Hydrolyzable Tannins: Gallotannins, Ellagitannins, and Ellagic Acid

Michael Jourdes, Laurent Pouységuy, Stéphane Quideau, Fulvio Mattivi, Pilar Truchado, and Francisco A. Tomás-Barberán

CONTENTS

20.1 Introduction .................................................................................................................................. 436
  20.1.1 Occurrence in Food and Medicinal Plants ............................................................................. 437
  20.1.2 Antioxidant Activity of Hydrolyzable Tannins and EA ...................................................... 437
20.2 HPLC Analysis ................................................................................................................................ 438
20.3 UV Spectrophotometry Detection ................................................................................................. 438
20.4 Quantitative Indirect Analysis of ETs Using HPLC-DAD after Acid Hydrolysis ....................... 440
  20.4.1 Procedure ................................................................................................................................ 440
    20.4.1.1 Standards and Solvents ................................................................................................. 440
    20.4.1.2 Sampling and Extraction of Polyphenols ..................................................................... 440
    20.4.1.3 Sample Preparation ....................................................................................................... 440
    20.4.1.4 Acid Hydrolysis ............................................................................................................ 441
  20.4.2 HPLC-DAD-ESI-MS Analysis ............................................................................................... 441
    20.4.2.1 HPLC-DAD Analysis .................................................................................................... 441
    20.4.2.2 HPLC-ESI-MS Analysis ................................................................................................ 441
  20.4.3 Molar Extinction Coefficients ............................................................................................... 441
    20.4.3.1 Molar Extinction Coefficient at Maximal Absorption in Methanol ............................. 441
    20.4.3.2 Molar Extinction Coefficient at 260 nm in Methanol .................................................. 442
    20.4.3.3 Molar Extinction Coefficient for HPLC Analysis ....................................................... 442
  20.4.4 UV Quantification .................................................................................................................. 442
  20.4.5 Principle and Application of the Method .............................................................................. 442
    20.4.5.1 Presence of ETs and EACs in Rubus Extracts ............................................................... 442
    20.4.5.2 Four Products Are Obtained from ETs Using Acid Hydrolysis in Methanol .............. 442
    20.4.5.3 Detailed Composition of Blackberries .......................................................................... 443
    20.4.5.4 Data Interpretation ....................................................................................................... 443
    20.4.5.5 Computation and Interpretation of mDP ..................................................................... 445
20.5 HPLC-MS-MS Analysis ............................................................................................................ 447
20.6 NMR Analysis ............................................................................................................................... 447
  20.6.1 Gallotannins .......................................................................................................................... 447
    20.6.1.1 Isolated Gallotannins ....................................................................................................... 448
    20.6.1.2 Enzymatically and Chemically Synthesized Gallotannins ....................................... 448
  20.6.2 Ellagitannins ......................................................................................................................... 450
    20.6.2.1 Absolute Configuration of ET Axially Chiral Biaryl Groups .................................. 450
    20.6.2.2 Determination of the Position of ET Galloyl-Derived Acyl Units ............................ 451
    20.6.2.3 Determination of the Absolute Configuration of the Anomeric Carbon .................. 451
20.1 Introduction

The chemical structures of the hydrolyzable tannins are basically composed of a central sugar core, typically a glucose unit, to which gallic acid moieties are esterified. β-Glucoagallin is the simplest glucosyl gallate known and serves as a galloyl unit donor in the biosynthesis of the fully galloylated β-d-glucopyranose (β-PGG), which is itself considered to be the immediate precursor of the two subclasses of hydrolyzable tannins, that is, gallotannins and ellagitannins (ETs) (Figure 20.1) (Gross 1999, 2008; Niemetz and Gross 2005; Quideau et al. 2011). Gallotannins are the result of further galloylations of β-PGG and are characterized by the presence of one or more meta-depsidic digalloyl moieties, as exemplified with the hexagalloylgucose 3-O-galloyl-1,2,4,6-tetra-O-galloyl-β-d-glucopyranose (1a in Figure 20.1). Alternatively, β-PGG can be subjected to intra- and intermolecular
Hydrolyzable Tannins

phenolic coupling processes that create connections between spatially adjacent galloyl residues by forming C–C biaryl and C–O diaryl ether bonds. The so-called hexahydroxydiphenoyl (HHDP) biaryl unit generated by intramolecular coupling is the structural characteristic that defines hydrolyzable tannins as ETs. The nature of the atropisomeric form of these chiral biaryl motifs, such as the (S)-HHDP unit of the tellimagrandins or the (R)-HHDP unit of geraniin, is determined by the position of the galloyl motifs on the glucopyranose core in either its 4C1- or its 1C4-conformation. Besides, the HHDP motif is susceptible to many additional transformations, among which its oxidation leads to the dehydrohexahydroxydiphenoyl (DHHDP) unit characteristic of the dehydroellagitannin natural products, such as geraniin (Quideau and Feldman 1996; Khanbabaee and van Ree 2001a; Feldman 2005; Pouységou et al. 2011).

ETs release ellagic acid (EA) upon hydrolysis. This occurs spontaneously in the gastrointestinal tract under physiological conditions (Larrosa et al. 2006). In addition, free EA and its glycoconjugated derivatives with sugars are also found in most ET-containing plants.

20.1.1 Occurrence in Food and Medicinal Plants

ETs are present in significant amounts in many berries, including strawberries, red and black raspberries (Zafrilla et al. 2001), blackberries, and nuts, including walnuts (Fukuda et al. 2003), pistachios, cashew nuts, chestnuts, oak acorns (Cantos et al. 2003), and pecans (Villarreal-Lozoya et al. 2007). They are also abundant in pomegranates (Gil et al. 2000), and muscadine grapes (Lee et al. 2005), and are important constituents of wood, particularly oak wood (Glabasnia and Hofmann 2006). ETs can be incorporated into several food products such as wines, and whiskies, through migration from wood to the food matrix during different aging processes. EA has also been found in several types of honey and this phytochemical has been proposed as a honey floral marker for heather honey (Ferreres et al. 1996). Free EA and different glycoside derivatives are also present in these food products, including glucosides, rhamnoses, arabinoses, and the corresponding acetyl esters (Zafrilla et al. 2001).

In a previous review, it was documented that there were no reliable figures available on the ET dietary burden but that it would probably not exceed 5 mg/day (Clifford and Scalbert 2000). Since then, a number of studies have shown that the ET content of several food products can be quite high. A glass of pomegranate juice can provide as much as 300 mg, a raspberry serving (100 g of raspberries) around 300 mg, a strawberry serving 70 mg, and four walnuts some 400 mg of ETs. As a result, the intake of dietary ETs can be much higher than previously estimated (Clifford and Scalbert 2000), especially if some of these ET-rich foods are regularly consumed in the diet.

20.1.2 Antioxidant Activity of Hydrolyzable Tannins and EA

ET-rich foods generally show a high free-radical scavenging activity evaluated in vitro. Especially relevant is the antioxidant activity of pomegranate juice (Gil et al. 2000). This study shows that pomegranate juice has twice the antioxidant activity of red wine and that this is due to the extraction of ETs from the fruit husk during juice manufacturing (Gil et al. 2000). This remarkable antioxidant activity has been the driving power of research on the biological activity of these powerful antioxidants from pomegranate, and is used by the food industry to market pomegranate juice products as super-antioxidant food. ETs are also responsible for a relevant part of the antioxidant activity observed in strawberries (Hannum 2004), raspberries (Zafrilla et al. 2001), blackberries, walnuts (Blomhoff et al. 2006), and pecans (Villarreal-Lozoya et al. 2007). This antioxidant activity can probably be related to the biological activity reported for these food products.

In parallel to those studies of the antioxidant activity of ET-rich food, clinical studies have also shown relevant biological activities that have been associated with these antioxidants, although no direct evidence of the biological activity of these polyphenols has been demonstrated. Several clinical studies have reported relevant biological activity after the intake of ET-rich foods, especially regarding the protective effect against cardiovascular diseases and cancer.
20.2 HPLC Analysis

ETs can be analyzed by high-performance liquid chromatography (HPLC) using reversed-phase columns with methanol, acetonitrile, and water gradients. The addition of 1% of formic or acetic acid to the water solvent helps increasing the resolution of the chromatograms through the separation in sharper peaks avoiding peak tailing. In general, complex ET mixtures are observed in the extracts. Some effects of the ET structure on the chromatographic retention and elution order have been reported (Salminen et al. 1999; Moilanen and Salminen 2008). In general, the occurrence of free galloyl groups in the ET molecule increases the retention times, while the formation of an HHDP in the hydrolyzable tannin molecule decreases the retention time. The opening of the glucopyranose ring, as it happens in some C-glycosyl ETs (i.e., vescalagin, castalagin), also decreases the retention time. ETs with a cyclic sugar, and without galloylation in C-1, produce two peaks corresponding to the α- and β-anomer, while galloylated ETs at C-1 produce only one chromatographic peak. This has been reported in punicalagin and punicalin, the characteristic ETs of pomegranate, that show two peaks for each ET corresponding to both α- and β-anomer (Gil et al. 2000). Acyclic epimers having hydroxyl groups at C-1 of the glucose can be distinguished from each other since the orientation of the hydroxyl group causes vescalagin-type ETs to elute before the castalagin-type ones (Moilanen and Salminen 2008).

EA is present in nature in a free state or in combination with different sugars and forming methyl ethers. EA hexosides (glucosides), deoxyhexosides (rhamnosides), and pentosides (xylosides and arabinosides) as well as glucuronides have been reported as Phase II metabolites present in biological fluids. In addition, acetylated derivatives of EA pentosides have been reported in raspberries (Zafrilla et al. 2001). Glucuronides are the first eluting metabolites, followed by hexosides (glucosides), deoxyhexosides (rhamnosides), and pentosides (xylosides first and arabinosides). Free EA elutes after the glucosides but earlier than the acetyl pentosides. EA methyl ethers and sulfates (that are often found in biological fluids after the intake of ETs and EA) elute with longer retention times than free EA, and the retention time increases with the number of methyl ethers introduced on the EA molecule.

The chromatographic behavior of the microbial metabolites of ETs and EA, known as urolithins (metabolites related to EA in which one of the lactone rings has been removed by the colonic microbiota), follows a similar trend as EA derivatives, increasing the retention time when decreasing the number of hydroxyl groups on the urolithin nucleus, and when increasing the number of methyl ethers (González-Barrio et al. 2011). Again, the introduction of a glucuronyl conjugation decreases the retention time, while the introduction of a sulfate residue increases the retention time. In addition, the chromatographic peaks corresponding to sulfate conjugates are broader, hence decreasing the chromatographic resolution.

20.3 UV Spectrophotometry Detection

The UV spectra of the different ETs, gallotannins, and EA derivatives are easily recorded in the analysis of the extracts by HPLC-diode-array detection (DAD) analysis. The occurrence of free galloyl groups in the ET molecule produces two absorption maxima in the spectrum, one around 270–280 nm and another around 210–220 nm. The higher the number of the galloyl groups with respect to the HHDP units, the steeper is the valley between the two maxima (Salminen et al. 1999), and the maximum for BI (the band between 270 and 290 nm) appears at higher wavelengths. In HHDP-rich tannins, the valley between the two absorption maxima even disappears from the UV spectrum, as is the case of bis-HHDP-glucopyranose, and no defined maximum is observed for the absorption band around 270–280 nm (Table 20.1).

The change from cyclic to acyclic sugars also has a substantial effect on the UV spectrum. Free EA shows a UV spectrum characterized by two absorption bands at 365–380 and 253–255 nm, with a characteristic shape that allows its easy detection and identification in the UV-DAD chromatograms. In general, substitution with pentoses, hexoses, and glucuronides produces shifts of the UV
maxima to shorter wavelengths. This effect is also observed when methyl ethers are introduced on the phenolic hydroxyls, and is particularly evident when a sulfate residue is introduced as the hypsochromic shifts in wavelengths are more marked. The same effects are observed for the urolithin derivatives. Urolithin A, the main ET and EA metabolite in mammals, has a UV spectrum close to that of EA, but it is clearly distinctive to allow the unambiguous differentiation of both compounds (Figure 20.2). The same effects on the UV spectrum described for the EA conjugation are valid for urolithin conjugations (González-Barrio et al. 2011). Urolithins with different hydroxylation patterns show characteristic UV spectra that can be used for the structural differentiation of the metabolites present in biological fluids using HPLC coupled to UV detection (DAD).

**TABLE 20.1**
UV Spectra of Ellagitannins. Effect of the Occurrence of Galloyl or Hexahydroxydiphenoyl Residues

<table>
<thead>
<tr>
<th>Compound</th>
<th>BI (nm)</th>
<th>BII (nm)</th>
<th>% of BI Respect BII</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td>276</td>
<td>219</td>
<td>38</td>
<td>42</td>
</tr>
<tr>
<td>Pentagalloxy-glucopyranose</td>
<td>285</td>
<td>220</td>
<td>42</td>
<td>27</td>
</tr>
<tr>
<td>Trigalloxy-HHDP-glucopyranose</td>
<td>283</td>
<td>215</td>
<td>27</td>
<td>23</td>
</tr>
<tr>
<td>Digalloxy-HHDP-glucopyranose</td>
<td>280</td>
<td>211</td>
<td>23</td>
<td>19</td>
</tr>
<tr>
<td>Galloyxy-bis-HHDP-glucopyranose</td>
<td>279</td>
<td>214</td>
<td>19</td>
<td>–</td>
</tr>
<tr>
<td>Bis-HHDP-glucopyranose</td>
<td>270i</td>
<td>205i</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>


**FIGURE 20.2** UV spectra of (a) ellagic acid and (b) urolithin A.
20.4 Quantitative Indirect Analysis of ETs Using HPLC-DAD after Acid Hydrolysis

Acid hydrolysis is the most practical and widely employed technique used to quantify the hydrolyzable tannins present in vegetable extracts. As an example, in the case of *Rubus*, the use of both HPLC analysis after hydrolysis (Vrhovsek et al. 2008) and direct HPLC analysis (Gasperotti et al. 2010) has demonstrated that the average structure of ETs is well conserved in the different genotypes, while differing in terms of absolute concentration. This means that for routine quantification of complex ETs, the method using analysis after hydrolysis is still appropriate and able to provide useful information.

However, care must be taken to choose the appropriate solvent for extraction and the whole essay, since these natural compounds are not easy to manage, and each step (extraction, hydrolysis, HPLC analysis, and data processing) should be carefully considered in order to provide consistent data. Moreover, EA is poorly soluble and can precipitate if not properly handled, escaping detection (Törrönen 2009). As a consequence, the ET content reported in the literature is highly variable, since different extraction and acid hydrolysis conditions significantly affect the yield of EA (Törrönen 2009). Earlier studies relied on the quantification of released EA and did not consider other phenolics formed during hydrolysis, which may provide helpful information on the chemical structure of ETs.

For this purpose we describe how to perform and interpret an optimized hydrolytic procedure (6 h with 4 M HCl), which includes quantification of all the four products of hydrolysis and provides a rationale for estimating the mean degree of polymerization (mDP) of *Rubus* ETs. The approach described below is essentially as reported by Vrhovsek et al. (2006), with some minor improvements in terms of quantification, in order to include all the products of hydrolysis in the computation of the mDP. The composition of eight blackberry samples is discussed as an example of the application of this method. Such an approach may also be extended to the analysis of hydrolyzable tannins from other botanical sources, provided that sufficient information on their structure is available (Koponen et al. 2007).

20.4.1 Procedure

20.4.1.1 Standards and Solvents

All chromatographic solvents should be HPLC grade: acetonitrile, methanol, diethyl ether, hexane, formic acid, acetic acid, and hydrochloric acid. EA standard (purity ≥ 96%) and methyl gallate standard (purity ≥ 98%) are both available from Fluka (Steinheim, Germany). Sanguisorbic acid (SA) and methyl sanguisorboate are not currently available, but their concentration can be estimated by applying the available molar extinction coefficients (see Section 20.4.3).

20.4.1.2 Sampling and Extraction of Polyphenols

Freshly collected samples of blackberries (*Rubus fruticosus*) from eight different cultivars were produced under standardized conditions in the experimental fields of the Edmund Mach Foundation (Vrhovsek et al. 2008). Polyphenols were extracted from freshly collected berries following the method of Mattivi et al. (2002) in which 60 g of fresh fruit are homogenized in a model 847-86 Osterizer blender at speed one in 250 mL of acetone/water mixture (70/30 v/v) for 1 min. Prior to extraction, the fruit and extraction solution should be cooled to 4°C to limit enzymatic and chemical reactions. The centrifuged extracts can be stored at −20°C until analysis, conditions under which the composition remains stable for a few months.

20.4.1.3 Sample Preparation

An aliquot (20 mL) of the extract is evaporated to dryness in a 100 mL pear-shaped flask by rotary evaporation under reduced pressure at 40°C. The sample is then brought back to 20 mL with methanol immediately prior to processing, due to the limited solubility of EA and its derivatives.
20.4.1.4 Acid Hydrolysis

A 6 h hydrolysis with 4 M HCl at 85°C has been shown to provide the maximal yield for the four hydrolysis products of Rubus ETs and has also been reported to be appropriate for strawberry extracts (Vrhovsek et al. 2006; Mertz et al. 2007). In order to carry out acid hydrolysis in 4 M HCl, 16.6 mL of 37% HCl are added to the sample prepared as above and the mixture is then diluted to 50 mL with methanol. After hydrolysis, the sample is brought back to its initial volume (50 mL) with methanol. An aliquot (10 mL) is then adjusted to pH 2.5 with 5 N NaOH and diluted to 20 mL with methanol. Finally, an aliquot (2 mL) is filtered with 0.22 μm, 13 mm polytetrafluoroethylene (PTFE) syringe-tip filters (Millipore, Bedford, MA) and transferred into LC vials for HPLC analysis.

20.4.2 HPLC-DAD-ESI-MS Analysis

20.4.2.1 HPLC-DAD Analysis

HPLC analysis before and after hydrolysis was carried out according to Vrhovsek et al. (2006) using a Waters 2690 HPLC system equipped with Waters 996 DAD (Waters Corp., Milford, MA), and Empower Software (Waters). Separation is carried out using a 250 × 2.1 mm i.d., 5 μm, endcapped reversed-phase Purospher Star column (Merck) and 4 × 4 mm, 5 μm, Purospher precolumn. The solvents are: A (1% formic acid in water) and B (acetonitrile). The gradients are as follows: from 0% to 5% B in 10 min, from 5% to 30% B in 30 min. The column is then washed with 100% of B for 2 min and equilibrated for 5 min prior to each analysis. The flow rate is 0.8 mL/min, the oven temperature set at 40°C, and the injection volume is 10 μL. EA and its derivatives are detected and quantified by UV detection at 260 nm. EA (RT = 30.8 min) is quantified following calibration with an EA standard (concentration range of 10–200 mg/L). Methyl sanguisorboate (RT = 34.6 min) and free SA (RT = 25.1 min) are quantified following calibration with the pure standard isolated according to Vrhovsek and co-workers (2006). Methyl gallate (RT = 21.8 min) is quantified following calibration with the corresponding standard compound within the concentration range 3–30 mg/L.

20.4.2.2 HPLC-ESI-MS Analysis

Detailed compound identification was carried out using the Micromass ZQ electrospray ionization-mass spectrometry (ESI-MS) system (Micromass, Manchester, UK). The mass spectrometry (MS) detector operated at capillary voltage 3000 V, extractor voltage 6 V, source temperature 105°C, desolvation temperature 200°C, cone gas flow (N₂) 30 L/h, and desolvation gas flow (N₂) 450 L/h. The outlet of the HPLC system was split (9:1) to the ESI interface of the mass analyzer. ESI-mass spectra ranging from m/z 100 to 1500 were taken in negative mode with a dwell time of 0.1 s. The cone voltage was set in scan mode at the values of 20, 40, and 60 V. Typical ions for the ESI-MS detection in negative mode are: EA, molecular ion at m/z 301; SA, molecular ion at m/z 469 and main fragment at m/z 301; methyl sanguisorboate, molecular ion at m/z 483 and main fragments at m/z 315 and m/z 301; and methyl gallate, molecular ion at m/z 183.

20.4.3 Molar Extinction Coefficients

Care must be taken in choosing the appropriate standard and comparing data obtained using different methods. Here we summarize a comprehensive list of the experimental values of the molar extinction coefficients reported in the literature (Vrhovsek et al. 2006; Gasperotti et al. 2010) and expressed as M⁻¹ cm⁻¹.

20.4.3.1 Molar Extinction Coefficient at Maximal Absorption in Methanol

In methanol, at maximal absorption, EA: ε₂₅₄ₙₘ = 40,704, ε₃₆₅ₙₘ = 8066; methyl sanguisorboate: ε₂₅₄ₙₘ = 58,543, ε₃₇₁ₙₘ = 13,022; methyl gallate: ε₂₇₄ₙₘ = 11,818.
20.4.3.2 Molar Extinction Coefficient at 260 nm in Methanol

In methanol, at 260 nm, EA: \( \varepsilon_{260\,\text{nm}} = 32,099 \), sanguinin H-6: \( \varepsilon_{260\,\text{nm}} = 72,070 \), lambertianin C: \( \varepsilon_{260\,\text{nm}} = 104,344 \).

20.4.3.3 Molar Extinction Coefficient for HPLC Analysis

In the conditions suggested for HPLC analysis with UV detection (Section 20.4.2), the molar extinction coefficient is as follows: EA: \( \varepsilon_{260\,\text{nm}} = 35,822 \) (solvent: 21% of acetonitrile in 1% formic acid in water; v/v), methyl sanguisorboate: \( \varepsilon_{260\,\text{nm}} = 45,114 \) (23.9% of acetonitrile in 1% formic acid in water; v/v).

In the slightly different conditions suggested for HPLC analysis by Gasperotti et al. (2010), where the separation is adapted to a C18 Luna column (solvent: 88% of acetonitrile and 12% of 1% formic acid in water; v/v): EA: \( \varepsilon_{260\,\text{nm}} = 28,266 \), sanguinin H-6: \( \varepsilon_{260\,\text{nm}} = 63,615 \), lambertianin C: \( \varepsilon_{260\,\text{nm}} = 95,744 \).

20.4.4 UV Quantification

To encourage the application of this method, overcoming the lack of a methyl sanguisorboate standard, the molar absorbivity of pure standards of methyl sanguisorboate and EA, measured at the optimal wavelength for UV detection in HPLC analysis, can be exploited. The ratio of the molar absorbivity at \( \lambda = 260 \) nm of methyl sanguisorboate vs. EA is 1259. This value is in agreement with the presence of three and two galloyl units in methyl sanguisorboate and EA, respectively, and has been found to be consistent with the experimental response of the two compounds in the HPLC analysis conditions reported above (Vrhovsek et al. 2006).

20.4.5 Principle and Application of the Method

20.4.5.1 Presence of ETs and EACs in Rubus Extracts

The 13 structures of Rubus ETs thus far described (Gasperotti et al. 2010) are in agreement with the assumption that Rubus oligomeric ETs contain only the sanguisorbyl linking ester group, besides the well-known EA and gallic acid moieties. All known Rubus oligomeric ETs share a common structure, originating in C–O oxidative coupling. More specifically, the linking unit in Rubus ETs comes from the donation of galloyl hydroxyl oxygen to form an ether linkage to an HHDP group, which produces the class of GOD-type ETs (Okuda et al. 2009). Blackberries were reported to contain on average 1080 mg/kg of ETs and 200 mg/kg of ellagic acid conjugates (EACs). Lambertianin C (Figure 20.3) is the main ET in blackberries, with an average lambertianin C/sanguinin H-6 ratio of 1.7 (range 0.9–3.4). It must be underlined that besides the 13 known ETs, Rubus extracts contain at least five other minor compounds, whose structures are still unknown (Gasperotti et al. 2010).

20.4.5.2 Four Products Are Obtained from ETs Using Acid Hydrolysis in Methanol

The presence of EA and one or two unidentified compounds with absorbance spectra very similar to that of EA after acid hydrolysis of red raspberry and strawberry samples has been reported by some authors (Rommel and Wrolstad 1993; Mattila and Kumpulainen 2002; Määttä-Riihinen et al. 2004). More recently, Vrhovsek et al. (2006) demonstrated the formation of methyl gallate, methyl sanguisorboate, and a minor unknown EA derivative, named “derivative 1,” during hydrolysis, in addition to EA. On the basis of UV and MS data already reported (Vrhovsek et al. 2006) and further confirmation by accurate MS and MS/MS, the latter was shown to be SA.

The updated scheme of the reaction is shown in Figure 20.3. Oligomeric ETs, such as in the example of lambertianin C, do not release only ellagic acid and methyl gallate. Also the sanguisorbyl linking ester groups are hydrolyzed, yielding methyl sanguisorboate as the main product, only a limited fraction
Hydrolyzable Tannins

of sanguisorbic unit escapes esterification and can be found after hydrolysis in its free form. Four compounds are quantified by HPLC after the hydrolysis.

20.4.5.3 Detailed Composition of Blackberries

Table 20.2 gives the quantitative composition of eight blackberry samples, analyzed using the HPLC-DAD method of Gasperotti et al. (2010). EA and ETs were quantified using UV detection at 260 nm. EA and its conjugates were quantified following calibration with EA standard. Sanguin H-6 and lambertianin C were quantified following calibration with the pure standard, and other ETs were quantified as equivalents of sanguin H-6. For each known structure, Table 20.2 also gives the theoretical number of the three moieties (ellagic, gallic, and SAs), that should be released from complete hydrolysis.

20.4.5.4 Data Interpretation

Under these conditions, the amount of EA measured after hydrolysis not only derives from the breakdown of ETs, but also includes free EA and the product of the hydrolysis of EA glycosides, usually present in Rubus extracts (Gasperotti et al. 2010). The interference of free EA with the assay can be avoided by using a blank analysis of the sample before hydrolysis. However, this makes it necessary to double the number of HPLC analyses and is therefore not usually performed. Other EACs, such as the methyl-EA glycosides (i.e., peaks 25 and 26 in Table 20.2), are expected to release the different isomers of methyl-EA, which do not interfere with the ET estimate, since under the suggested conditions they elute as separate peaks (two peaks with molecular ion at m/z 315 in the case of blackberries) after methyl sanguisorboate.

FIGURE 20.3 Updated scheme for hydrolysis, which accounts for the presence of oligomers. Besides ellagic acid and methyl gallate, the sanguisorbyl linking ester groups are released mainly as methyl sanguisorboate. A limited fraction of sanguisorbic unit escapes esterification and can be found after hydrolysis in its free form.
### TABLE 20.2

Quantification of Ellagitannins and Ellagic Acid Conjugates in Blackberries, Expressed in mg/kg, Quantified Using the Direct HPLC-DAD Method Suggested

| Cultivar        | 1   | 2   | 3   | 4   | 5   | 6   | 9   | 10  | 13  | 14  | 15  | 16  | 17  | 18  | 19  | H-2 | C   | ETs | EACs |
|-----------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Apache          | 14.2| 10.0| 13.6| 11.0| 10.6| 13.3| 155.3| 26.2| 34.9| n.d.| 36.2| 163.1| n.d.| 9.5 | 16.1| 205.1| 152.5| 74.1| 86.3| 68.4| 872 | 229 |
| Black satin     | 19.0| 9.1 | 18.7| 19.1| 12.3| 14.7| 213.9| 25.3| 42.5| n.d.| 9.0 | 16.7 | 222.4| 10.4| 12.8| 18.0 | 353.0| 324.9| 45.6| 75.6| n.d.| 1342| 121 |
| Cacak           | 13.9| 10.2| 17.9| n.d.| n.d.| 14.1| 83.4 | 28.6| n.d.| 10.2| 42.8| 95.3 | 8.9 | 15.2| n.d.| 671.7 | 256.2| 61.9| 96.9| 53.3| 1268| 212 |
| Hull tornless   | 29.6| 10.3| 27.3| n.d.| 10.1| 17.5| 99.5 | 20.5| 28.7| n.d.| 9.3 | 19.5 | 97.9 | 14.5| 19.2| 20.5 | 559.2| 550.5| 103.5| 108.7| 69.0| 1534| 281 |
| Kotata          | 16.8| 9.7 | 24.2| n.d.| 15.4| 164.3| n.d.| 24.8| n.d.| n.d.| 179.5| n.d.| 11.1| n.d.| 671.5 | 257.6| 85.0| 93.1| 90.4| 1375| 269 |
| Lochness G      | 17.2| 9.0 | 16.4| 13.6| 16.3| 15.4| 149.8| 36.2| 21.3| 8.6 | 11.2| 26.2 | 149.4| 11.9| 12.0| 10.8 | 299.7| 292.5| 50.4| 73.4| n.d.| 1118| 124 |
| Lochtay         | 15.7| 10.6| 21.8| n.d.| 12.5| 111.8| 14.0| 23.0| n.d.| 18.1| 132.5| 9.5 | 13.3| 10.9| 756.1 | 354.4| 68.3| 102.2| 79.2| 1504| 250 |
| Triple crown    | 16.8| 11.4| 20.9| n.d.| 11.5| 81.8 | 30.0| n.d.| 17.9| 57.1| 91.2 | 9.2 | 14.8| 10.9| 615.7 | 279.4| 72.7| 115.4| 39.3| 1269| 227 |
| Molecular size  | 2   | 2   | 3   | ?   | 2   | 3   | ?   | 2   | ?   | 3   | ?   | ?   | 3   | 2   | 3   | 1   | 3   | 2   | 0   | 0   | 0   |
| n° of ellagic   | 2   | 3   | 3   | 2   | 3   | 2   | 3   | 2   | 3   | 3   | 2   | 3   | 1   | 4   | 3   | 1   | 0   | 0   | 0   | 0   |
| acid units      | 1   | 0   | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 0   | 0   | 0   | 0   |
| n° of gallic     | 1   | 1   | 2   | 1   | 2   | 1   | 3   | 2   | 2   | 2   | 1   | 2   | 1   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
| acid units      | n° of sanguisorbic acid units | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |

It should be highlighted that the absolute values obtained with the two independent methods, that is, indirect HPLC-DAD measurement of total ETs plus EACs after hydrolysis, externally calibrated with the building units (EA, methyl sanguisorboate, methyl gallate, and SA) are only comparable in terms of the order of magnitude, but do not overlap with the more precise HPLC data obtained separately for ETs and EACs using the direct quantification method (Gasperotti et al. 2010), where sanguin H-6 and lambertianin C were used as external standard for the ETs, and EA for EACs. The indirect method was reported to have a much lower repeatability, with CV% around 12% for the two major compounds and 18% for the minor compounds (Vrhovsek et al. 2008), and can be considered as an acceptable and cheaper way of providing an estimate of the total quantity of EA derivatives.

20.4.5.5 Computation and Interpretation of mDP

The simultaneous quantification of the four main hydrolytic products of Rubus ETs provides direct measurement of the relative molar abundance of the building units of ETs. According to the method developed by Vrhovsek et al. (2006), and also including the presence of free SA in the updated method, the estimate of the mDP of Rubus ETs can be theoretically derived from the molar ratio between the sanguisorboyl units (methyl sanguisorboate plus SA) and the EA produced in the reaction, $R_{[MS+SA]/[EA]}$. The experimental value is highly reproducible, with a CV% of around 2% (Vrhovsek et al. 2008). Taking into consideration the structure of the major known Rubus ETs (Gasperotti et al. 2010), and assuming complete hydrolysis, this ratio is expected to increase from 0 for the monomers (galloyl-bis-HHDP-glucosides) up to a value of 0.60 for tetrameric lambertianin D, with intermediate values for dimeric sanguin H-6 and trimeric lambertianin C (Vrhovsek et al. 2006). For oligomeric compounds such as sanguin H-6 and lambertianin C, which have been shown to account for 67% (range 41–83%) of ETs in blackberries (Gasperotti et al. 2010), as well as for the other lambertianin oligomers, this ratio is expected to increase according to the equation $R_{[MS+SA]/[EA]} = (DP – 1)/(DP + 1)$. In conclusion, the value of $R_{[MS+SA]/[EA]}$ can be obtained experimentally from HPLC analysis of the hydrolytic products of raw Rubus extract and can be used for computation of the mDP of Rubus ETs, which can be derived from the following equation: $mDP = (R_{[MS+SA]/[EA]} + 1)/(1 – R_{[MS+SA]/[EA]})$.

Table 20.3 gives an example of practical workflow. From the experimental values obtained from the HPLC run after hydrolysis, expressed in mg/L, the data can be converted into mg/kg in order to give the concentration in the berries and can be converted in mmol/L, which are used for the computation of $R$ and mDP.

The application of this method to the eight raspberry samples in our example gives an average mDP of ca. 1.9 (Table 20.3). This value is slightly higher than reported in a previous survey (Vrhovsek et al. 2008), also due to the inclusion of the contribution of free SA in the updated formula. The correction is not major since the latter is on average ca. 7 times less concentrated than methyl sanguisorboate (last column in Table 20.3). An mDP value close to 2 suggests that the sum of oligomers with DP > 2 (such as trimer lambertianin C), or with a lower content of EA (such as the peaks of dimers 1, 5, and 10, as well as of the trimers 3, 6, 17, and 19 in Table 20.2) or with a higher presence of SA (such as the monomer sanguin H-2, in addition to the peaks of dimer 18 and trimer 14 in Table 20.2) roughly balance the sum of the monomers and free EA in terms of concentration. Such a result is in acceptable agreement with the detailed HPLC data of ETs and EACs reported in Table 20.2.

It should be kept in mind that mDP computed according to this method is estimated, which could lead to misleading values if directly applied to hydrolyzable tannins of a different nature (Koponen et al. 2007). However, sufficient data are available to support its application to the analysis of ETs in botanical species characterized by the presence of GOD-type ETs, as in the case of Rubus (e.g., raspberries, blackberries, boysenberries), Sanguisorba, and strawberry samples.

After careful characterization of hydrolysis products, it could also in principle be extended to other sources of ETs, once enough structural information on the chemical structure of ETs and the products of degradation is available.
TABLE 20.3
Quantification of Ellagitannins and Ellagic Acid Conjugates in Blackberries, Quantified Using HPLC-DAD after Acid Hydrolysis

<table>
<thead>
<tr>
<th>Methylgallate (mg/L)</th>
<th>SA (mg/L)</th>
<th>EA (mg/L)</th>
<th>Methylsanguisorboate (mg/L)</th>
<th>Methylgallate (mmol/L)</th>
<th>SA (mmol/L)</th>
<th>EA (mmol/L)</th>
<th>Methylsanguisorboate (mmol/L)</th>
<th>Methylgallate (mg/kg)</th>
<th>SA (mg/kg)</th>
<th>EA (mg/kg)</th>
<th>Methylsanguisorboate (mg/kg)</th>
<th>R = [\text{MS} + \text{SA}/\text{EA}]</th>
<th>mDP = R + \sqrt{1 - R}</th>
<th>Sum ETs mg/kg</th>
<th>Ratio MS/SA</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>5.0</td>
<td>138.3</td>
<td>62.7</td>
<td>0.035</td>
<td>0.011</td>
<td>0.457</td>
<td>0.129</td>
<td>27.0</td>
<td>20.7</td>
<td>576.0</td>
<td>261.3</td>
<td>0.306</td>
<td>1.882</td>
<td>901</td>
<td>12.3</td>
</tr>
<tr>
<td>12.3</td>
<td>11.3</td>
<td>168.4</td>
<td>69.2</td>
<td>0.067</td>
<td>0.024</td>
<td>0.557</td>
<td>0.143</td>
<td>51.1</td>
<td>47.2</td>
<td>701.9</td>
<td>288.5</td>
<td>0.300</td>
<td>1.856</td>
<td>1130</td>
<td>5.9</td>
</tr>
<tr>
<td>16.9</td>
<td>15.8</td>
<td>277.7</td>
<td>125.4</td>
<td>0.092</td>
<td>0.034</td>
<td>0.919</td>
<td>0.259</td>
<td>70.2</td>
<td>65.9</td>
<td>1156.9</td>
<td>522.6</td>
<td>0.318</td>
<td>1.934</td>
<td>1875</td>
<td>7.7</td>
</tr>
<tr>
<td>16.6</td>
<td>16.4</td>
<td>287.6</td>
<td>121.1</td>
<td>0.090</td>
<td>0.035</td>
<td>0.952</td>
<td>0.250</td>
<td>69.3</td>
<td>68.5</td>
<td>1198.5</td>
<td>504.5</td>
<td>0.299</td>
<td>1.855</td>
<td>1903</td>
<td>7.2</td>
</tr>
<tr>
<td>9.8</td>
<td>15.8</td>
<td>189.9</td>
<td>94.7</td>
<td>0.053</td>
<td>0.034</td>
<td>0.628</td>
<td>0.196</td>
<td>40.9</td>
<td>65.7</td>
<td>791.3</td>
<td>394.6</td>
<td>0.364</td>
<td>2.147</td>
<td>1352</td>
<td>5.8</td>
</tr>
<tr>
<td>14.6</td>
<td>10.9</td>
<td>179.1</td>
<td>65.4</td>
<td>0.079</td>
<td>0.023</td>
<td>0.593</td>
<td>0.135</td>
<td>60.7</td>
<td>45.5</td>
<td>746.3</td>
<td>272.5</td>
<td>0.267</td>
<td>1.729</td>
<td>1165</td>
<td>5.8</td>
</tr>
<tr>
<td>18.7</td>
<td>15.9</td>
<td>261.0</td>
<td>97.3</td>
<td>0.101</td>
<td>0.034</td>
<td>0.864</td>
<td>0.201</td>
<td>77.7</td>
<td>66.3</td>
<td>1087.7</td>
<td>405.4</td>
<td>0.272</td>
<td>1.746</td>
<td>1697</td>
<td>5.9</td>
</tr>
<tr>
<td>20.9</td>
<td>26.7</td>
<td>295.4</td>
<td>135.5</td>
<td>0.113</td>
<td>0.057</td>
<td>0.977</td>
<td>0.280</td>
<td>87.0</td>
<td>111.3</td>
<td>1230.7</td>
<td>564.4</td>
<td>0.344</td>
<td>2.050</td>
<td>2097</td>
<td>4.9</td>
</tr>
</tbody>
</table>

Note: From the experimental values obtained from the HPLC run after hydrolysis of the extract, expressed in mg/L, the data can be converted into mmol/L, which are used for the computation of R and mDP, and into mg/kg in order to give the concentration in the berries.
20.5 HPLC-MS-MS Analysis

To obtain information of the molecular masses of ETs and EA derivatives preliminarily detected by HPLC-DAD, HPLC-ESI-MS analyses of the crude extracts, or fractions obtained from them, can be carried out. However, the chromatographic conditions have to be changed as the acid composition of the mobile phase for HPLC-MS analysis must be milder than those for HPLC-DAD analysis, and 0.1–0.4% formic acid should be used instead (Salminen et al. 1999). MS analysis in the negative mode provides more information on the ET and EA conjugate structures than those in the positive mode.

For most ETs, it is possible to obtain \( m/z \) values corresponding to \([\text{M}–\text{H}]^–\), \([\text{M}–2\text{H}]^2–\), or \([2\text{M}–\text{H}]^–\) depending on the mass of the compound. Often, both monomeric and dimeric ETs give the same \( m/z \) values, but the \([\text{M}–\text{H}]^–\) and the \([\text{M}–2\text{H}]^2–\) signals can be separated by their isotopic \( m/z \) values. For \([\text{M}–\text{H}]^–\), the isotopic signals differ in 1 m.u., while for the \([\text{M}–2\text{H}]^2–\), the isotopic differences are only 0.5 m.u.

ET fragmentation is produced by the sequential losses of galloyl residues (\( m/z \) 152, 169, or 170) and HHDP (hexahydroxydiphenic) residues (\( m/z \) 301). The galloyl units attached to phenolic hydroxyls of other galloyl molecules are more cleavable in the negative ESI-MS than the galloyl units attached directly to the glucose core (Salminen et al. 1999). This means that in the first case, losses of 152 units are found in the MS spectrum while in the last ones, losses of 169 or 170 units are observed instead. In the case of ETs containing catechin moieties, the characteristic cleavage releases an \( m/z \) of 289 from the flavan-3-ol residue.

For acyclic epimers having hydroxyl groups at C–1, they can be distinguished by the loss of water \([\text{M}–\text{H}_2\text{O}]^–\) from the vescalagin-type ETs, while this is hardly observed from castalagin-type ETs (Moilanen and Salminen 2008).

For ET derivatives with catechins, it is possible to show the place of substitution. When catechin is added to vescalagin-type ETs, as is the case of hippophaenin B, it is linked at C-1 of the glucose residue, as the \([\text{M}–\text{H}_2\text{O}]^–\) signal is not observed and the \([\text{M}–\text{catechin}–\text{H}]^–\) is observed instead.

In addition, it has been shown that the catechin addition did not occur at the carboxyl (–COOH) group, since the MS data showed both the cleavage of catechin and carboxyl group \([\text{M}–\text{catechin}–\text{H}–\text{COOH}]^2–\).

Characteristic MS fragmentation of vescalagin are: 1103 \([\text{M}–\text{H}]^–\); 1085 \([\text{M}–\text{H}_2\text{O}–\text{H}]^–\); 1041 \([\text{M}–\text{H}_2\text{O}–\text{COOH}]^–\); 529 \([\text{M}–\text{H}–\text{COOH}]^–\); and 520 \([\text{M}–\text{H}_2\text{O}–\text{H}–\text{COOH}]^–\).

Characteristic MS fragmentation of hippophaenin B are: 1375 \([\text{M}–\text{H}]^–\); 1085 \([\text{M}–\text{catechin}–\text{H}]^–\); 687 \([\text{M}–2\text{H}]^2–\); 665 \([\text{M}–\text{H}–\text{COOH}]^–\); 520 \([\text{M}–\text{catechin}–\text{H}–\text{COOH}]^2–\); and 289 \([\text{catechin}–\text{H}]^–\).

Oligomeric ETs can be detected and characterized by LC/ESI-MS, by examination of the multicharged ions, and by looking at the isotopic ions (Karonen et al. 2010).

For EA conjugates, the molecular masses are easily observed in the negative mode of the HPLC-ESI-MS as \([\text{M}–\text{H}]^–\) pseudomolecular ions (\( m/z \) 463 for EA-hexosides; \( m/z \) 447 for EA-rhamnosides, and \( m/z \) 433 for EA-pentosides), and the loss of the sugar residue (M-162 for hexosides, M-146 for rhamnosides, and M-132 for pentosides) released the EA molecule (\( m/z \) 301).

In addition, HPLC-ESI-MS allows the detection of ET oligomers, dimers and trimers being quite common (i.e., enothein A and B, respectively), and even hexameric and heptameric ETs have been recently evidenced (Karonen et al. 2010). For the identification of these oligomers, the use of high-resolution MS, and the analysis of the isotopic patterns are essential for the detection of the pentamers, hexamers, and heptamers (Karonen et al. 2010).

20.6 NMR Analysis

20.6.1 Gallotannins

As far as the gallotannins are concerned, most studies concern the detection and identification of these polygalloylglucoses in various natural sources (e.g., the traditional Chinese herb *Galla chinensis*) using
analytical techniques (Mueller-Harvey 2001) such as HPLC-MS (Salminen et al. 1999; Tian et al. 2009) or MALDI-TOF mass spectrometry protocols (Xiang et al. 2007), or relying on degradation studies, for example, using tannases (i.e., galloyl esterase) (Mingshu et al. 2006) or performing mild methanolysis in methanolic acetate buffer (pH 5.5) (Haslam et al. 1961), followed by mass spectrometry analysis of the residues. Several gallotannins, from hexa- to tetradeagalloyl glucose, were thus observed in such investigations, but no structural determination of the different isomers could be achieved. In contrast, pure gallotannins, which were obtained either through isolation from natural sources or by enzymatic or chemical synthesis, could be successfully characterized by nuclear magnetic resonance (NMR) spectroscopy.

### 20.6.1.1 Isolated Gallotannins

In the early 1980s, Nishioka and coworkers reported the isolation of several pure gallotannins from various medicinal plant extracts such as *Rhus semialata* (Chinese gall), *Quercus infectoria* (Turkish gall), *Paeonia albiflora* (syn. *P. lactiflora*), and *Moutan cortex* (Nishizawa et al. 1980a,b, 1982, 1983a,b). Each component could be separated from the plant extract according to the degree of galloylation using a combination of Sephadex® LH-20 column chromatography and normal-phase HPLC. The structures of the resulting individual gallotannins were next determined mainly by 1H and 13C NMR spectroscopy. The position of digalloyl depside motifs onto the glucopyranose core was determined by comparison of the 13C NMR chemical shifts recorded in acetone-δ6 of both the glucose carbon atoms and the carbonyl atoms of the galloyl groups with those of β-PGG. Typically, a downfield shift of 0.4–0.6 ppm is observed for a glucose carbon atom linked to a digalloyl depside, which is in accordance with the difference measured between the ester methyl carbons of methyl meta-digallate and methyl gallate (δ 52.2 and 51.9 ppm, respectively). In addition, the carbonyl carbon signals of the proximal depsidically linked galloyl moieties were observed at ca. 0.6 ppm upfield. These 13C NMR spectroscopic analyses allowed Nishioka and coworkers to demonstrate that the digalloyl depside moiety of the hexagalloylglucose isolated from the root of *Paeoniae albiflora* (i.e., *P. radix*) and *Moutan cortex* was attached to the C-6 position of the glucopyranose (i.e., 1d), but that of *Galla chinensis* was randomly distributed among the C-2, C-3, and C-4 positions (i.e., 1b, 1a, and 1c, respectively) (Nishizawa et al. 1980a). Similar 13C NMR spectra analysis led to the structural determination of six heptagalloylglucose, featuring either two digalloyl groups (i.e., 2a–e) or one trigalloyl motif (i.e., 2f), and even one octagalloylglucose bearing three digalloyl groups (i.e., 3) (Figure 20.4). Moreover, the investigations carried out by Nishioka and coworkers also revealed that the previously reported meta-depsidic digalloyl units (Fischer 1914; Nierenstein et al. 1925) are in fact equilibrium mixtures of meta- and para-depsidically linked galloyl units resulting from intramolecular transesterification (Nishizawa et al. 1982). This conclusion was supported by the analysis and comparison of 13C NMR spectra, run in acetone-δ6, of pure gallotannins with those of methyl meta- and para-digallate, notably by carefully examining both the carbonyl and the aromatic regions.

### 20.6.1.2 Enzymatically and Chemically Synthesized Gallotannins

Within the framework of their studies on the biosynthesis of hydrolyzable tannins, Gross and coworkers intensively investigated enzymatic synthesis of gallotannins (Gross 1999, 2008; Niemetz and Gross 2005). Experiments carried out *in vitro* with cell-free extracts from leaves of staghorn sumac (*Rhus typhina*) and β-PGG as a standard acceptor substrate led to the isolation of β-glucogallin-dependent galloyltransferases (Niemetz and Gross 2005; Gross 2008). It was found that none of these enzymes displayed high substrate specificity, but some of them preferentially acylated β-PGG to give the 2-, 3-, or 4-O-depsidic digalloylated hexagalloylglucoses 1a–c, while others preferentially catalyzed the galloylation of hexa- and heptagalloylglucoses to furnish, for example, 3-O-trigalloyl-1,2,4,6-tetra-O-galloyl-β-d-glucopyranose (2f) and higher galloylated gallotannins (Figure 20.4). Structural determination of 1a–c and 2f was accomplished mainly by 1H NMR spectroscopy, and further comparison with the materials isolated by the Nishioka group. The 1H NMR spectra recorded
in acetone-$d_6$ were compared with that of β-PGG, and meta-depsidic digalloyl moieties were typically detected from the diagnostic appearance of doublets for the aromatic protons of the proximal galloyl group, instead of the initially observed set of two singlets, as the result of the disymmetry introduced by the formation of the $m$-depsidic link. In addition, these signals were shifted from the 7.00–7.10 ppm region (singlets) to significantly higher $\delta$ values of 7.25–7.55 ppm (Hofmann 1996; Gross 1999). In contrast, the aromatic hydrogens of newly introduced distal galloyl residues generally displayed the expected sharp singlets at 7.10 ppm (Hofmann 1996; Gross 1999). It is worth noting that no NMR evidence of meta/para-depsidic digalloyl equilibrium mixtures was reported by the Gross group.

To the best of our knowledge, despite a few chemical studies on depside motifs carried out in the early 1900s (Fischer 1914; Nierenstein et al. 1925), no total chemical synthesis of “complex” (or “true”) gallotannins, as opposed to “simple” gallotannins, that is, their mono- to pentagalloylglucose precursors, has been reported thus far. The chemical elaboration of meta-depsidic digalloyl units acylating a glucose core has been reported only by Romani and coworkers in their synthesis of the 2,3-bis-$O$-digalloylgucose (Arapitsas et al. 2007). They also showed that UV–visible spectra of compounds featuring the $m$-depsidic digalloyl moiety display a characteristic shoulder at 300 nm (Arapitsas et al. 2007). In order to determine the influence of the gallotannin depsidic link on the biological activities of hydrolyzable tannins, Quideau and coworkers recently engaged efforts in the total synthesis of the hexagalloylgucose 1a, the heptagalloylgucose 2f, as well as the decagalloylgucose, referred to as “tannic acid” and whose commercial sample is known for not being a structurally well-defined gallotannin but rather a complex mixture of various gallotannin species and derivatives thereof (Mueller-Harvey 2001; Romani et al. 2006). Full characterization of the chemically pure synthetic gallotannins 1a, 2f, and tannic acid (Figure 20.5), as well as their $\alpha$-anomeric analogs, was accomplished by 1D and 2D NMR experiments (i.e., $^1$H and $^{13}$C NMR, COSY H–H, heteronuclear multiple quantum coherence, and heteronuclear multiple bond
coherence [HMBC]) and mass spectrometric analysis (Sylla 2010). The meta/para-depsidic equilibrium was confirmed by $^1$H and $^{13}$C NMR, and could even be evaluated as a ca. 2:1 ratio by $^1$H NMR spectroscopic analysis.

### 20.6.2 Ellagitannins

The ETs constitute the second subclass of hydrolyzable tannins, which is today composed of nearly 1000 members that have been identified and fully characterized following their isolation from various plants (Haslam and Cai 1994; Quideau and Feldman 1996; Okuda et al. 2009; Yoshida et al. 1992, 2009). The chirality of their characteristic HHDP (i.e., 6,6′-dicarbonyl-2,2′,3,3′,4,4′-hexahydroxybiphenyl) units is a consequence of the prevention of free rotation around their biaryl axis, which is impeded by the presence of the phenolic hydroxyl groups and the glucose-esterified carboxyl groups ortho-positioned relatively to that carbon–carbon bond axis (Quideau and Feldman; 1996; Khanbabaee and van Ree 2001b). The determination of the configuration ($R$ or $S$) of this axial chirality or atropisomerism is an essential step of the structural characterization of ETs (Figure 20.6), together with the determination of the regiochemistry of all galloyl and galloyl-derived units on the glucose core and that of the stereochemistry at the anomeric position of the latter.

#### 20.6.2.1 Absolute Configuration of ET Axially Chiral Biaryl Groups

Until 1982, the axial chirality of ET HHDP biaryl units was determined by a chemical degradation procedure beginning with a methylation step using dimethyl sulfate and potassium carbonate (Tanaka et al. 1986; Yoshida et al. 2000). The permethylated biaryl unit was then cleaved from the glucopyranose core by methanalysis using sodium methoxide in methanol. The chirality of the hexamethoxydiphenic acid derivative thus released under its native atropisomeric form was then determined by comparison with atropisomerically pure standards, the main drawback of this procedure being the degradation of the samples.

However, since 1982, a more suitable and nondestructive procedure has been developed using circular dichroism spectroscopy to establish the absolute stereochemistry of HHDP units linked to the ET glucopyranose core. Okuda et al. (1982a,b, 1984) showed that the Cotton effects observed near 220–230 and

**FIGURE 20.5** Structure of tannic acid.
250–260 nm were correlated with the absolute configuration of the HHDP units. An (S)-configured HHDP group is characterized by a positive and a negative Cotton effect at 220–230 and 250–260 nm, respectively, whereas an (R)-configured HHDP group exhibits instead a negative and a positive effect at the same values, respectively. Similar Cotton effects are also observed in the case of other HHDP-derived groups such as the DHHD, chebuloyl, and sanguisorboyl groups, as well as for the C-glucosidic ET nonahydroxyterphenoyl (NHTP) group (Yoshida et al. 2000). Moreover, this characteristic Cotton effect is not influenced by the position of the biaryl group or by the presence of galloyl groups on the glucopyranose core, making this procedure applicable to any ETs (Okuda et al. 1982a, b). Interestingly, ET HHDP units linked to the 2,3- or 4,6-positions of a 4C4-glucopyranose core, or to the 1,6-positions of a 1C4-glucopyranose core, are predominantly, and to a large extent, (S)-configured, while those linked to the 2,4- or 3,6-positions of a 1C4-glucopyranose core are essentially always (R)-configured (Quideau and Feldman 1996; Khanbabaee and van Ree 2001b).

20.6.2.2 Determination of the Position of ET Galloyl-Derived Acyl Units

Proton and carbon NMR analyses, together with partial hydrolysis procedures under mild conditions, provide useful structural information on ETs, such as the nature and number of galloyl and galloyl-derived acyl groups esterified to the glucopyranose core (e.g., galloyl, HHDP, DHHD, sanguisorboyl groups). However, the crucial step in the structural determination of monomeric or oligomeric ET is to establish the position of each acyl group. For this purpose, 2D long-range HMBC experiments provide a straightforward assignment by establishing two key three-bond correlations; one between the ester carbonyl carbon and an aromatic proton of the acyl unit, and another one between the same ester carbonyl carbon and the proton on the glucopyranose position at which the ester bond is connected, as shown on the punicalagin structure in Figure 20.7.

20.6.2.3 Determination of the Absolute Configuration of the Anomeric Carbon

The determination of the absolute configuration at the anomeric position of the glucopyranose core is another important point in the structural elucidation of ETs, especially since in some cases both α- and β-anomer can be in solvent-dependent equilibrium, like in the case of punicalagin (Figure 20.7) (Lu et al. 2008). Furthermore, in some dimeric ETs, for example, each glucopyranose moiety can possess an anomeric center with a different configuration. For example, in sanguin H-6, the glucose moiety “1” displays a β-configuration at that locus (i.e., equatorial orientation of the sanguisorboyl group), whereas the glucose “2” shows an α-configuration (Figure 20.8) (Tanaka et al. 1985; Gasperotti et al., 2010; Kool et al. 2010).
The stereochemistry at the anomeric position can be easily determined by measuring, on the $^1$H NMR spectrum, the coupling constant between the anomeric proton H-1 and the adjacent proton H-2. A large coupling constant ($J_{H-1,H-2} > 5$ Hz) generally indicates a β-glucosidic configuration (i.e., substituent with an equatorial orientation), whereas a small coupling constant ($J_{H-1,H-2} = 0–5$ Hz) indicates an α-glucosidic configuration (i.e., substituent with an axial orientation). The presence or absence of a galloyl or galloyl-derived acyl group at the anomeric position has only a minor influence on the value of this H-1, H-2 coupling constant (Table 20.4). Moreover, the chemical shift of the anomeric proton H-1 of an α-anomer usually presents a lower-field shift compared to that of the corresponding β-anomer (Hatano et al. 1988). These differences are also observed in oligomeric ellagitannins such as the dimers Sanguin H-6 and rubusuarin B, and in the trimers lambertianin C and rubussuarin C (Gaspertotti et al., 2010). In contrast, the chemical shifts of the glucopyranosic carbons C-1, C-2, C-3, and C-5 of an α-anomer are up-fielded compared to those of its corresponding β-anomer. For example, the chemical shifts of the carbons C-1, C-2, C-3, and C-5 of the α-punicalagin (i.e., with an axially oriented hydroxyl group at the anomeric position) are shifted upfield by 4.2, 1.9, 2.4, and 4.8 ppm, respectively, compared with the corresponding signals of the β-punicalagin (Tanaka et al. 1986).

**FIGURE 20.7** Structure of punicalagin.

**FIGURE 20.8** Structure of sanguin H-6.
20.6.2.4 About the C-Glucosidic Ellagitannins

The C-glucosidic ETs constitute an important subclass of ETs with the structural particularity of having a highly characteristic C–C linkage between the carbon C-1 of an open-chain glucose core and the carbon C-2 of the O-2 galloyl-derived moiety of a 2,3,5-NHTP unit (also known as the flavogalloyl group), such as in vescalagin and castalagin. This characteristic C–C linkage on an open-chain glucose core can also occur with a 2,3-HHDP unit, such as in casuarinin and stachyurin (Figure 20.9). Vescalagin and its C-1 epimer castalagin were the first members of this ET subclass to be isolated from Castanea (chestnut) and Quercus (oak) species in 1971 (Mayer et al. 1967, 1969, 1971). However, their structures, as well as those of stachyurin and casuarinin (Okuda et al. 1981, 1982c, 1983), were fully determined much later when Nishioka’s group revised the assignment of the configuration at the C-1 position of all C-glucosidic ETs (Nonaka et al. 1990). This structural revision was based on the observation of spatial correlations between the protons H-1 and H-3 by Nuclear Overhauser Effect Spectroscopy (NOESY) in the corresponding spectra of vescalagin and stachyurin. These correlations are not observed for castalagin and casuarinin, for which these protons H-1 and H-3 are oriented toward a different side of the molecule (Figure 20.9). Furthermore, the observation of such diagnostic cross-peaks on the 2D NOESY spectra of

![Chemical Structures](image)

**TABLE 20.4**

<table>
<thead>
<tr>
<th></th>
<th>α-Anomer</th>
<th>β-Anomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gemin D&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.31 (d, $J = 4.0$ Hz)</td>
<td>4.78 (d, $J = 7.5$ Hz)</td>
</tr>
<tr>
<td>Tellimagrandin I&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.57 (d, $J = 4.0$ Hz)</td>
<td>5.13 (d, $J = 8.0$ Hz)</td>
</tr>
<tr>
<td>Pedunculagin&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.50 (d, $J = 3.5$ Hz)</td>
<td>5.09 (d, $J = 8.0$ Hz)</td>
</tr>
<tr>
<td>Punicalagin&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.34 (d, $J = 3.5$ Hz)</td>
<td>5.01 (d, $J = 8.0$ Hz) (see casuarictin)</td>
</tr>
<tr>
<td>Potentilline&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.63 (d, $J = 4.0$ Hz) (see potentillin)</td>
<td>6.22 (d, $J = 9.0$ Hz)</td>
</tr>
<tr>
<td>Sanguin H-6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.50 (d, $J = 3.5$ Hz) (glucose 2)</td>
<td>6.01 (d, $J = 8.5$ Hz) (glucose 1)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Hatano et al. (1988).
<sup>b</sup> Tanaka et al. (1986).
<sup>c</sup> Okuda et al. (1984).
<sup>d</sup> Kool et al. (2010).

**FIGURE 20.9** Structures of main monomeric C-glycosidic ellagitannins vescalagin, castalagin, casuarinin, and stachyurin.

*Hydrolyzable Tannins*
These experiments revealed that for camellia tannin E in which the epicatechin unit is connected by its phenylcyclopentenone motif (also known as the dihydrofuran group). Recently, new types of colored flavan-3-ols, procyanidines, and anthocyanins. In these flavano-ET hybrids, the two moieties are connected via C-C linkage between the carbon C-1 of the C-glucosidic ET and the carbon C-8 or C-6 of the A ring of the flavanoid. Depending on the nature of each moiety, these complex tannins present a large diversity of structures such as the catechin-based stenophyllanins A/B (Nonaka et al. 1985) and acutissimins A/B (Ishimaru et al. 1987) or the epicatechin-based camelliatannins A, B, and F, and malabathrins A and E (Hatano et al. 1991; Okuda et al. 2009; Yoshida et al. 2009). Moreover, these complex tannins can be further transformed by oxidative means to generate different types of derivatives such as the mongolicaicins A/B (Nonaka et al. 1988), which contain a\textit{o,m}-hydroxyphenylcyclcopentenone motif (also known as the dihydrofuran group). Recently, new types of colored flavano-ETs, named anthocyanos-ETs, were obtained by hemisynthesis in aqueous acidic media from vescalagin and the anthocyanin oenin or its malvidin aglycone (Quideau et al. 2005; Jourdes et al. 2009; Chassaing et al. 2010). All of the isolated and characterized flavano-ETs to date present a β-oriented linkage between the flavanoid unit and the C-glucosidic ET moiety. These β-configurations were determined by the observation of a small coupling constant between the proton H-1 and the proton H-2 ($J_{\text{H}1\text{-H}2} ≈ 0–2 \text{ Hz}$). Such a stereoselectivity was recently rationalized through molecular modeling studies by the Quideau group (Quideau et al. 2003, 2005).

After having characterized the C-glucosidic ET moiety using some of the strategies highlighted above, the crucial element of the structural elucidation of a flavano-ET structure is to establish by which A ring carbon (C-8′ or C-6′) the flavanoid unit is connected to the carbon C-1 of the C-glucosidic ET unit (Okuda et al. 2009; Yoshida et al. 2009). This connectivity can be established by the observation of two- and three-bond HMBC correlations between proton H-1 and carbons C-7′, C-8′, and C-8′a in the case of a C-8′/C-1 linkage, whereas a C-6′-linked flavanoid unit will show correlations between proton H-1 and carbons C-5′, C-6′, and C-7′ (Figure 20.10) (Jourdes et al. 2009; Quideau et al. 2003, 2005). Moreover, in order to establish this connectivity unambiguously, the HMBC data can be supported by those of Rotational Nuclear Overhauser Effect Spectroscopy (ROESY) experiments that reveal, for example, through-space connectivities between the proton H-2′ and H-6′ of the B-ring of the flavanoid unit and the proton H-1, H-2, and H-3 of the C-glucosidic ET unit. Such spatial correlations are only possible in the case of the C-8′/C-1 linkage between the flavanoid and C-glucosidic ET units (Figure 20.10). Such ROESY experiments were used to confirm the structure of the camelliatannins A and B (Hatano et al. 1991), as well as that of the hemisynthesized anthocyanos-ETs (Chassaing et al. 2010).

Hatano et al. (1995) also used a different strategy to establish the connectivity between an epicatechin unit and a C-glucosidic ET unit (i.e., 5-O-desgalloylstachyurin-derived unit) in the case of the camelliatannins C and E isolated from Camellia japonica leaves (Figure 20.11). After methylation of all phenolic hydroxyl groups using dimethyl sulfate and potassium carbonate in acetone, NOE experiments were performed by successively irradiating the flavanoid A-ring methoxy groups at positions O-5′ and O-7′. These experiments revealed that for camelliatannin E in which the epicatechin unit is connected by its C-8′ center to the 5-O-desgalloylstachyurin-derived unit, the irradiation of both methoxy groups at O-5′ and O-7′ results in NOE signals with the A-ring aromatic proton H-6′ (Hatano et al. 1995). In contrast,
in the case of camelliataannin C, only the irradiation of the methoxy group at O-7' gave an NOE signal with an A-ring aromatic proton (i.e., H-8'). thus establishing that the epicatechin unit is linked to the carbon C-1 of the C-glucosidic ET unit by its A-ring carbon C-6' (Figure 20.11).

ACKNOWLEDGMENTS

We are grateful to Urska Vrhovsek, Domenico Musaero, and Mattia Gasperotti for their collaboration in this research, and to Lara Giongo and Marcella Grisenti for providing the blackberry samples. This work has been supported by the Spanish MICINN (Consolider Ingenio 2010- Fun-C-Food CSD2007-0063) and Fundación Seneca de la Region de Murcia (grupo de excelencia GERM 06, 04486). Pilar Truchado holds a PhD grant from the Seneca Foundation (Murcia, Spain). Stéphane Quideau also wishes to thank the Conseil Interprofessionnel du Vin de Bordeaux and the Conseil Régional d’Aquitaine (Bordeaux, France) for their financial support of his team’s research on hydrolyzable tannins.
REFERENCES


Hydrolyzable Tannins


