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# Clarifying the Identity of the Main Ellagitannin in the Fruit of the Strawberry, *Fragaria vesca* and *Fragaria ananassa* Duch.

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Supporting Information

**ABSTRACT:** Although the composition of strawberry fruit has been extensively studied, especially for the most abundant phenolic compounds, agrimoniin has never been univocally identified as one of the most abundant phenolic compounds in the fruit. In this study agrimoniin was isolated in the fruit of *Fragaria vesca* and its structure characterized. Furthermore, its presence was definitively established to be the main ellagitannin in both *F. vesca* and *Fragaria ananassa* D. fruit. The presence of sanguiin H-6 and lambertianin C as minor compounds was confirmed in both *F. vesca* and *F. ananassa* D. samples. For the first time here is reported the full NMR assignments for agrimoniin. These data should represent a point of reference for NMR analysis of this and other structurally related ellagitannins. Finally, the establishment of an HPLC protocol for separation provided information making it possible to avoid confusion with sanguiin H-6, the main ellagitannin in *Rubus* species, which is also present in strawberries but at a much lower concentration.

**KEYWORDS:** *Fragaria, strawberry, ellagitannins, agrimoniin, NMR, mass spectrometry, circular dichroism, UV*

## INTRODUCTION

Of the most commonly consumed berries, strawberries (*Fragaria ananassa* Duch.) are the most popular choice with consumers, being eaten both fresh and frozen, as well as in different processed products such as desserts, juice, nectar, puree, jam, syrup, and wine. Strawberries are indeed one of the most important crops worldwide. The statistical database of the Food and Agriculture Organization of the United Nations[1] reports a global production area of 254 523 ha distributed over 77 countries on all of the continents in 2009, with estimated worldwide production of 4 178 152 tons. The major production areas are located in Europe (1 353 149 t), North America (1 289 882 t), eastern Asia (401 990 t), North Africa (332 239 t), and western Asia (326 548 t). With a rounded figure of 600 g per year theoretically available for each of the 7 billion inhabitants on our planet, strawberries are the most commonly consumed berries and one of the most important sources of polyphenols in the human diet, with a strong influence on human health.[2−6]

The nutritional quality of strawberries is correlated to the presence of soluble sugars, organic acids, amino acids, vitamins, and important secondary metabolites such as polyphenols.[7] Strawberries are a rich source of bioactive compounds, including proanthocyanidins, anthocyanins, flavonols, phenolic acids, and ellagitannins.[8] Their chemical composition and putative influence on the healthy properties of the fruit have been studied.[9] The three major classes of phenolic compounds in the fruit are, in decreasing order, proanthocyanidins, anthocyanins, and ellagitannins. Proanthocyanidins and flavan-3-ols are the main classes, and a recent paper reported the profiling of different oligomeric forms and the degree of polymerization in different strawberry cultivars.[8] Anthocyanins are another important group of polyphenols responsible for fruit pigmentation and which have also been suggested contribute to the inhibition of ethanol-induced ulcers in rats fed strawberry extracts.[6]

Ellagitannins are a class of compounds present only in some fruit and nuts (e.g., strawberries, raspberries, blackberries, pomegranates, muscadine grapes, and walnuts). The major sources of ellagitannins in the Western diet are strawberries, raspberries, and blackberries.[9] Their structural complexity is a major limiting step that prevents their study at the molecular level. Due to the considerable diversity of ellagitannins, they still represent a challenge to food science and are a source of discussion in relation to their correct identification.[10] Correct identification of the structure is clearly a prerequisite for understanding their bioavailability, bioactivity, and metabolism.

The structural elucidation of ellagitannins is a difficult task, because they are made up of the same building blocks (including but not limited to glucose, ellagic and gallic acid, and hexahydroxydiphenoyl (HHDP) units), which are organized into an impressive number of different but similar structures.[11] As a consequence, many structurally related ellagitannins display characteristic, but very similar or sometimes almost identical, mass spectra.[12] This issue, together with the lack of commercially available standards, makes their accurate identification and quantitation very demanding. In the case of

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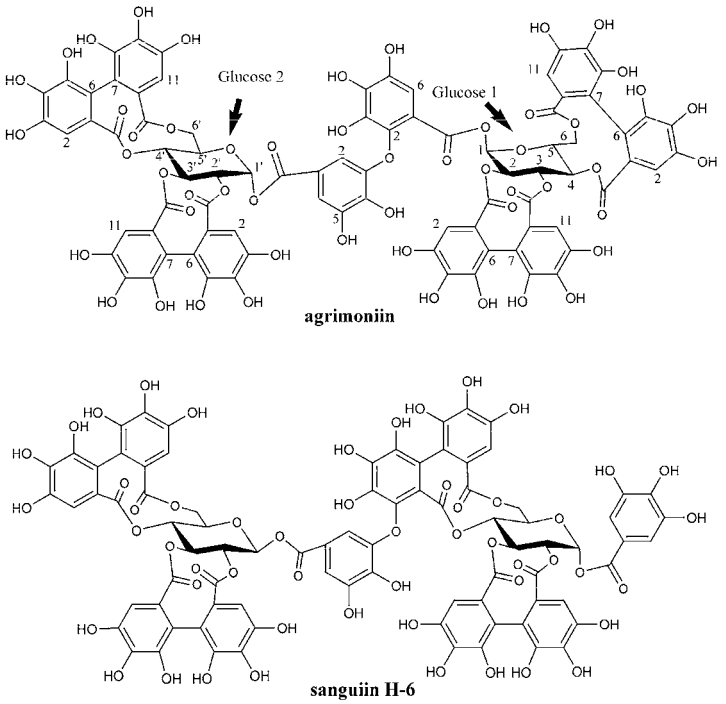


**Figure 1.** Molecular structures of agrimoniin (atom numbering used in NMR spectra assignments in Table 1) and sanguiin H-6.

strawberries, there is fundamental disagreement about the identification of the main ellagitannin in the fruit.

The ellagitannin composition of the fruit has not yet been reported, even in the most comprehensive food databases.[13] Reports on the presence of agrimoniin (Figure 1) in strawberry fruit are scarce and contradictory. In strawberry fruit the most abundant ellagitannins reported so far are lambertianin C, sanguiin H-6 (Figure 1), and galloyl-bis-HHDP-glucose.[8,14] The question of whether the major ellagitannin was agrimoniin or sanguiin H-6, because the fragmentation patterns matched both, was left open.[15] The ellagitannins from strawberries were reported to be structurally different from those of cloudberries and raspberries, with the predominance of casuarictin-like and/or potentillin-like (galloyl diHHDP glucose) structures.[16] The mass 1870 Da was observed, but the identity of the molecule was not completely assigned.[15] The presence of agrimoniin was described in green strawberry fruit.[5] In conclusion, most of the papers dealing with the identification of native ellagitannins in strawberries have reported sanguiin H-6 as the main ellagitannin, probably due to the lack of the reference standards and the similarity of the mass spectrum with agrimoniin.

Identification is more consistent in studies considering other parts of the strawberry, especially the leaves. Agrimoniin was reported to be the main soluble phenol in strawberry leaves, accounting for up to 40% of total soluble phenols[17] in strawberry leaves[18] and Fragariae folium extracts.[19] Three different isomers of agrimoniin were reported to be present in the leaves of *Fragaria* sp.[18,20,21] Agrimoniin was also found in different parts of strawberry flowers[12] and suggested to be present in both achenes and receptacles during fruit development.[22]

The purpose of this study was to isolate and characterize the structure of agrimoniin in woodland strawberries (*Fragaria vesca*) and also to confirm its presence in cultivated strawberries (*F. ananassa* D.).

## MATERIALS AND METHODS

**Standards and Solvents.** All of the chromatographic solvents were of HPLC grade or LC-MS grade for the MS experiments. Acetonitrile, acetone, methanol, diethyl ether, hexane, and formic acid 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 H-6 and lambertianin C were isolated as described previously.[23]

**Plant Material.** One kilogram of woodland strawberries (*F. vesca*) and 60 g of strawberries (*F. ananassa* D. cv. Darselect) were grown in an experimental field in Vigalzano (Trento, Italy). All of the plants were grown under the same conditions to minimize the effect of environmental and agronomic factors. Woodland strawberries and strawberries were harvested at maturity and were frozen at −20 °C and then transported to the laboratory for solvent extraction. The extraction of polyphenols was carried out as reported in Mattivi et al.[24] with an acetone/water mixture (70:30 v/v). Before extraction, the fruit and extraction solution were cooled to 4 °C to limit enzymatic and chemical reactions. Sixty grams of fresh fruit was homogenized in an 847-86 model Osterizer blender at speed 1, in 2 × 100 mL of a mixture of acetone/water (70:30 v/v) for 1 min and made up to 250 mL with the same solvent.

**Isolation of Agrimoniin from Woodland Strawberries (*F. vesca*).** Aqueous acetone strawberry extracts (1 kg of fruits extracted in 4 L of acetone/water mixture (70:30 v/v)) were evaporated until dryness in a pear-shaped flask, using rotary evaporation under reduced pressure at 37 °C. The sample was diluted to 1 L with methanol/water mixture (30:70 v/v) and filtered using a Durapore 0.45 μm filter (Millipore, Vimodrone, Italy). Isolation of agrimoniin was carried out in two consecutive steps using 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.

*Step 1.* The first step in isolation was purification of the methanolic extract for the removal of anthocyanins from the sample. After this step, the sample for isolation was made up of only the ellagitannin fraction of the woodland strawberries. Purification was done using Sephadex LH-20 and carried out with a slightly modified version of our protocol.[23] In particular, the purification method was changed to

scale-up the size. A column cartridge (10 × 4 cm) was packed with Sephadex LH-20 resin, connected to a vacuum line to speed elution, prewashed with 50 mL of methanol, and equilibrated with 100 mL of methanol/water (30:70 v/v). An aliquot of 50 mL of the aqueous methanol berry extract was loaded, and anthocyanins were washed off with 500 mL of methanol/water (30:70 v/v). The yellowish fraction containing the ellagitannins was eluted from the cartridge using 350 mL of acetone/water (70:30 v/v). The ellagitannin fraction was combined together for each purification step and then dried using rotary evaporation under reduced pressure at 37 °C and reconstituted in 50 mL of methanol for the next isolation step.

*Step 2.* The chromatographic isolation of agrimoniin was performed using preparative HPLC with a 250 × 50 mm, 10 μm, Discovery HS C18 column (Supelco, Bellefonte, PA, USA). The column was protected by using a 2 μm PEEK filter (Gilson, Milano, Italy). The sample obtained from the purification step (50 mL in methanol) was evaporated using rotary evaporation under reduced pressure at 40 °C, reconstituted with water (2 L), and loaded into the column using a solenoid valve. The injection volume with the solenoid valve was 44 mL for each consecutive injection. The mobile phases were distilled water (solvent A) and acetonitrile (solvent B). The column was conditioned for 10 min with 10% B. Separation was achieved using a linear gradient from 10 to 30% B in 120 min, at a flow rate of 35 mL/min, and the elution was monitored in UV at 260 nm. The peaks of interest were manually collected in a separate flask, checking the chromatographic run and their retention times for each injection before the fractions were pooled. After separation, agrimoniin was dried using rotary evaporation under reduced pressure and then dissolved in the smallest possible volume of methanol, diluted with diethyl ether and hexane. The pure isolated compound (ca. 200 mg) was recovered by filtration and precipitation from *n*-hexane as an amorphous pale rose powder, which was further characterized by NMR and MS. When heated in a Kofler melting point microscope (model Reichert Thermovar, USA), it showed decomposition at temperatures >200 °C, leading eventually to a gummy dark material at 250 °C.

**NMR.** NMR spectra ($^1$H NMR, COSY, NOESY, HSQC, and HMBC) for agrimoniin were recorded in both hexadeuterated acetone (99.90% $CD_3COCD_3$) and tetradeuterated methanol (99.90% $CD_3OD$) at 298 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 ($\delta$) were calibrated on the residual signal of protonated acetone at $\delta_H$ 2.040 and $\delta_c$ 29.80 for spectra taken in $CD_3COCD_3$ and on the residual protonated signal of methyl group at $\delta_H$ 3.310 and $\delta_c$ 49.00 for spectra taken in $CD_3OD$.

Molecular mechanics calculations were carried out by the computer program GMMX as implemented in PCMODEL 7.0 (PCMOD 7.0/GMMX version 1.5, Serena Software, Bloomington, IN, USA).

**UV and Circular Dichroism (CD).** The UV spectra of agrimoniin were recorded both in methanol and in ethanol, on a Hitachi U-2000 spectrometer (Tokyo, Japan). The following molar extinction coefficients were observed: in methanol, $\varepsilon_{260\ nm}$ = 58178 $M^{-1}$ $cm^{-1}$; in ethanol, $\varepsilon_{260\ nm}$ = 67508 $M^{-1}$ $cm^{-1}$.

The CD spectra of agrimoniin 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 ($\lambda$) were observed: Θ = +4.9 × $10^5$ (240 nm), Θ = −1.8 × $10^5$ (264 nm), Θ = +1.3 x$10^5$ (284 nm), Θ = −4.5 x$10^4$ (310 nm).

**Mass Spectrometry.** Separation was carried out with a Waters Acquity UPLC system equipped with a UV−vis Waters PDA (Waters Corp., Milford, MA, USA) under the same conditions described for HPLC analysis. Detailed compound characterization was carried out using a Waters HDMS-QTOF Synapt mass spectrometer with electrospray ionization system (ESI) and MassLynx 4.1 software. 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.

## RESULTS AND DISCUSSION

**Isolation of Agrimoniin.** Agrimoniin was the first dimeric hydrolyzable tannin isolated from a plant, *Agrimonia pilosa*.[25] In our study the isolation of agrimoniin was performed from *F. vesca* fruits. After purification of the extract on a Sephadex resin with the scope of eliminating anthocyanins, the compounds were isolated using preparative HPLC. Figure 2 shows good separation of agrimoniin from other compounds in a preparative chromatographic run. The retention time for agrimoniin was 65 min in these elution conditions. Good separation allowed relatively straightforward isolation of this compound. After chromatographic isolation, a good grade of precipitation was required for further characterization of the compounds isolated. The protocol used was the same as the one developed for the main *Rubus* ellagitannins.[23] The solution with the isolated compound was dried, dissolved in the smallest possible volume of methanol, and then diluted with diethyl ether. In the same way, hexane was then also added, but in much larger volumes. As a result of this and its insolubility in hexane, the solution quickly formed precipitate of the pure compound. The final agrimoniin yield obtained from 1 kg of *F. vesca* was ca. 200 mg, with a purity of 98%, as assessed by NMR.

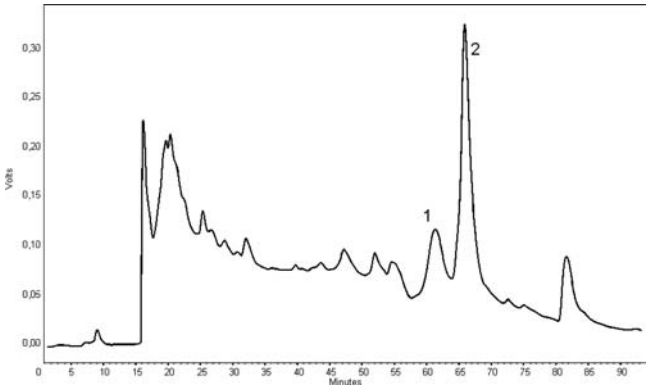


**Figure 2.** Chromatogram of preparative HPLC-DAD for isolation, detected at 260 nm. Peaks: 1, ellagic acid (Rt 60 min); 2, agrimoniin (Rt 65 min).

**Structural Elucidation of Agrimoniin by NMR and CD.** Heteronuclear, $^3J$ (H, C) optimized, direct (HSQC) and long-range correlation (HMBC)-2D NMR measurements allowed us to assign and to report here for the first time (see Table 1) all of the $^1$H and $^{13}$C NMR resonances of agrimoniin. Because only partial descriptions of the NMR agrimoniin spectra are present in the literature,[25,26] these data should represent a point of reference for NMR analysis of other structurally related ellagitannins.

Due to the structural resemblance of agrimoniin with sanguiin H-6[23] (Figure 1), the two structures share the same general NMR features, namely, the presence in their corresponding $^1$H NMR spectra (Table 1; Figure 3) of sharp singlets attributable to protons on HHDP groups and meta-coupled doublets for protons on galloyls in the aromatic region, besides the presence, at higher field, of a series of sugar multiplets. On the other hand, distinctive NMR resonances and possibly different glucose−proton *J* coupling patterns were promptly detected, due to the specific structural differences

**Table 1. NMR (400 MHz, 298 K) Assignments for Agrimoniin in $CD_3COCD_3$**

| moiety | carbon no. | $^1$H NMR $\delta_H$ | $^{13}$C NMR $\delta_C$ |
|---|---|---|---|
| α-glucose 1 | 1 | 6.56 (d, $J_{1,2}$ = 3.9) | 90.6 d |
| | 2 | 5.35 (dd, $J_{1,2}$ = 3.9, $J_{3,2}$ = 9.5) | 73.8 d |
| | 3 | 5.48 (dd, $J_{3,2}$ = 9.5, $J_{3,4}$ = 10.2) | 75.6 d |
| | 4 | 5.15 (t, $J_{4,3} \approx J_{4,5}$ = 10.2) | 68.7 d |
| | 5 | 4.48 (dd, $J_{4,5}$ = 10.2, $J_{5,6}$ = 6.2) | 71.0 d |
| | 6 | 3.68 (d, $J_{gem}$ = 13.2) | 62.9 t |
| | | 5.23 (dd, $J_{gem}$ = 13.2, $J_{5,6}$ = 6.2) | |
| α-glucose 2 | 1′ | 6.54 (d, $J_{1',2'}$ = 4.1) | 90.8 d |
| | 2′ | 5.36 (t, $J_{1',2'}$ = 4.1 $J_{3',2'}$ = 9.5) | 73.7 d |
| | 3′ | 5.54 (dd, $J_{3',2'}$ = 9.5, $J_{4',3'}$ = 10.2) | 75.7 d |
| | 4′ | 5.19 (t, $J_{4',3'} \approx J_{4',5'}$ = 10.2) | 68.8 d |
| | 5′ | 4.64 (dd, $J_{6'5'}$ = 6.5, $J_{4',5'}$ = 10.2) | 70.7 d |
| | 6′ | 3.78 (d, $J_{gem}$ = 13.3) | 62.9 t |
| | | 5.30 (d, $J_{gem}$ = 13.3, $J_{6'5'}$ = 6.5) | |
| 2-O,3,4,5-trihydroxybenzoate on O−C(1) of glucose 1 | 1 | | 114.6 s |
| | 2 | | 137.5 s |
| | 3 | | 140.1 s |
| | 4 | | 140.9 s |
| | 5 | | 143.4 s |
| right part of DHDG | 6 | 7.29 s | 109.6 d |
| | 1-OC═O | | 162.8 s |
| 3-O,4,5-dihydroxybenzoate on O−C(1″) of glucose 2 | 1 | | 125.4 s |
| | 2 | 7.38 (d, $J_{2,6}$ = 2.0) | 112.1 d |
| | 3 | | 146.5 s |
| | 4 | | 140.9 s |
| | 5 | | 148.1 s |
| | 6 | 6.93 (d, $J_{2,6}$ = 2.0) | 108.2 d |
| left part of DHDG | 1′-OC═O | | 164.9 s |
| 2,3 HHDP on glucose 1 | 1 | | 126.6 s |
| | 2 | 6.43 s | 107.0 d |
| | 3 | | 145.1 s |
| | 4 | | 136.4 s |
| | 5 | | 144.9 s |
| | 6 | | 114.7 s |
| | 7 | | 114.2 s |
| | 8 | | 144.9 s |
| | 9 | | 136.1 s |
| | 10 | | 145.0 s |
| | 11 | 6.33 s | 107.1 d |
| | 12 | | 126.6 s |
| | 2-OC═O | | 168.3 s |
| | 3-OC═O | | 169.3s |

| moiety | carbon no. | $^1$H NMR | $^{13}$C NMR (HSQC and HMBC) |
|---|---|---|---|
| 4,6 HHDP on glucose 1 | 1 | | 125.8 s |
| | 2 | 6.60 s | 107.7 d |
| | 3 | | 145.1 s |
| | 4 | | 136.6 s |
| | 5 | | 144.4 s |
| | 6 | | 116.1 s |
| | 7 | | 115.7 s |
| | 8 | | 144.4 s |
| | 9 | | 136.3 s |
| | 10 | | 145.1 s |
| | 11 | 6.65 s | 108.1 d |
| | 12 | | 126.1 s |
| | 4-OC═O | | 167.7 s |


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**Table 1. continued**

| moiety | carbon no. | $^1$H NMR | $^{13}$C NMR (HSQC and HMBC) |
|---|---|---|---|
| | 6-OC═O | | 168.1 s |
| 2′,3″ HHDP on glucose 2 | 1 | | 126.6 s |
| | 2 | 6.55 s | 107.2 d |
| | 3 | | 145.1 s |
| | 4 | | 136.5 s |
| | 5 | | 144.1 s |
| | 6 | | 114.9 s |
| | 7 | | 114.2 s |
| | 8 | | 144.1 s |
| | 9 | | 136.1 s |
| | 10 | | 145.0 s |
| | 11 | 6.34 s | 107.1 d |
| | 12 | | 126.5 s |
| | 2′-OC═O | | 168..3 s |
| | 3′-OC═O | | 169.2 s |
| 4′,6′ HHDP on glucose 2′ | 1 | | 125.7 s |
| | 2 | 6.59 s | 107.7 d |
| | 3 | | 145.1 s |
| | 4 | | 136.6 s |
| | 5 | | 144.3 s |
| | 6 | | 116.1 s |
| | 7 | | 115.6 s |
| | 8 | | 144.3 s |
| | 9 | | 136.3 s |
| | 10 | | 145.1 s |
| | 11 | 6.64 s | 108.1 d |
| | 12 | | 126.1 s |
| | 4′-OC═O | | 167.7 s |
| | 6′-OC═O | | 168.0 s |

between these two dimeric ellagitannins. First of all, both the glucose moieties in agrimoniin are involved in $\alpha$ relative configurations, whereas in sanguiin H-6 glucose 2 presents a $\beta$ linkage to its acetal center. In the corresponding $^1$H NMR spectra (Table 1), this is reflected by the presence of identical coupling constants ($J \sim 4$ Hz) for acetal protons at both the glucose units in agrimoniin and quite different $J$ values for the corresponding protons in sanguiin H-6 (4 Hz for glucose 1 and 8 Hz for glucose 2). A significant solvent effect on these acetal protons is shown by the $^1$H NMR spectrum of agrimoniin in methanol (Figure 4B) in comparison with acetone (Figure 4A) . Curiously, it shifts them in opposite directions, leading to an upfield effect on the H−C(1) of glucose 1 and a downfield effect on H−C(1′) of glucose 2. This outcome is worth noting, because it allows good spectroscopic resolution even for spectra obtained using low-field NMR instruments. It is important to note here that the assignment of acetal protons in ellagitannins is a *conditio sine qua non* for correctly assigning the relative configurations of the chiral centers. Another striking structural/biogenetic difference between agrimoniin and sanguiin H-6 is the different linking unity between monomers, this being a dehydrodigalloyl group (DHDG) in the former and a sanguinsorboyl group in the latter. As a consequence, the small NMR differences detected in agrimoniin at its corresponding structural sites are due only to the asymmetry imposed by the central DHDG.

Two sets of resonances for ester groups are present in agrimoniin, one set at $\delta_C$ about 167−169, attributable to −COO groups linked to HHDP moieties, and a second set at $\delta_C$ about 163−165 for −COO groups linked to DHDG linking groups. The shielded values for the latter can be explained by taking into account the fact that these two carboxyl groups should assume a conformation wherein they are almost coplanar with the corresponding aromatic rings (galloyl), thus allowing better conjugation and leading to an overall upfield effect. For carboxyl groups linked to HHDP units, the adoption of this conformation is strongly hindered by the conformational constraints imposed by the carbon−carbon bond linking the two aromatic rings in the HHDP moiety. As previously reported for sanguiin H-6,[23] molecular mechanics (MM) calculations carried out through extended geometry optimization of agrimoniin (Figure 5) confirm that the dihedral angle formed by −C═O with the HHDP aromatic rings in all the groups is about 50°, whereas it is much lower (∼20°) in both the galloyls embedded in the DHDG linking unit. More importantly, MM calculations strongly suggest that both 10-membered rings (defined by 2,3 junctions of HHDP) and 11-membered rings (defined by the 4,6 junction of HHDP) adopt a twisted chairlike conformation, within which the two ester carbonyl groups are in *anti* orientation (dihedral angle between the two ester O—C═O groups estimated to be ∼170°) with the diaryl units resulting heavily twisted (∼60°).

The absolute configuration of the four chiroptical HHDP groups on the $^4C_1$ glucose core was unambiguously established to be *S* by the positive sign of the strong Cotton effect at $\lambda$ 240 nm and the negative Cotton effect at $\lambda$ 266 nm.[27]

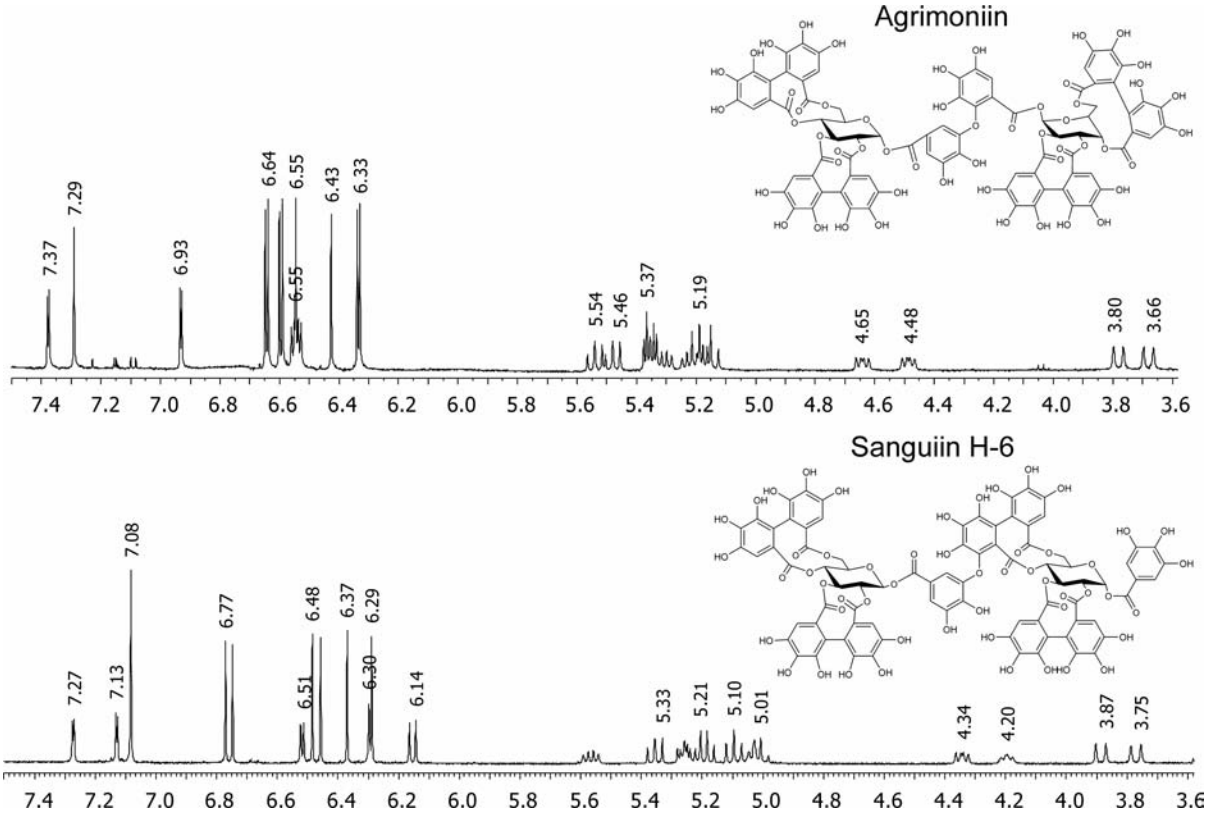


**Figure 3.** $^1$H NMR spectra in acetone-$d_6$ of agrimoniin (top) and sanguiin H-6 (bottom).

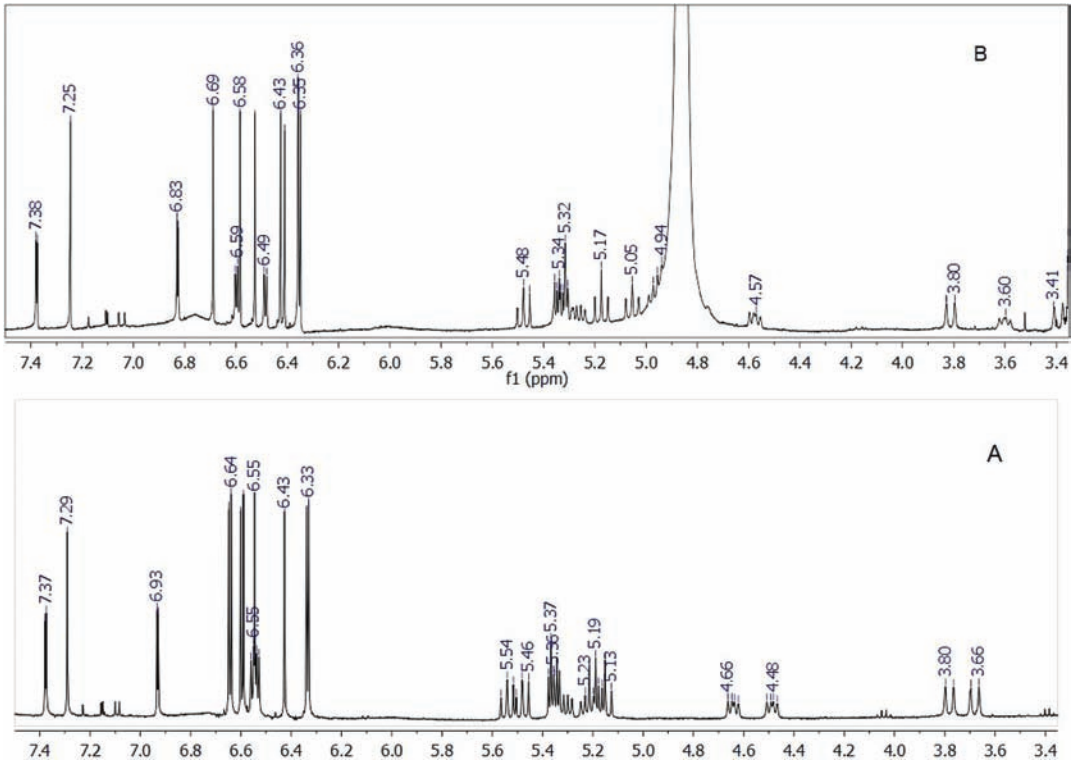


**Figure 4.** $^1$H NMR spectra of agrimoniin in (A) acetone-$d_6$ and (B) methanol-$d_4$.

**ESI(−) High-Resolution Mass Measurements and Mass Spectrum Interpretation.** Sanguiin H-6 and agrimoniin are two isomers built out of the same monomeric units, galloyl-bis-HHDP-glucose (Figure 1), and consequently their negative ion mode ESI-MS spectra are very similar (Figure 6). In both spectra the parent ion $[M - H]^-$ can be observed, for agrimoniin at $m/z$ 1869.146 and for sanguiin H-6 at $m/z$ 1869.154 (theoretical monoisotopic mass of both isomeric


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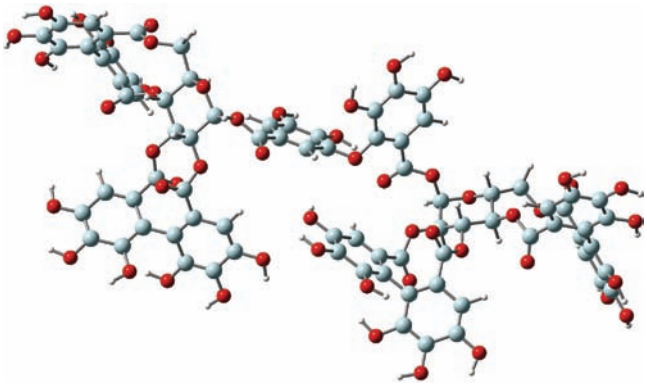

**Figure 5.** Energy-minimized structure of agrimoniin, as obtained from molecular mechanics calculations.

forms calculated for $C_{82}H_{53}O_{52}$ is 1869.151). Characteristic fragment ions (Figure 7) correspond to the loss of some common building blocks in ellagitannins (ellagic, HHDP, or HHDP-glucoside units). In particular, the low intensity ion at *m/z* 1567.151 (**1**, Figure 7) derives from the loss of one HHDP unit followed by 2H transfer from HHDP to glucose (calculated theoretical monoisotopic mass for $C_{68}H_{47}O_{44}$ of **1** is 1567.145), whereas the ion at *m/z* 935.079 (**3a**, Figure 7) must result from the break of the C−O bond linking the two monomers, thus affording the galloyl-bis-HHDP-glucose 1 negative ion (theoretical monoisotopic mass calculated for $C_{41}H_{27}O_{26}$ of **3a** is 935.079). After 2H transfer, the HHDP moiety is prone to give the free ellagic acid (**2**, Figure 7), which in ESI(−) ion mode presents itself as an anion at *m/z* 300.998 (theoretical monoisotopic mass calculated for $C_{14}H_5O_8$ of **2** is 300.999). Through the loss of an HHDP unit and 2H transfer from the HHDP toward glucose 1 unit, **3a** leads to the anion fragment **4a** at *m/z* 633.072 (theoretical monoisotopic mass calculated for $C_{27}H_{21}O_{18}$ is 633.073). Isobaric ion daughter ions (**3b** and **4b**, Figure 7) can, however, be generated along a different route through symmetrical bond breaking. Because the ESI(−)/MS fragmentation pattern of sanguiin H-6 is almost superimposable (Figure 6) to that of agrimoniin, there is a clear indication that the central diaryl ether function is the weakest

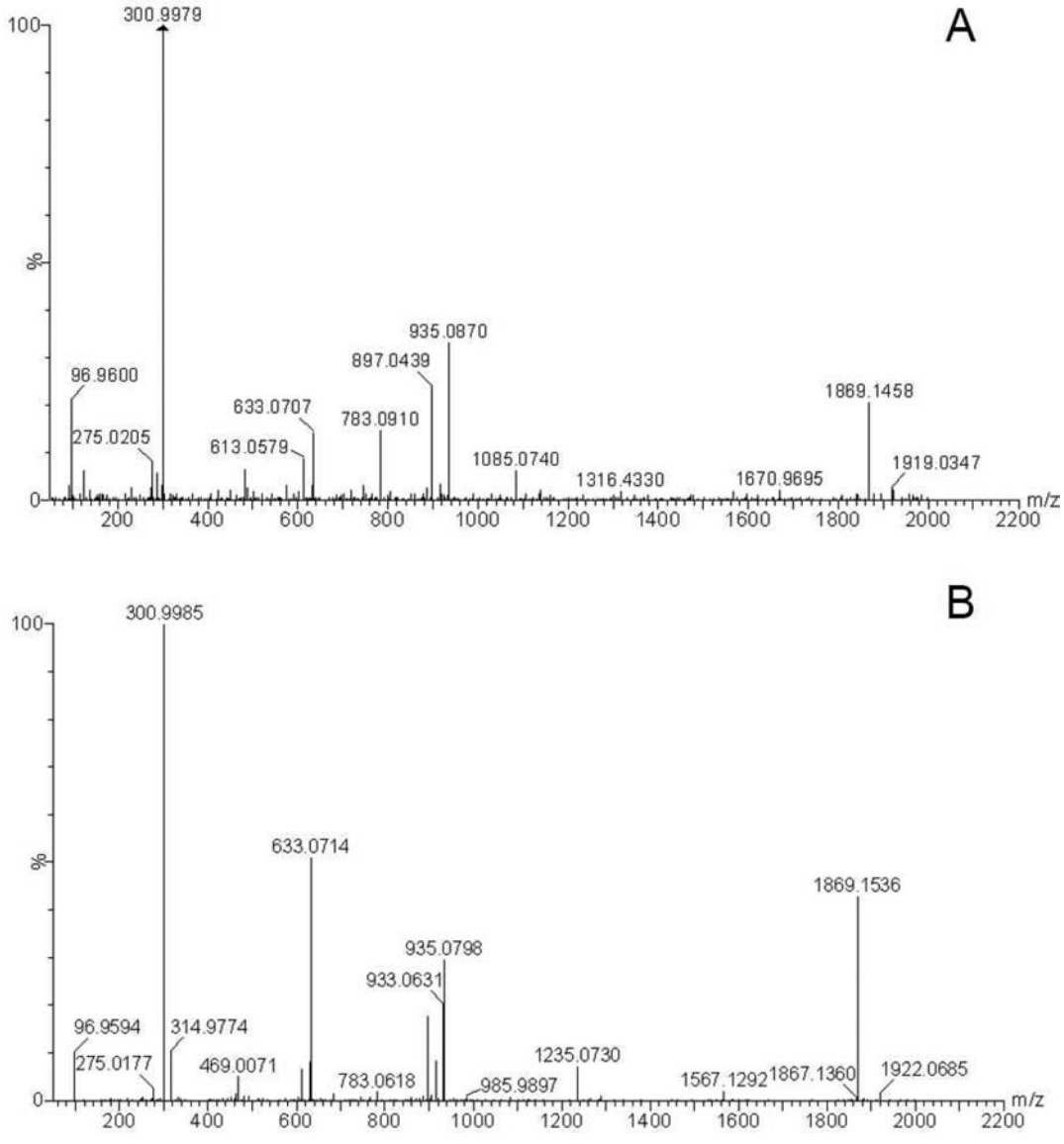


**Figure 6.** High-resolution ESI(−) mass spectra of agrimoniin (A) and sanguiin H-6 (B).


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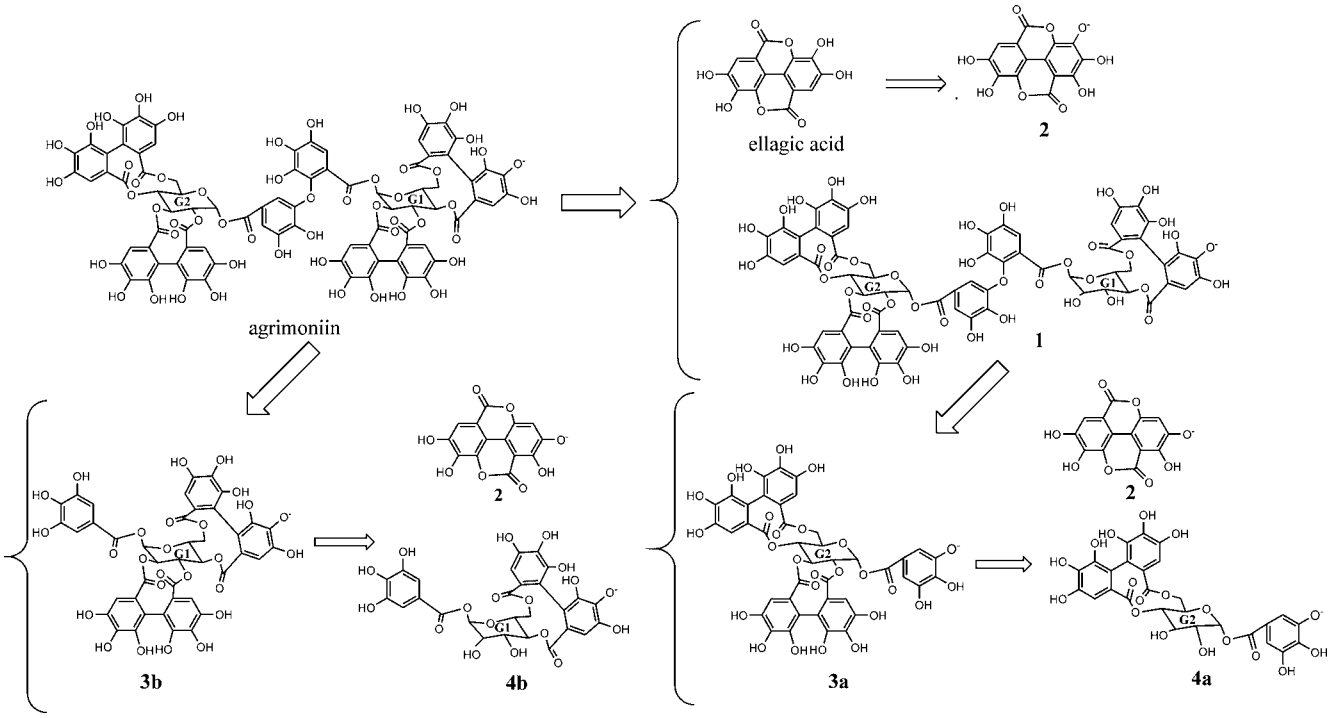


**Figure 7.** Fragmentation routes in the high-resolution ESI(−)-MS spectrum of agrimoniin.

bond in dimeric ellagitannins, thus undergoing significant dissociation in the ESI source.

By comparison of the chromatographic runs of *F. vesca*, *F. ananassa* Duch, and standard mix (Figure 8) it was demonstrated that the main ellagitannin in *Fragaria* has the same molecular mass as sanguiin H-6. However, on the basis of the retention time, it was possible to assign the main compound as agrimoniin, which elutes later during the gradient, because its polarity is considerably lower. With our separation method on a reversed phase C18 column, sanguiin H-6 elutes at 26.7 min and agrimoniin at 36.4 min, whereas the retention time of ellagic acid was 28.5 min (Figure 8). To properly and uniquely assign the identity to the compounds, the availability of true standards is crucial to compare the retention times of the two compounds. In the event of a lack of reference standards, the elution order of the compounds should be carefully evaluated to tentatively assign the main strawberry ellagitannins. We suggest that ellagic acid, widely available, should be taken as a reference on the C18 column, because sanguiin H-6 elutes before and agrimoniin after it (Figure 8).

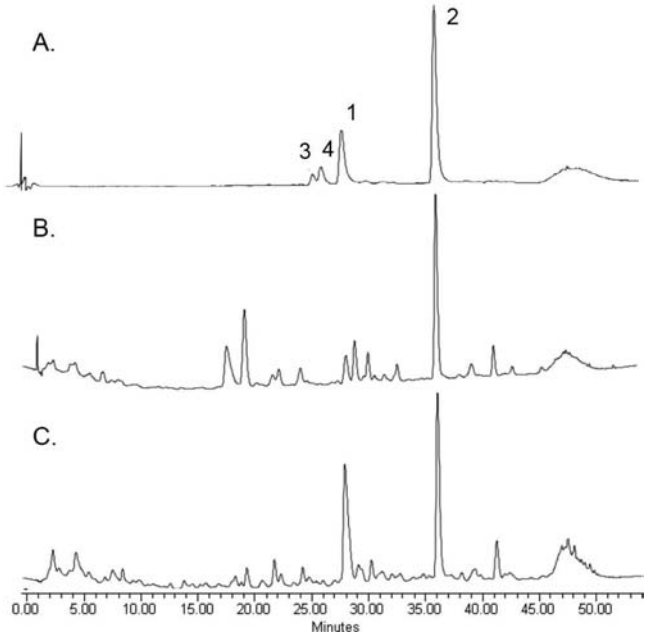


**Figure 8.** HPLC-DAD (260 nm) chromatograms: (A) ellagitannin standard mix; (B) woodland strawberry; (C) strawberry cv. Darselect. Peaks (in the standard mix): 3, lambertianin C; 4, sanguiin H-6; 1, ellagic acid; 2, agrimoniin.

After apples, strawberries were recently reported to be the fruit contributing most to polyphenol intake, estimated in the SU.VI.MAX French cohort.[28] Of fruit containing ellagitannins, strawberries are the most widely consumed, and agrimoniin is therefore expected to be one of the most widely present ellagitannins in the human diet. Due to the many healthy properties associated with ellagitannins, agrimoniin should play an important, yet still largely unexplored, role in the beneficial health effects associated with the consumption of strawberries by humans. It is known that agrimoniin has been used for treatment of diarrhea and hemorrhaging.[26,27,29] It has also been reported to have antitumor properties.[30−32]

Although the composition of strawberry fruit has been extensively studied, especially for the most abundant phenolic compounds, agrimoniin has never been unequivocally identified as one of the most abundant phenolic compounds in the fruit. To our knowledge, this is the first time that agrimoniin has been isolated and its structure characterized in the fruit of *F. vesca* and its presence reported as the main ellagitannin in both *F. vesca* and *F. ananassa* D. fruit. The presence of sanguiin H-6 and lambertianin C as minor compounds was confirmed in both *F. vesca* and *F. ananassa* D. samples.

## ■ ASSOCIATED CONTENT

### Supporting Information

Figure SM1. HSQC spectrum of agrimoniin in acetone-$d_6$. Figure SM2. HMBC spectrum of agrimoniin in acetone-$d_6$. Figure SM3. COSY spectrum of agrimoniin in acetone-$d_6$. 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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## NOTE ADDED AFTER ASAP PUBLICATION

There was an error in Figure 7 of the version of this paper published March 1, 2012. The correct version published March 2, 2012.