, the main ellagitannin in *Fragaria* spp.[15] The purpose of this study was isolation of a further two ellagitannins which are particularly abundant in strawberries and woodland strawberries, along with agrimoniin. Furthermore, accurate quantification of the main ellagitannins and ellagic acid conjugates, using their respective standards in 6 different strawberry cultivars and 2 woodland strawberry types at four different ripening stages was carried out. Evaluation of the content and profile at different stages was used to understand the behavior of this important class of polyphenols during fruit ripening, from the green stage to overripeness.

■ MATERIALS AND METHODS

Chemicals and Reagents. All the chromatographic solvents were HPLC grade or LC−MS grade for the MS experiments. Acetonitrile, acetone, methanol, and diethyl ether were purchased from Sigma Aldrich (Milan, Italy). Hexane and formic acid were purchased from Carlo Erba (Milan, Italy). Ellagic acid standard (purity >96%) was purchased from Fluka (Steinheim, Germany). Sanguiin H6 and lambertianin C were isolated as described in Gasperotti et al.[10] and agrimoniin as described in Vrhovsek et al.[15]

Plant Material. For isolation of ellagitannins and ellagic acid conjugates, 1 kg of woodland strawberries (*Fragaria vesca*) and 5 kg of strawberries (*Fragaria × ananassa* D. cv. Darselect) were used.

For analysis of ellagitannins in different cultivars at different ripening stages, the experimental design consisted of 96 samples (6 cultivars and 2 accessions × 3 repetitions × 4 stages). Six *Fragaria × ananassa* strawberry cultivars (Alba, Clery, Eva, Elsanta, Darselect, Portola) and 2 accessions (one red and one white) of *Fragaria vesca* were grown in the same experimental field in Vigalzano (Trentino, Italy; 520 AMSL) during the 2011 season. All cultivars were produced under standardized conditions in order to minimize the effect of environmental and agronomic factors. The plants were cultivated using the soilless technique, in an adequate quantity to obtain three biological replicates for each cultivar. For each cultivar analyzed 3 repeats of 6 plants (18 plants in total) were considered. This meant that plants of the same cultivar were obtained vegetatively from the same mother. The agronomical system involved the use of plastic pots (50 × 25 × 11 cm) filled with an inert substratum (peat). The fertirrigation drip system consisted of a proportional liquid dispenser with a flow meter, where the fertilizers and the microelements were premixed. Soil acidification (pH 5.2 to 5.8) was carried out using nitric acid. The flux was regulated on the basis of the soil volume, in order to have 25 cm³ of nutrient solution for each liter of pit. Irrigation took place daily and was manually timed for 12 h, on the basis of climate changes, with cycles of a few minutes each 0.5−1 h.

For each cultivar and each time stage 250 g of strawberries at 4 phenological stages (1, green; 2, veraison; 3, ripe; 4, overripe) were collected in triplicate (biological replicates) from different plants. Strawberries have staggered flowering, and this results in the evolution of nonsimultaneous formation of fruits on the plant. Moreover the fruit developing from primary inflorescence is bigger than the fruit developing from secondary, tertiary, or quaternary inflorescence.

Consequently the number of achenes is determined by the flower order.[28] To avoid sampling variability, all fruits were sampled from any single plant belonging to the same lot of six plants in two different production periods. Then the fruits were divided according to their phenological stage into the 4 homogeneous groups mentioned above. Once collected the samples were stored in a freezer at −20 °C until extraction. The extraction of strawberry polyphenols was performed as reported in Mattivi et al.[29] with an acetone/water mixture (70/30 v/v), avoiding the addition of acids in order to prevent chemical hydrolysis.

Total Soluble Solids (TSS), Total Titratable Acidity (TTA), and pH. Samples (40 g) were homogenized using an Ultraturrax (Ika, Staufen, Germany) for 3 min at 13500 rpm and then centrifuged at 4 °C at 8000 rpm for 5 min. An aliquot was used to determine pH and TSS. TSS was expressed as ° Brix with a refractometer (RFM 81, Bellingham & Stanley Ltd., U.K.). TTA was measured following the AOAC Official Method 942.15[30] using an automatic titrator, Compact Tritator (Crison, Barcellona, Spain). Results were expressed as g of citric acid kg⁻¹ of fresh weight.

Agronomical Parameters. The strawberry cultivars were evaluated by measuring the following descriptors in 10 fruits for each cultivar: color, firmness, weight, and size at ripening stage. Fruit color was measured using a Minolta spectrophotometer (CM-3600d) equipped with the light source D65 and observation angle of 10°. This instrument measures the following CIELAB variables:[31] $L*$, $a*$, $b*$. Chroma ($C*ab$) was calculated using the equation: $C*ab = [(a*)^2 + (b*)^2]^{1/2}$. Flesh firmness was measured by using a digital fruit firmness tester (TR Turoni srl, Forlì, Italy) on the equatorial part of the fruit with a 6 mm probe. The weight was determined with a digital fruit firmness tester (KERN EW1500-2M) at ripening stage 3 for all varieties and at each ripening stage for Elsanta, for which the percentage of weight increase at the different stages was calculated. Fruit size was determined using a caliper (Sylvac model S 235 PAT), measuring the diameter at the equatorial part of the fruit and the height. The descriptors' mean value was calculated for each strawberry.

Isolation of Ellagitannins from *Fragaria vesca* and *Fragaria × ananassa* D. Isolation was performed as described in detail by Vrhovsek et al.[15] In short, for the isolation of methyl ellagic acid rhamnoside, 1 kg of red woodland strawberries, and for the isolation of galloyl-bis-HHDP glucose, 5 kg of Darselect cv. strawberries were used. Isolation of the two compounds was carried out with a preparative HPLC Shimadzu SCL-10 AVP equipped with a Shimadzu SPD-10 AVP UV/vis detector, 8A pumps, and Class VP Software (Shimadzu Corp., Kyoto, Japan). The UV signal was recorded at 260 nm. The first phase of isolation involved purification of the anthocyanins from the sample using Sephadex LH20. The second phase in the isolation of compounds was performed using preparative HPLC with a 250 × 50 mm 10 μm Discovery HS C18 column (Supelco, Bellefonte, PA, USA). The pure isolated compounds were recovered by precipitation from *n*-hexane as an amorphous pale rose powder, which was further characterized by NMR, UV, and MS.

NMR and Circular Dichroism (CD) Analysis. NMR spectra (¹H NMR, COSY, NOESY, HSQC, and HMBC) were recorded in tetradeuterated methanol (99.90% CD₃OD) or in hexadeuterated acetone (99.9% CD₃COCD₃) at 300 K on a Bruker-Avance 400 MHz NMR spectrometer by using a 5 mm BBI probe with 90° proton pulse length of 9.1 μs at a transmission power of 0 dB. The chemical shift scales (δ) were calibrated on the residual signal of methyl group of methanol at δ_H 3.310 ppm and δ_C 49.00 ppm for spectra acquired in CD₃OD solutions (casuarictin). The residual signal of methyl group of acetone at δ_H 2.04 ppm and δ_C 29.00 ppm was used for calibration for spectra acquired in CD₃COCD₃ solutions (methyl ellagic-rhamnoside).

The CD spectra of casuarictin (2.1×10^{-5} M) and ellagic derivative (3.0×10^{-5} M) were recorded in methanol (1.8×10^{-6} M) on a Jasco J-40AS dichrograph. The following Cotton effects expressed in molar ellipticity Θ (mol⁻¹ L cm⁻¹) at the corresponding wavelengths (λ) were observed: for casuarictin, $\Theta = +1.8 \times 10^4$ (240 nm), $\Theta = -4.1 \times 10^3$ (268 nm), $\Theta = -1.8 \times 10^3$ (310 nm); for ellagic derivative, $\Theta = +3.5 \times 10^3$ (240 nm).
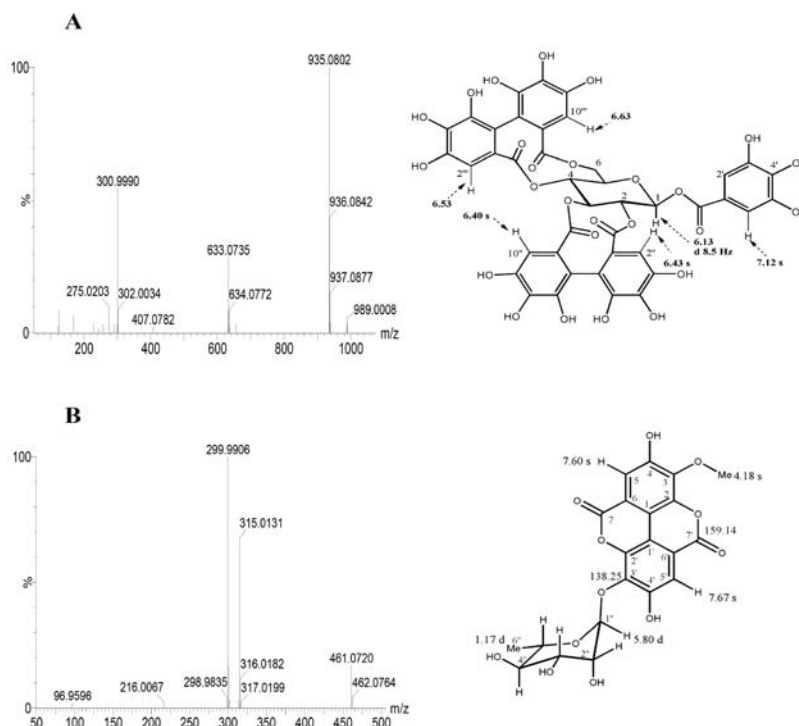
Figure 1. HRMS spectra and selected ^{1}H NMR resonances of casuarictin (A) and methyl ellagic acid rhamnoside (B) in CD3OD at 300 K.

UV Measurement. The UV spectra of methyl ellagic acid rhamnoside and casuarictin were recorded both in methanol and ethanol, on a Hitachi U-2000 spectrometer (Tokyo, Japan). The following molar extinction coefficients were observed. In methanol: methyl ellagic acid rhamnoside, $\varepsilon_{260nm} = 15500$ M^{-1} cm^{-1}; casuarictin, $\varepsilon_{260nm} = 29200$ M^{-1} cm^{-1}. In ethanol: methyl ellagic acid rhamnoside, $\varepsilon_{260nm} = 15600$ M^{-1} cm^{-1}; casuarictin: $\varepsilon_{260nm} = 31800$ M^{-1} cm^{-1}. The reported molar extinction coefficient for casuarictin[32] in methanol was $\varepsilon_{260nm} = 38900$.

Structural Elucidation Using UPLC-Q-TOF-HDMS Analysis. Chromatographic separation was carried out with a Waters Aquity UPLC system equipped with UV−vis Waters PDA (Waters Corp., Milford, MA) under the same conditions described for quantitative HPLC analysis. Detailed compound characterization was carried out using a Waters HDMS-QTOF Synapt (Waters Corp., Milford, MA) mass spectrometer with electrospray ionization system (ESI) and MassLynx Software 4.1 (Waters Corp., Milford, MA). HDMS analysis was performed in negative mode in the following conditions: capillary voltage 3 kV, sampling cone 40 V, extraction cone 3 V, source temperature 100 °C, desolvation temperature 350 °C, cone gas flow (N_2) 50 L/h, desolvation gas flow (N_2) 800 L/h. The m/z range was 50−3000 Da.

The MS was calibrated using sodium formate, and leucine enkephalin was used as the lock mass. The experimental m/z values reported were detected and accepted within ±7 ppm of the monoisotopic m/z for the exact theoretical structures.

Sample Preparation for Quantitative HPLC Analysis. Anthocyanins are the main source of interference in HPLC analysis of ellagitannins, and their elimination is essential for obtaining high quality MS spectra of minor ellagitannins. The purification of ellagitannins from anthocyanins using the Sephadex LH-20 was performed following the same protocol described in Gasperotti et al.[10] However, unlike the *Rubus* analysis reported in the paper, for the purification and subsequent quantification of strawberries, an aliquot of 40 mL of extract was used, due to the different concentration range of target compounds.

Quantitative Analysis Using HPLC-DAD. The purified extract was evaporated until dryness in a 100 mL pear-shaped flask using rotary evaporation under reduced pressure at 40 °C. Then the dry sample was diluted to 1 mL with methanol. HPLC analysis was carried

out using a Waters 2690 HPLC system equipped with Waters 996 DAD (Waters Corp., Milford, MA), and Empower Software (Waters) as described in Gasperotti et al.[10] All the compounds were quantified using UV detection at 260 nm. Calibration curves such as ranges and limits of detection (LOD) and quantification (LOQ) for standard casuarictin, lambertianin C, sanguiin H-6, ellagic acid, agrimoniin, and methyl ellagic acid rhamnoside are reported in detail in Table S1 in the Supporting Information. Casuarictin, lambertianin C, sanguiin H-6, ellagic acid, agrimoniin, and methyl ellagic acid rhamnoside were quantified following calibration with their respective pure isolated standards and expressed as mg/kg FW. Other ellagitannins were quantified as equivalents of agrimonin and the ellagic acid glycosides as equivalent of ellagic acid, expressed as mg/kg of fresh fruit.

Statistical Analysis. Statistical analysis was carried out using the STATISTICA s/w data analysis software system (version 9, StatSoft, Tulsa, OK, USA). Cluster analysis of the results obtained for the 3 batches of fruits from the 6 cultivars of *Fragaria* × *ananassa* and two *Fragaria vesca*, based on 27 variables describing the individual concentration of the 26 different ellagitannins and ellagic acid conjugates plus their total content, was performed separately for each of the four stages, based on the single linkage, Euclidean distance. The combined effects of the two genotype and ripening stage factors were explored using multivariate repeated measure ANOVA analysis on the same 27 variables used for cluster analysis.

RESULTS AND DISCUSSION

Agronomical Parameters. Detailed knowledge of agronomical parameters is the starting point for better understanding the differences between the cultivars analyzed at the chemical level. Indeed, as previously reported,[33−35] most of these (color, size, sweetness, sourness, etc.) are correlated with polyphenol content.

The strawberry cultivars investigated were selected from those most widely cultivated in both Italian and European temperate zones (Belgium, The Netherlands, Germany, and English-speaking areas) and are listed in Table S2 in the Supporting Information. Morphological trait values such as color (chroma) and firmness were different for each selected

cultivar with increasing values from Elsanta to Portola (Table S2 in the Supporting Information). Glossiness ranged from 26.2 (Clery) to 35.0 $L*$ (Darselect) respectively. The weight, diameter, and fruit height size parameters ranged from 10.4 g (Clery) to 15.8 g (Portola), from 28.1 mm (Alba) to 33.2 mm (Darselect), and from 33.5 mm (Clery) to 40.3 mm (Darselect) respectively (Table S2 in the Supporting Information). For the Elsanta variety the fresh fruit mean weight increased by ∼200% from the green stage to veraison and by only ∼7% between veraison and ripeness (Table S3 in the Supporting Information). The pH value was relatively high at the green stage, decreased during veraison, and increased at the overripe stage for the *F. vesca* selections. The pH increment was inconsistent for Portola as compared to the other *Fragaria* × *ananassa* varieties. Citric acid content was highest in the Darselect cultivar but fell drastically during ripening. In the red *F. vesca* TTA was high at the green stage, increased at veraison, and decreased during the last two stages, whereas in the white selection the amount was low at the green stage but increased at veraison and the ripe stage, to then decrease at the overripe stage. Sugar content increased proportionally in all samples, except for Alba and Eva, which turned from green to red quite quickly, showing a small drop at veraison, and was significantly higher at the ripe and overripe stages in the white *vesca* (Table S4 in the Supporting Information).

These data, in conjunction with productivity (not reported), show some of the main agronomical characteristics behind the development and commercial use of these cultivars,[36] confirming the representativeness of the genotypes included in the study.

Structural Assignment of Isolated Compounds.
Methyl Ellagic Acid Rhamnoside. In this study a high concentration of methyl ellagic acid rhamnoside was found in the red and white woodland strawberry for the first time. It had previously been found in the bark of *Eucalyptus globulus*,[37] in *Punica granatum* heartwood,[38] and in the fruit of *Caraipa densifolia* Mart.[39] To date, a few ellagic acid glycosides have been reported in strawberries, but without specific knowledge of the type of glycoside conjugated.[40,41] Using high resolution mass spectrometry (HRMS) analysis (Figure 1) the isolated compound (t_R 39 min) showed a $[M − H]^{-1}$ ion at m/z 461.0720, suggesting an anionic species with the molecular formula $C_{21}H_{17}O_{12}$, whose calculated molecular mass (461.0725) is in excellent agreement ($\Delta m = 0.2$ ppm) with experimental measurements. The two main daughter ions detected at 315.0131 and 299.9906 suggested species with the molecular formulas $C_{15}H_7O_8$ (calculated mass 315.0146) and $C_{14}H_4O_8$ (calculated mass 299.9912); the former derives from the loss of a deoxy-hexose moiety and the latter from further loss of a methyl group. All these findings can be accounted for by assuming a structure where the methyl ellagic acid is linked to a deoxy-hexose monosaccharide.

The nature of this deoxy-hexose moiety and the structural details of this compound were established using 1D and 2D NMR measurements carried out in CD_3COCD_3. In particular, its ^1H NMR spectrum showed resonances (doublet signal at δ_H 1.17 ppm coupled to an axial proton signal at δ_H 4.30 ppm) attributable to a 6-deoxy monosaccharide, whereas analysis of the homo- and heteronuclear correlations were in agreement with the structure of an α-rhamnose moiety. 2D NMR analysis indicated that the anomeric carbon of rhamnose was linked to the 3′-OH group of 3-*O*-methyl ellagic acid; thus, peak 25

(Figure 2) was unambiguously established to be 3-*O*-methyl ellagic acid 3′-*O*-α-rhamnopyranoside.
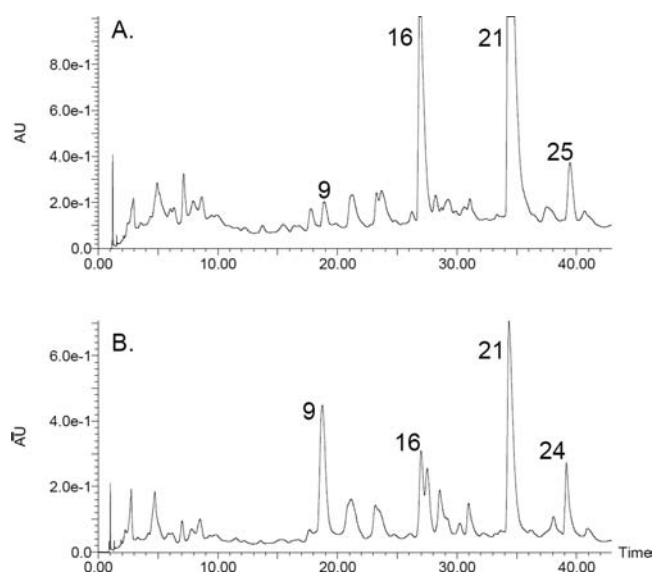

Figure 2. PDA chromatogram (260 nm) of ellagitannin profiling in woodland strawberries (A) and the strawberry cv. Clery (B) Peak numbers refer to casuarictin (**9**), ellagic acid (**16**), agrimoniin (**21**), lambertianin C like (**24**), and methyl ellagic acid rhamnoside (**25**).

Casuarictin (Galloyl-bis-HHDP Glucose). Casuarictin represents one of the monomers frequently found as constituents of the oligomeric ellagitannins found in *Rubus* spp., *Fragaria* spp., and other natural sources.[42] It is also known as galloyl-peduncalagin or casuarictin/potentillin, on the basis of the configuration of the glucose core. Specifically, if it is in α position, the monomer is potentillin, whereas it is casuarictin if in the beta position.[43] From the biosynthetic point of view, galloyl-bis-HHDP glucose comes from the oxidation of the vicinal galloyl units of pentagalloylglucose, leading to the formation of the 2 HHDP units.[44−46]

In HRMS structural analysis (Figure 1) the isolated compound (t_R 18.4 min) showed a $[M − H]^{-1}$ ion at m/z 935.0802, suggesting an anionic species with the molecular formula $C_{41}H_{27}O_{26}$ whose calculated molecular mass (935.0796) is in excellent agreement ($\Delta m = 0.6$ ppm) with the experimental measuremets. The two main daughter ions detected at 633.0735 and 300.9990 suggested single charged anionic species with the molecular formulas $C_{27}H_{21}O_{18}$ (calculated mass 633.07333) and $C_{14}H_5O_8$ (calculated mass 300.9990); the former comes from the loss of a HHDP unit followed by proton transfer and the latter by internal rearrangement of the HHDP itself, both being common fragmentation patterns of polymeric ellagitannins. Consequently, on the basis of the fragmentation pattern and the molecular ion, the compound was identified as the monomer galloyl-bis-HHDP glucose, with a monoisotopical mass of 936.0868 ($C_{41}H_{28}O_{26}$).

NMR analysis carried out in deuterated methanol using 1D and 2D techniques suggested the presence of a pentaacyl-substituted glucose. In particular, the characteristic ^{13}C sugar signals were found in the HSQC-NMR spectrum, coupled with a series of multiplets at δ_H 3.9−5.2 ppm, while the anomeric C1 (δ_C 91.9 ppm) was shown to be coupled with the deshielded H1 at δ_H 6.13 ppm. On the other hand, the strong $^3J(C,H)$

Table 1. Characterisation and Tentative Identification of Ellagitannin and Ellagic Acid Conjugates Found in Strawberries and Woodland Strawberries Using UPLC-Q-TOF

peak no.	t_R (min)	m/z ESI(−)	tentative structural assignment	MM[a]	Δ mass (ppm)[b]	cultivar
1	4.5	[783.0685]⁻¹, [633.0741]⁻¹, [481.0617]⁻¹, [300.9992]⁻¹	pedunculagin	784.0759	−0.6	strawberry, Elsanta cv.
2	6.1	[951.0735]⁻¹, [783.0685]⁻¹, [631.0582]⁻¹, [481.0627]⁻¹, [300.9986]⁻¹	unknown ellagitannin	952.0818	0.4	woodland strawberry, red type
3	8.0	[633.0634]⁻¹, [481.0477]⁻¹, [331.0544]⁻¹, [300.9970]⁻¹	strictinin	634.0806	−1.1	strawberry, Elsanta cv.
4	9.9	[933.0657]⁻¹, [466.0246]⁻², [300.9982]⁻¹	castalagin isomer	934.0712	−2.6	woodland strawberry, red type
5	13.4	[933.0670]⁻¹, [466.0257]⁻², [301.0000]⁻¹	castalagin isomer	934.0712	−4.0	woodland strawberry, red type
6	14.0	[933.0671]⁻¹, [466.0253]⁻², [301.0002]⁻¹	castalagin isomer	934.0712	−4.4	strawberry, Portola cv.
7	17.4	[785.0861]⁻¹, [615.0650]⁻¹, [392.0355]⁻², [300.9984]⁻¹, [169.0141]⁻¹	digalloyl-HHDP-glucose	786.0915	−3.2	woodland strawberry, red type
8	18.0	[1103.0848]⁻¹, [951.0808]⁻¹, [933.0637]⁻¹, [783.0696]⁻¹, [633.0737]⁻¹, [300.9983]⁻¹	sanguiin H-2	1104.0927	0.0	woodland strawberry, red type
9	18.4	[935.0809]⁻¹, [633.0739]⁻¹, [463.0519]⁻¹, [301.0004]⁻¹	casuarictin	936.0868	−2.1	strawberry, Elsanta cv.
10	19.5	[1567.1527]⁻¹, [933.0659]⁻¹, [783.0694]⁻², [633.0746]⁻¹, [300.9983]⁻¹	sanguiin H-10	1568.1518	−5.6	strawberry, Elsanta cv.
11	21.1	[1235.0723]⁻¹, [933.0806]⁻¹, [633.0744]⁻¹, [617.0284]⁻², [300.9995]⁻¹	unknown ellagitannin	1236.0775	−2.2	strawberry, Elsanta cv.
12	22.9	[1869.1497]⁻¹, [1567.1431]⁻¹, [1235.0636]⁻¹, [934.0724]⁻², [933.0567]⁻¹, [783.0611]⁻¹, [633.0667]⁻¹, [300.9987]⁻¹	sanguiin H-6 isomer	1870.1581	−0.3	strawberry, Elsanta cv.
13	23.3	[1103.0811]⁻¹, [951.0764]⁻¹, [933.0662]⁻¹, [783.0716]⁻¹, [633.0734]⁻¹, [300.9982]⁻¹	sanguiin H-2 isomer	1104.0927	3.4	woodland strawberry, red type
14	24.8	[1401.1096]⁻², [1235.0768]⁻¹, [933.0657]⁻¹, [933.0567]⁻¹, [783.0702]⁻¹, [633.0757]⁻¹, [300.9984]⁻¹	lambertianin C	2804.2293	2.1	woodland strawberry, red type
15	25.8	[1869.1560]⁻¹, [1567.1537]⁻¹, [1235.0636]⁻¹, [934.0739]⁻², [933.0665]⁻¹, [783.0730]⁻¹, [633.0743]⁻¹, [300.9986]⁻¹	sanguiin H-6	1870.1581	−3.0	woodland strawberry, red type
16	26.2	[300.9984]⁻¹, [257.0085]⁻¹, [229.0139]⁻¹	ellagic acid	302.0062	−0.3	woodland strawberry, red type
17	26.7	[447.0563]⁻¹, [300.9990]⁻¹, [299.9914]⁻¹	ellagic acid deoxyhexose	448.0641	−0.2	strawberry, Elsanta cv.
18	28.4	[2037.1661]⁻¹, [1018.0735]⁻², [1567.1521]⁻¹, [1235.0841]⁻¹ [933.0655]⁻¹, [783.0684]⁻¹, [300.9997]⁻¹	sanguiin H-6 with galloyl moiety	2038.1639	0.5	strawberry, Elsanta cv.
19	30.7	[2501.2153]⁻¹, [1869.1633]⁻¹, [1567.1493]⁻¹, [1250.1063]⁻², [933.0693]⁻¹, [783.0734]⁻¹, [633.0746]⁻¹, [300.9993]⁻¹	lambertianin C without ellagic moiety	2502.2230	−2.2	strawberry, Elsanta cv.
20	32.5	[1085.0804]⁻¹, [542.0308]⁻², [301.0008]⁻¹	galloyl-castalagin	1086.0822	−5.6	woodland strawberry, red type
21	33.8	[1869.1555]⁻¹, [1567.1484]⁻¹, [1265.1418]⁻¹, [934.0709]⁻², [933.0670]⁻¹, [783.0685]⁻¹, [633.0732]⁻¹, [300.9985]⁻¹	agrimoniin	1870.1581	−2.8	strawberry, Elsanta cv.
22	35.2	[1085.0787]⁻¹, [542.0303]⁻², [300.9985]⁻¹	galloyl-castalagin isomer	1086.0822	−4.1	woodland strawberry, red type
23	37.5	[939.1123]⁻¹, [483.0210]⁻¹, [300.9996]⁻¹, [169.0130]⁻¹	pentagalloyl glucose	940.1181	−2.2	woodland strawberry, red type
24	38.7	[1401.1008]⁻², [933.0692]⁻¹, [783.0727]⁻¹, [633.0737]⁻¹, [481.0657]⁻¹, [300.9992]⁻¹	lambertianin C like	2804.2293	4.2	strawberry, Elsanta cv.
25	39.0	[461.0720]⁻¹, [315.0138]⁻¹, [299.9896]⁻¹	methyl ellagic acid rhamnoside	462.0798	−0.2	woodland strawberry, red type
26	41.0	[2019.1527]⁻¹, [1567.1472]⁻¹, [1235.0774]⁻¹, [1009.0682]⁻², [933.0688]⁻¹, [783.0714]⁻¹, [633.0747]⁻¹, [300.9991]⁻¹	unknown ellagitannin	2020.1606		strawberry, Elsanta cv.

[a]MM: theoretical molecular monoisotopic mass of the putative metabolite. [b]Δ mass (ppm): deviation of the observed ion mass from the corresponding theoretical monoisotopic mass.

heterocorrelations of the carbonyl groups with the corresponding protons on the sugar moiety detected in the HMBC-NMR spectrum made it possible to clearly define the overall substitution pattern of the sugar moiety. Of the five resonances detected for these 5 ester groups, four at δ_C about 168−170 ppm are attributable to −COO groups linked to HHDP moieties, and one at δ_C 165.2 ppm to the −COO group linked to the galloyl group at the anomeric C-1 of the glucose. Moreover, the coupling pattern of the deshielded H1 proton (δ_H 6.13 ppm, ^3J(H1,H2) = 8.5 Hz) indicated that galloyl group was in the β position (see Figure 1 and Figure S1 in the

Supporting Information). Therefore peak 11 (Figure 2) was unambiguously established to be casuarictin.[47] The absolute configuration of the four chiroptical HHDP groups on the $^4C_1$ glucose core was established to be S by the positive sign of the strong Cotton effect at λ 240 nm and the negative Cotton effect at λ 268 nm.

Identification of Ellagitannins and Ellagic Acid Glycosides in the Different Strawberry Cultivars. Use of electrospray ionization high resolution mass spectrometry (ESI-HRMS) has been shown to be a useful technique for profiling ellagitannins and their structural characterization in

raspberries and blackberries.[10] In the same way, the profiles of ellagitannins in different strawberry cultivars and wild strawberry types were identified. ESI-HRMS analysis of different strawberry cultivars and wild strawberry types allowed characterization of 26 compounds and their tentative identification, with an accuracy of within 7 ppm in relation to the exact monoisotopical molecular mass. The compounds identified are given in Table 1 with their monoisotopical molecular ions, the most characteristic fragments, their tentative identification, and the ppm difference from the exact monoisotopical mass. The MS features of the ellagitannins and ellagic acid conjugates reported in this paper were verified in all the different cultivars investigated, and the exact molecular ions for each are given in Table S5 in the Supporting Information.

Ellagitannin Structural Profiling. The compounds were first identified using a UV–vis detector (Figure 2) and then analyzed with HRMS, checking their monoisotopical molecular ions and characteristic fragmentation pattern. The fragmentation pattern is often similar in different ellagitannins, due to their structural similarity.

Compound **1** (t_R 4.45 min) showed a $[M - H]^{-1}$ ion at m/z 783 and main fragments at m/z 481 (loss of HHDP) and 301 (ellagic acid). On the basis of these fragments and the difference in ppm in relation to molecular ion, this compound was identified as bis-HHDP-glucose or pedunculagin, 784 Da.[40] Compound **2** (t_R 6.1 min) showed a $[M - H]^{-1}$ ion at m/z 951 and fragments at m/z 783 (loss of galloyl fragment), 481, 301. This compound showed the same molecular ion already reported as an unknown ellagitannin.[40] The compound can be tentatively identified as sanguisorboyl-HHDP-glucose. Compound **3** (t_R 8 min) showed a $[M - H]^{-1}$ ion at m/z 633 and main fragments at m/z 481 (loss of gallic acid), 331 (loss of HHDP), 301. On the basis of these fragments and the molecular ion, compound **3** was identified as galloyl-HHDP glucose or strictinin, 634 Da. Compounds **4**, **5**, and **6** (t_R 9.9, 13.4, and 14 min respectively) showed the same $[M - H]^{-1}$ ions at m/z 933 and $[M - H]^{-2}$ at m/z 466. They also had the common fragment at m/z 301. These 3 compounds were in accordance with previous findings,[4,6] where they were identified as unknown ellagitannins. It was also suggested that these fragmentation patterns could be related to the presence of different isomeric forms of castalagin, 934 Da.[24] Compound **7** (t_R 17.4 min) showed a $[M - H]^{-1}$ ion at m/z 785 and $[M - H]^{-2}$ at m/z 392, fragments at m/z 615, 301, and 169 (gallic acid). This compound was tentatively identified as digalloyl-HHDP-glucose.[40] Compounds **8** and **13** (t_R 18 and 23.3 min) showed a $[M - H]^{-1}$ ion at m/z 1103. On the basis of these molecular ions and the fragmentation of 951, 933, 783, 633, and 301, the compounds were identified as sanguiin H-2 isomers, 1104 Da. Compound **9** (t_R 18.4 min) showed a $[M - H]^{-1}$ ion at m/z 935 and fragments at m/z 633, 301. On the basis of this information and the results of NMR analysis, after isolation of the peak, the compound was identified as the characteristic ellagitannin monomer casuarictin. Compound **10** (t_R 19.5) showed a $[M - H]^{-1}$ ion at m/z 1567 and $[M - H]^{-2}$ at m/z 783. The compound was identified as sanguiin H-10, 1568 Da, and the fragmentation patterns were made by the fragment at m/z 933, 783, 633, 301. Compound **11** (t_R 21.1 min) showed a $[M - H]^{-1}$ ion at m/z 1235 and $[M - H]^{-2}$ at m/z 617, giving a molecular mass of 1236, and has already been previously reported as unknown ellagitannin in the strawberry.[40] It has also been reported with the common name davuriicin M1.[48] Compound **12** (t_R 22.9 min) showed a $[M -$

$H]^{-1}$ ion at m/z 1869 and $[M - H]^{-2}$ at m/z 934 with fragments at m/z 1567, 1235, 933, 783, 633, 301. On the basis of this information this compound was identified as an isomer of sanguiin H-6. This isomeric form of sanguiin H-6 had a different retention time as compared to sanguiin H-6. Compound **14** (t_R 24.8) was in accordance with the retention time of lambertianin C previously isolated in the raspberry[10] and showed $[M - H]^{-2}$ at m/z 1401, giving a molecular mass of 2804. In the light of this evidence, the same retention time, and the double charged signal, this compound was identified as lambertianin C, also due to the characteristic fragmentation pattern of the polymeric ellagitannins in *Rubus* (933, 783, 633, 301). Compound **15** (t_R 25.8) was in accordance with the retention time of sanguiin H-6 previously isolated in the raspberry[10] and showed $[M - H]^{-2}$ at m/z 934, giving a molecular mass of 1870, and also had the same characteristic fragmentation pattern. Compound **18** (t_R 28.4 min) showed a $[M - H]^{-1}$ ion at m/z 2037 and $[M - H]^{-2}$ at m/z 1018 with fragments at m/z 1567, 1235, 933, 783, 301. On the basis of this information and the literature[10,41,48] this compound was tentatively identified as sanguiin H-6 with galloyl moiety. Compound **19** (t_R 30.7 min) showed a $[M - H]^{-1}$ ion at m/z 2501 and $[M - H]^{-2}$ at m/z 1250 with fragments at m/z 1869, 1567, 933, 783, 633, 301. The compound was tentatively identified as lambertianin C without an ellagic moiety, 2502 Da.[10] Compounds **20** and **22** (t_R 32.5 and 35.2 min) showed a $[M - H]^{-1}$ ion at m/z 1085 and $[M - H]^{-2}$ at m/z 542. These two compounds had previously been reported in a paper[40] as unknown ellagitannins or as putative galloyl-castalagin, with a mass of 1086 Da, but had never previously been reported in the strawberry.[24] Compound **21** (33.8 min) was in accordance with the retention time of agrimoniin previously isolated and clarified in the strawberry[15] and showed $[M - H]^{-1}$ at m/z 1869 and a $[M - H]^{-2}$ at m/z 934, giving a molecular mass of 1870. Compound **23** (t_R 37.5 min) showed a $[M - H]^{-1}$ ion at m/z 939 and fragments at m/z 483 (loss or 3 galloyl moieties), 301, 169. On the basis of these observations, compound **23** was identified as pentagalloyl-glucose. Compound **24** (t_R 38.7 min) showed a $[M - H]^{-2}$ ion at m/z 1401 and fragments at m/z 933, 783, 633, 481, 301. This compound, with a monoisotopical mass of 2804, was identified as an isomer of lambertianin C on the basis of the double charged signal and may be formed by the polymerization of agrimoniin with another galloyl-bisHHDP-glucose. Compound **26** (t_R 41 min) showed a $[M - H]^{-1}$ ion at m/z 2019 and $[M - H]^{-2}$ at m/z 1009, giving a molecular mass of 2020 Da, with fragments at m/z 1567, 1235, 933, 783, 633, 301. This compound was identified as an unknown ellagitannin, as already reported.[41]

Ellagic Acid and Ellagic Acid Glycosides. Ellagic acid and related glycosides can be distinguished in UV–vis by their characteristic spectra at 260 and 360 nm as compared to ellagitannin spectra. In the literature, only a few ellagic acid glycosides have been reported in strawberries.[14,17,41]

Compound **16** (t_R 26.2) showed an intense $[M - H]^{-1}$ ion at m/z 301 and fragments at m/z 257 and 229, typical of ellagic acid. The compound was confirmed with the injection of the pure standard. Compound **17** (t_R 26.7) showed a $[M - H]^{-1}$ ion at m/z 447 and fragment at m/z 300. The compound was tentatively identified as an ellagic acid deoxyhexose.[14,40] Compound **25** (t_R 39 min) showed a $[M - H]^{-1}$ ion at m/z 461 and fragments at m/z 315 and 300. This compound was identified as methyl ellagic acid rhamnoside after isolation from

the woodland strawberry and structural elucidation carried out using HRMS and NMR.

Qualitative Variability in the Ellagitannin Profile. Variability in the composition was observed in different cultivars (Table S5 in the Supporting Information). The variability was clearly not due to analytical errors, since it was confirmed in all 12 individual analyses for each genotype.

The quantitative and qualitative differences between cultivars are shown in the results of cluster analysis of the composition of berries at the same stage of maturity. The results of cluster analysis were similar for stages 3 and 4, while the separation was limited using the data for unripe samples (stage 1). The example of cluster analysis at stage 3 (ripe) is reported in the hierarchical tree plot of Figure 3, while at stages 2 and 4 in
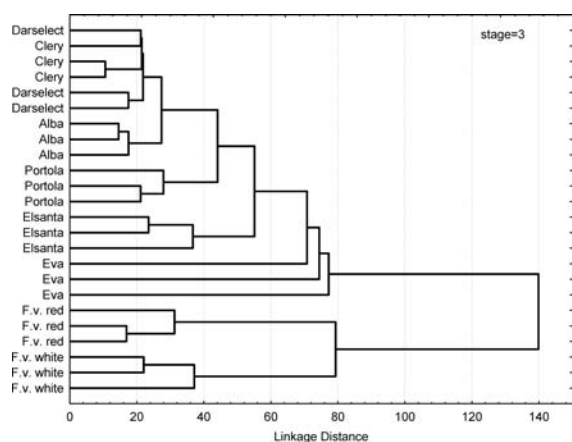

Figure 3. Cluster analysis based on the concentration of 26 different ellagitannins and ellagic acid conjugates plus their total content in strawberry cultivars and woodland strawberry types at stage 3 (ripe). The same figure at stages 2 and 4 is given as Supporting Information.

Figure S2 in the Supporting Information. The separation of the samples was due to the qualitative and quantitative differences in the presence and concentration of 26 different ellagitannins and ellagic acid conjugates, described below.

The two *Fragaria vesca* genotypes clustered apart both at the ripe and overripe stages, due to higher concentrations of ellagitannins, and the two genotypes were well separated, due to their different profile, while Darselect, Clery, Alba, and Portola appeared as nearest neighbors on the opposite site of the cluster. The Elsanta and Eva cultivars were intermediate, the latter in particular being closer to the *Fragaria vesca* samples, possibly in the light of a relatively high concentration of ellagitannins at full ripeness. Such grouping remained qualitatively comparable even at stage 2, with the peculiarity that at veraison the samples of Eva were the most distant among all the studied genotypes, including the *F. vesca*.

The two groups of *Fragaria × ananassa* cultivars and *Fragaria vesca* accessions were qualitatively different, since as many as five different ellagitannins (**4**, **7**, **10**, **13**, **20**) and methyl ellagic acid rhamnoside (**25**) were exclusively found in the two *Fragaria vesca* genotypes.

In the red woodland strawberry, 22 of the 26 compounds were present, with the exception of compounds **6** (castalagin isomer), **17** (ellagic acid deoxyhexose), **24** (lambertianin C like), and **26** (unknown ellagitannin). The ellagitannin profile was less complex in the white woodland strawberry. Indeed, when comparing the two types, red and white, four ellagitannins were absent in the white genotype: compounds

2 (sanguisorboyl-HHDP glucose), **5** (castalagin isomer), **8** (sanguiin H-2 isomer), and **22** (galloyl-castalagin isomer). The latter four compounds (**2**, **5**, **8**, **22**) were also not detected in the berries of all six *Fragaria × ananassa* cultivars.

In strawberries, ellagic acid and 10 ellagitannins (compounds **1**, **3**, **9**, **11**, **12**, **14**, **15**, **18**, **19**, **21**) were present in all the cultivars/accessions included in the study. Specifically, the main ellagitannins, casuarictin and agrimoniin, were present in all of them. It was more interesting to observe the variability in the cultivars, where some ellagitannins were either absent or present in only one of them. Ellagitannin **26** was present only in the Elsanta and Clery cvs. Compound **23** (pentagalloyl-glucose) was found only in the berries of the cv. Clery. Furthermore, compound **6**, a castalagin isomer, was detected in a single cultivar of *Fragaria × ananassa*, Portola; and compound **17** was present in all the *Fragaria × ananassa* cultivars with the exception of Alba, while it was not detected in the woodland strawberries. Only the 2 cultivars Darselect and Eva showed the same qualitative profile in terms of ellagitannins and ellagic acid glycosides.

Quantitative Variability in the Concentration of Ellagitannin and Ellagic Acid Conjugates. Quantification of ellagitannins as the equivalent of other commercially available compounds, such as gallic acid or ellagic acid, may reveal the inadequacy of the approach. Molar extinction factors of 10600[49] and 58200[15] M^{-1} cm^{-1}, respectively, at a 1 to 5 ratio were observed at 260 nm in methanol for gallic acid and agrimoniin. This ratio is related to the number of chromophores present in the molecules, and better quantification will be possible with an equivalent compound more similar to the analytes. The same can be observed in relation to the quantification of casuarictin, with a gallic acid ratio of 1 to 3. Consequently, correct quantification of ellagitannins can only take place with proper reference compounds. To support this conclusion in Table S6 in the Supporting Information we list other ellagitannins with a comparable number of chromophores (ellagic acid and gallic acid moieties) and their ratio between molar extinction factors and molecular weight.

The content of ellagitannins and ellagic acid conjugates in strawberries was found to be rather variable, ranging from 84.8 to 1636 mg/kg, with an average of 637 mg/kg, depending on the species, cultivar, and ripening stage (Table 2). It is worth taking a closer look at these values, since this is the first attempt to give an accurate quantitative estimate of their individual concentrations.

Considering the amounts at ripeness, the average results are substantially in accordance with one of the recent papers on strawberry and ellagitannin quantification in their native form, at least in terms of the order of magnitude,[50] although we would emphasize that it is not entirely accurate to compare quantitative data expressed as equivalent of ellagic acid for all the ellagitannins with our data, in which quantification was carried out using isolated ellagitannin standards.

The 11 ellagitannins and ellagic acid conjugates found in all strawberry samples (Table 2) showed a range of concentrations and average concentration in fruits respectively in the following decreasing order: agrimoniin, **21** (25.0−747 mg/kg; 223 mg/kg); casuarictin, **9** (19.0−386 mg/kg; 100 mg/kg); sanguiin H-6 isomer, **12** (1.5−133 mg/kg; 40.2 mg/kg); sanguiin H-6 with a galloyl mojety, **18** (6.4−95.2 mg/kg; 40.0 mg/kg); unknown ET, **11** (3.9−95.8 mg/kg; 30.4 mg/kg); lambertianin C without one ellagic acid mojety, **19** (2.2−82.6 mg/kg; 28.6 mg/kg); pedunculagin, **1** (5.7−108 mg/kg; 27.3 mg/kg); ellagic acid, **16**

Table 2. Quantification of Ellagitannin and Ellagic Acid Conjugates at Different Ripening Stages in Different Strawberry Cultivars and Woodland Types[a]

		1 peduncalagin	2 unknown ET	3 strictinin	4 castalagin	5 castalagin isomer	6 castalagin isomer	7 digalloyl-HHDP-glucose	8 sanguiin H-2	9 casuarictin	10 sanguiin II-10	11 unknown ET	12 sanguiin H-6 isomer	13 sanguiin H-2 isomer	14 lambertianin C	15 sanguiin II-6	16 ellagic acid	17 ellagic acid deoxyhexose	18 sanguiin H-6 with galloyl moiety	19 lambertianin C without ellagic moiety	20 galloyl-castalagin	21 agrimoniin	22 galloyl-castalagin isomer	23 pentagalloyl glucose	24 lambertianin C like	25 methyl ellagic acid rhamnoside	26 unknown ET	total amount
	tR	4.6	6.1	8	9.9	13.4	14	17.4	18	18.4	19.5	21.1	22.9	23.3	24.8	25.8	26.2	26.7	28.4	30.7	32.5	33.8	35.2	37.5	38.7	39	40.7	
Strawberry cultivar																												
Darselect	green	29.7	nd	12.5	nd	nd	nd	nd	nd	266	nd	56.4	80.4	nd	11.9	17.7	15.9	21.3	70.2	66	nd	419.6	nd	nd	78.8	nd	nd	1146.5
	sd	1.2		1.5						31		13.8	6.6		4.1	4.6	2.7	3.4	17	18.3		90.3			18.3			212.8
	veraison	13.6	nd	4.9	nd	nd	nd	nd	nd	112.1	nd	22.6	40.1	nd	3.6	6	8.4	10	40.3	21.1	nd	156.2	nd	nd	23.6	nd	nd	462.7
	sd	3.9		0.5						5.3		1.1	2.3		0.8	0.7	1	0.6	1.6	1.2		19.6			0.9			39.7
	ripe	11.6	nd	4	nd	nd	nd	nd	nd	87.1	nd	17.9	24.1	nd	1.8	8.2	5.5	7	31.7	15	nd	136.4	nd	nd	18.8	nd	nd	369.3
	sd	1.5		0.3						7.5		2	2.2		0.8	7.5	0.5	0.9	1.9	1.5		13.8			2.2			42.6
	overripe	9.9	nd	4.3	nd	nd	nd	nd	nd	68.9	nd	14.9	20.6	nd	2.3	3.8	6.4	7.2	21.9	12.7	nd	111	nd	nd	13	nd	nd	296.9
	sd	0.6		2.5						8.5		1.9	1.9		0.4	0.2	1.1	1	7.9	2.2		14.4			3.2			45.8
Alba	green	20	nd	6.5	nd	nd	nd	nd	nd	84.5	nd	26.2	31.1	nd	5.9	5.7	18.7	nd	36.3	26.9	nd	132	nd	nd	35.7	nd	nd	429.5
	sd	4.6		2.4						22.7		6.7	3.5		2.8	2.4	6.5		10.8	7.7		33.9			11.5			115.3
	veraison	19.8	nd	4.6	nd	nd	nd	nd	nd	78.3	nd	32	33.8	nd	7.1	7.1	23.8	nd	47.8	29.6	nd	163	nd	nd	36.3	nd	nd	483.1
	sd	4.2		1.9						15.3		7.7	7.1		1.4	1.3	4.6		9.1	6.4		32.9			9.8			101.7
	ripe	16.1	nd	4.2	nd	nd	nd	nd	nd	52.9	nd	13.5	18.2	nd	3.4	3.9	17	nd	27.1	13.8	nd	78.2	nd	nd	20.3	nd	nd	268.8
	sd	1.8		1.3						7.7		3.3	1.8		0.1	0.1	1.8		0.5	0.7		9.8			0.9			29.8
	overripe	8.3	nd	2.2	nd	nd	nd	nd	nd	28.3	nd	5.4	4.6	nd	0.7	1.3	11.2	nd	12.1	5.4	nd	38.1	nd	nd	8	nd	nd	125.4
	sd	2.1		0.3						6.2		1.3	2.6		0.1	0.4	1.3		5.4	2.8		12.5			5.4			40.6
Portola	green	24.7	nd	10.2	nd	nd	3.6	nd	nd	242.2	nd	46.7	119.4	nd	13.5	12.9	17	15.5	38.3	52.5	nd	217.1	nd	nd	54.6	nd	nd	868.2
	sd	2		1.3			0.8			24.6		8.5	12.3		1.3	1.9	2.4	1.4	6	9.5		42.9			6.9			121.6
	veraison	18.1	nd	5.6	nd	nd	2.3	nd	nd	136.1	nd	31.9	63.8	nd	7.6	8.1	12.5	12.4	28.3	31.4	nd	132.7	nd	nd	28.7	nd	nd	519.4
	sd	3.1		1			0.6			10.2		5.1	6.8		1.3	0.9	1.8	1	3.5	3.2		12.1			2.3			53
	ripe	10.8	nd	4.6	nd	nd	1.8	nd	nd	102.2	nd	20.4	36.2	nd	4.6	5.3	10.8	8.8	17	14.5	nd	60.6	nd	nd	13.4	nd	nd	311
	sd	2.4		2			0.6			18.3		4.8	8.3		2	1.7	1.3	2.1	3	1.9		5.6			2.9			56.9
	overripe	8.4	nd	3.5	nd	nd	1.1	nd	nd	77	nd	14.2	26.1	nd	2.3	2.9	8.5	7.4	10.5	7	nd	42.6	nd	nd	7.5	nd	nd	219.1
	sd	1.4		0.6			0.8			17.1		3	5.4		0.7	0.8	1.3	1.6	2	1.3		8.7			2			46.7
Eva	green	25.9	nd	7.6	nd	nd	nd	nd	nd	322	nd	63.6	94.6	nd	12.9	11.5	15.3	19.2	42.6	48.7	nd	346	nd	nd	56.2	nd	nd	1066
	sd	2.1		1.9						61.2		30.4	29		2.3	4.4	4.2	4.5	10.5	14.2		100			18.3			283
	veraison	29.1	nd	9.7	nd	nd	nd	nd	nd	265.5	nd	55.3	80.9	nd	9	12.6	17.8	20.2	46.7	44	nd	286.7	nd	nd	45.8	nd	nd	923.4
	sd	7.7		2.6						33.4		11	15.3		1.5	2.1	4	4.1	9.2	7.3		31			8.2			137.5
	ripe	20.8	nd	6.4	nd	nd	nd	nd	nd	202.8	nd	38.3	58.7	nd	5.7	7.9	14.2	14.6	39	28.7	nd	190.8	nd	nd	41.1	nd	nd	668.7
	sd	4		2.1						41.6		14.3	21.5		3	3.2	1	1.5	10.4	10.3		52.4			12.4			177.8
	overripe	12.8	nd	2.9	nd	nd	nd	nd	nd	154.3	nd	26.3	41	nd	5.2	6	12.4	11.1	31.1	19.1	nd	138.3	nd	nd	31.1	nd	nd	491.6
	sd	1.3		0.2						10.7		3.4	3.8		0.2	0.9	2.4	2.3	4.5	3.5		31.7			2.8			67.6
Clery	green	38.6	nd	13.2	nd	nd	nd	nd	nd	210.4	nd	47.2	83.7	nd	10.3	10.8	16.3	19.4	66.6	51.1	nd	294.5	nd	28.4	84.7	nd	19.1	994.6
	sd	6.4		1.9						32.2		8.2	16.3		1.9	2	2.6	3.9	11.1	10		70.9		5.9	19.5		6.8	199.7
	veraison	20.3	nd	5.1	nd	nd	nd	nd	nd	112.3	nd	27.5	38.1	nd	5.4	6	13	12.3	38.9	25	nd	151.2	nd	13.2	36.2	nd	9.6	514.1
	sd	0.6		0.3						14.8		1.4	3.6		1.4	0.8	2.4	1.1	3.4	2.7		22.1		1.7	5.3		1.9	63.6
	ripe	16.1	nd	3.8	nd	nd	nd	nd	nd	81.5	nd	18.7	27.7	nd	3.9	4.7	12.9	10.3	29.6	15.1	nd	101.5	nd	7.4	21.1	nd	6.6	361
	sd	1.9		0.5						8.2		0.5	1.6		0.3	0.5	3.4	0.7	2	2.6		10.4		0.9	1.8		1.2	36.4
	overripe	12.3	nd	2.7	nd	nd	nd	nd	nd	66.7	nd	14.7	17.6	nd	2.8	3.4	11.6	10.8	26.6	12.4	nd	98.9	nd	6.3	15.6	nd	7.2	309.6
	sd	4.8		0.9						20.1		7.3	10.4		1.8	1.8	1.8	2.5	7.9	6.8		25.6		3.4	6.9		3.1	105.2
Elsanta	green	20.6	nd	6.8	nd	nd	nd	nd	nd	56.1	nd	33.3	49.2	nd	6.4	9.5	14.9	14	37.8	33.4	nd	406.1	nd	nd	36.9	nd	16.8	741.9
	sd	6.4		2.5						12.3		6.4	13.8		1.2	1.3	2.3	2.9	6.6	5.7		82.9			12.7		5.2	162.2
	veraison	13.4	nd	4.4	nd	nd	nd	nd	nd	40.3	nd	24.1	34.1	nd	4.8	7	12.8	10.8	27.7	22.6	nd	284.2	nd	nd	23.8	nd	10.1	520.1
	sd	1.6		0.2						3.3		2	4.5		0.6	0.9	2.3	1.9	1.9	1.4		37.9			0.8		0.3	59.4
	ripe	7.5	nd	2	nd	nd	nd	nd	nd	21.4	nd	12.2	14.9	nd	0.9	2.6	7.4	6	15.3	9.6	nd	146.6	nd	nd	10.8	nd	5.5	262.5
	sd	1		0.4						3.1		2.1	3.2		0.5	0.3	1.8	0.8	2.7	2.5		29.4			2.5		1.2	51.5
	overripe	6.4	nd	1.6	nd	nd	nd	nd	nd	21.1	nd	12.8	14.6	nd	2.1	3.4	7.4	5.9	15.3	10.4	nd	139.9	nd	nd	10.1	nd	5.4	256.2
	sd	0.6		0.4						1.7		1.4	1.2		0.8	0.9	1.1	0.8	1.8	1.3		8.8			0.8		0.6	22.1
Woodland strawberries																												
Red type	green	75.4	14	34.4	28.7	7.4	nd	14.5	11.1	53.6	14.1	59	35.2	52.6	16.1	20.8	66.7	nd	76.4	46.4	49.4	442.6	54.4	22.3	nd	86.7	nd	1281.9
	sd	19.1	3.1	19.9	4.4	1.1		1.6	0.5	8.7	1.9	8.3	6.1	12.6	1.6	2	10		14.8	16	10.7	83.7	10.3	7.1		26.3		269.9
	veraison	81.7	19.2	39.1	24	7.4	nd	15.1	11.3	47.7	13.7	45.9	30.4	35.7	13.1	16.7	58.3	nd	67.3	42.1	40.5	305.2	39	17.7	nd	91.4	nd	1062.4
	sd	23.5	5.6	13.1	6.4	2		4.3	3.2	10.8	4.1	8.7	5.9	6.6	1.2	2.4	7.7		15.1	13	4.4	77.5	4.4	5.9		37.2		263
	ripe	64.7	15.5	33.3	20.7	4.9	nd	13.5	7.6	33.7	11.7	33.3	24.2	23.7	8.7	13.5	52	nd	56.1	41.6	33.7	244.1	35.7	15.5	nd	71.6	nd	859.3
	sd	5.3	2	5.5	2.1	1.6		4.2	3	1.6	0.6	1.4	0.5	3.5	1.8	2.8	3.1		5.4	4.4	4.1	15.4	2.2	2.3		3.5		76.1
	overripe	46.5	13.4	29.5	19.4	3.8	nd	15.9	8.1	37.3	11.8	44.3	25.6	25	10.9	15.6	67.1	nd	59	41.8	43	264.9	43.7	18.9	nd	68.1	nd	913.7
	sd	8	4	5.4	3.6	1.6		6.9	5.1	11.8	6.4	6	6.8	10	3.9	4.5	6.6		11.2	7.5	8.1	36.6	7.1	3		15.4		179.7
White type	green	64.8	nd	33.1	23.6	nd	nd	9.7	nd	47.1	13.4	40.8	40.4	57.5	9.7	15.2	67.9	nd	72.6	44.7	39.9	625.9	nd	33.9	nd	55.3	nd	1295.5
	sd	15.7		13.4	5.3			4.6		18.1	6.7	13.9	15.3	11.4	3.5	4.7	30.2		20.7	9.1	15	121.8		12.9		12.1		334.4
	veraison	48.9	nd	37	21.1	nd	nd	7.2	nd	34.3	11.9	29.5	30.1	41.6	7.5	10	50.3	nd	62.5	29.8	31.1	408.8	nd	21.6	nd	46.9	nd	930.3
	sd	9.1		14.2	3.2			2.6		7.1	3	3.4	2.4	9.1	1.4	4.9	5.5		2.9	2.4	2.2	16		0.8		15		105.1
	ripe	42.2	nd	23	15.7	nd	nd	6.5	nd	28.8	11.1	24.4	24.4	27.1	7.2	6.6	41.7	nd	47.1	27.9	24.4	307.9	nd	13.9	nd	41.3	nd	719.5
	sd	9.6		0.6	1			3.7		4.1	1.2	5.9	2.4	1.2	1	1.5	1.4		2.8	3.2	8.7	18.8		3.5		1.4		72.1
	overripe	34.7	nd	19.6	13.5	nd	nd	3.7	nd	33.6	9.9	21.3	23.4	28.3	5.8	10.2	49.5	nd	41.6	24.1	19.7	279.7	nd	18.7	nd	55.3	nd	697.1
	sd	7.5		2.6	1.8			3.7		13.7	1	3.3	1	2	1.5	2.8	1.4		4.4	1.8	2.8	16.5		0.2		4.6		69.6

[a]The values reported are the mean value of the three biological replicates in mg/kg of fresh weight fruit and their respective standard deviations (sd).

(4.9−97.8; 23.9 mg/kg) strictinin, **3** (1.2−54.2 mg/kg; 12.0 mg/kg); sanguiin H-6, **15** (0.8−23.0 mg/kg; 8.6 mg/kg); lambertianin C, **14** (0.3−17−9 mg/kg; 6.7 mg/kg).

In conclusion, agrimoniin was confirmed to be the main ellagitannin in strawberries, as suggested by Vrhovsek et al.,[15] and casuarictin, for the first time reported as a natural constituent of strawberries, was the second compound in terms of quantitative importance among ellagitannins in *Fragaria × ananassa* fruits alone, reaching a concentration as high as that of agrimoniin in some cultivars, such as Eva, Portola, and Darselect (Table 2).

Of the ellagitannins specific to *Fragaria × ananassa*, only the lambertianin C-like ellagitannin, **24**, was found to be among the main ellagitannins (range 2.3−98.8 mg/kg, average 31.3 mg/kg), while **6**, **17**, and **26**, when present, were minor constituents (Table 2).

The *Fragaria vesca* samples were in general richer in ellagitannins and ellagic acid conjugates (range 658−1636 mg/kg, average 970 mg/kg), once again having agrimoniin as the main ellagitannin, and with a significant presence and at similar levels as **25**, **18**, **1**, and **16** (Table 2). In particular, the compound 3-O-methyl ellagic acid 3′-O-α-rhamnopyranoside, **25** (33−132 mg/kg, average 64.6 mg/kg), was found to be the main ellagic acid conjugate in *Fragaria vesca* samples.

Concentration of Ellagitannins and Ellagic Acid Glycosides in Different Cultivars and at Different Stages.
It is well-known from the literature that different strawberry cultivars differ in terms of their phenolic composition and concentration, but few papers have described the ellagitannin profile and content in detail.[6,14,20,50,51] Moreover the influence of fruit maturity on the content of ellagitannins in their native form in soft fruits is not particularly well documented. An interesting paper published by Aaby et al.[14] studied the influence of three stages of maturity within the phase considered suitable for consumption on the concentration of different polyphenol classes, including ellagitannins, in three *Fragaria × ananassa* cvs. The changes in ellagitannins observed in this study were ambiguous and marginal as compared to important changes in anthocyanins and hydroxycinnamates.

Our results for quantitative HPLC-DAD analysis of ellagitannins are reported in Table 2, which gives the means, with standard deviations, for each of the 26 ellagitannins in the 3 biological replicates. The variability observed is relatively reasonable, considering that these were biological (not technical) replicates, which were treated as separate individual samples from plant growth up to harvest, sample extraction, and analysis.

Changes in the Amount and Pattern of Ellagitannins During Fruit Ripening.
The combined effects of the two genotype and ripening stage factors were analyzed using multivariate ANOVA analysis for repeated measures on 27 variables. These included the individual concentration of 26 different ellagitannins and ellagic acid conjugates in the 3 batches of fruit from the 6 *Fragaria × ananassa* cultivars and two *Fragaria vesca* analyzed at four ripening stages, for a total of 96 observations.

Both the genotype and stage factors and the interaction factor (genotype × stage) were found to be highly significant, as shown in Table S7 in the Supporting Information. It is important to note that the genotype had a bigger influence than the ripening stage.

Figure 4 highlights the general trend, namely, a huge drop in the mean total concentration of ellagitannins during the early
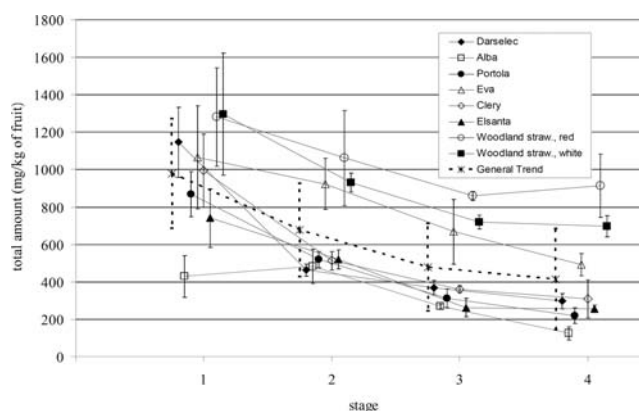

Figure 4. Behavior of total ellagitannins and ellagic acid conjugates during the ripening stages for strawberry cultivars and woodland strawberry types and general trend as an average of all samples, per fresh weight. Error bars refer to the standard deviation of the 3 biological replicates. The symbols are slightly split to avoid any overlapping of error bars. Stages are numbered accordingly to 1, green; 2, veraison; 3, ripe; 4, overripe.

phase of berry ripening (stages 1, 2, 3), common to all genotypes, with more limited changes during the two final stages (3, 4). The average total concentration of ellagitannins remaining in the berry at maturity (stage 3) was half that measured in green fruit (stage 1). The huge drop in ellagitannins is comparable with results reported in some other papers in which a strong decrease was evaluated using colorimetric assay[52] or ellagitannin hydrolysis.[53] However, the use of these two strategies meant that any molecular background or individual variability was lost. Only a few ellagitannins were reported and described individually during fruit development by Fait et al.,[24] but not quantified and expressed only in terms of relative peak response area.

Indeed, the overall effect was consequently a major drop in the concentration of ellagitannins. This was caused by a significant drop in most of the ellagitannins, including the major peaks in Figure 2 (casuarictin **9**, agrimoniin **21**, and the isomer of lambertianin C, **24**) as well as other ellagitannins with intermediate concentrations (peaks 1, 4, 11, 12, 14, 15, 17, 18, 19), which all showed similar behavior. However, this behavior was not general. Observing the univariate results (data not reported), some of the ellagitannins found only in *Fragaria vesca* (**2**, **7**, **8**, **10**), as well as ellagic acid **16** and methyl ellagic acid rhamnoside **25**, did not show any statistically significant changes in their concentration with the ripening stages.

Ellagitannins are generally synthesized early during fruit development and tend to decline in terms of fresh weight during the ripening stages, as described for the first time in the strawberry by Cheng and Breen,[54] considering the mechanism for regulating anthocyanin and phenolic production. Subsequently, the biosynthetic processes in strawberry development were also described by comparing the levels of gene transcript and enzyme activity, while a biosynthetic decline in terms of metabolite levels as the one observed in our study was reported so far only for ellagic acid.[51]

We observed a significant effect of the genotype × stage interaction factor, which was shown to be variable in terms of the drop in ellagitannins during fruit ripening. Figure 4 also shows the drop in total ellagitannin content for the different genotypes. Another paper observed a strong genotype variability in terms of bioactive compounds in strawberry but

comparing the environmental effect and not the fruit development.[55] The two *Fragaria vesca* genotypes showed a less pronounced drop, which was partly explained by a relatively large amount of ellagic acid and methyl ellagic acid rhamnoside. Of the *Fragaria × ananassa* cultivars, Eva retained a higher concentration of ellagitannins at ripening, being similar to Clery and Darselect at stage 1, but retaining a higher amount of ellagitannins at stages 3 and 4.

To conclude, we observed major qualitative and quantitative differences in the amount and profile of ellagitannins and ellagic acid conjugates in *Fragaria × ananassa* and *Fragaria vesca* species, as well as several qualitative differences in some minor ellagitannins in the *Fragaria × ananassa* cultivars. This information suggests that the ellagitannin profile could also be interesting for characterizing cultivars.

The working hypothesis derived from this study is therefore that genotype is a major factor in defining ellagitannin concentration and pattern in strawberries, and that in the context of a major drop in ellagitannins during ripening, variable behavior of the genotypes still exists, which could also be considered in order to retain the optimal concentration of ellagitannins in fruit at the ripening stages most suitable for consumption.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figure S1: ^{1}H NMR spectrum in CD3OD of casuarictin. Figure S2: Cluster analysis based on the concentration of 26 different ellagitannins and ellagic acid conjugates plus their total content in strawberry cultivars and woodland strawberry types, respectively at stages 2 (veraison) and 4 (overripe). Table S1: Analytical parameters used for quantitative analysis. Table S2: Summary of variety kinship, provenance and agronomical parameters. Table S3: Average weight of Elsanta cultivar strawberries at different stages. Table S4: Summary of agronomical analysis. Table S5: Ellagitannin and ellagic acid conjugates profiling of different strawberry cultivars and woodland strawberry types using UPLC-Q-TOF. Table S6: Molar extinction factors reported in the literature for some ellagitannins, in relation to their molecular weight and characteristic number of chromophore residues. Table S7: Multivariate tests of significance for the multivariate repeated measures ANOVA aimed to evaluate the influence of the two genotype and ripening stage factors on the concentration of ellagitannins and ellagic acid conjugates in strawberries (sigma-restricted parametrization; effective hypothesis decomposition). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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