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Mass Spectrometry-Based Applications in Tannin Analytics: From Qualitative and Quantitative Analyses to Biological Activity

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ABSTRACT

Tannins are widespread specialized plant metabolites that contribute significantly to the polyphenol content of plant-based diets. Their effects on human and animal health vary depending on their structure, with potential benefits including antioxidative, antimicrobial, anthelmintic, and anticarcinogenic properties. Understanding tannin composition and quantity in plant products is essential, as their bioactivities are influenced by their functional groups. Mass spectrometry-based techniques excel in tannin analysis, offering both qualitative and quantitative insights. Combining ultrahigh-performance liquid chromatography with electrospray ionization and high-resolution and triple quadrupole mass analyzers is optimal for comprehensive tannin profiling. Such an approach enables precise analysis and helps predict tannin bioactivities. This review highlights the mass spectrometric analysis of proanthocyanidins and hydrolysable tannins, addressing ionization techniques, interpretation of multiply charged ions, characteristic fragmentations, and reaction monitoring. Applications related to tannin bioactivities are also briefly discussed, demonstrating the utility of mass spectrometry in tannin analysis in complex sample matrices.

1 | Introduction

Tannins are a widely distributed heterogeneous group of plant specialized metabolites. They occur ubiquitously in the plant kingdom, for example, in bark, phloem, wood, stems, leaves, seeds, fruits, fruit pods, berries, and nuts. Tannins can precipitate proteins, and historically, they are among the first natural products utilized industrially, that is, as tanning agents for transformation of fresh hides into leather (L. J. Porter 1989a; L. J. Porter 1989b). Nowadays, plant tannins are known to have many beneficial bioactivities, for example, antioxidant, anticarcinogenic, anthelmintic, and antimicrobial activities (Engström et al. 2019; Engström, Karonen et al. 2016; Gaudin et al. 2016; Gong et al. 2018; Gontijo et al. 2019; Hoste et al. 2006; Karonen et al. 2020; Kolodziej et al. 1999; López-Andrés et al. 2013; Puljula et al. 2020; Quijada et al. 2015; Reddy et al. 2007; Scalbert 1991; Soorkia et al. 2020). Tannins can also be modified or transformed, for example, by alkaline or aerobic oxidation (García et al. 2016; Imran et al. 2021; Karonen et al. 2021; Petit et al. 2013), by synthetic approaches

Abbreviations: APCI, atmospheric pressure chemical ionization; APPI, atmospheric pressure photo ionization; CI, chemical ionization; DART, direct analysis in real time; DESI, desorption electrospray ionization; EI, electron ionization; ESI, electrospray ionization; FT-ICR, Fourier transform ion cyclotron resonance mass analyzer; HPLC, high-performance liquid chromatography; HT, hydrolysable tannin; LC, liquid chromatography; MALDI, matrix assisted laser desorption/ionization; MRM, multiple reaction monitoring; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PA, proanthocyanidin; Q, quadrupole; QqQ, triple quadrupole; SRM, single reaction monitoring; TOF, time-of-flight mass analyzer; UHPLC, ultrahigh-performance liquid chromatography.

(Cobo et al. 2024; Deffieux et al. 2011; Laitila et al. 2023; Malik et al. 2012; Petit et al. 2019; Pouységu et al. 2011; Quideau et al. 2011; Sylla et al. 2015) or during wine aging (He et al. 2010; Jourdes et al. 2011; Laitila and Salminen 2020; Quideau et al. 2003; Rasines-Perea et al. 2019; Saucier et al. 2006).

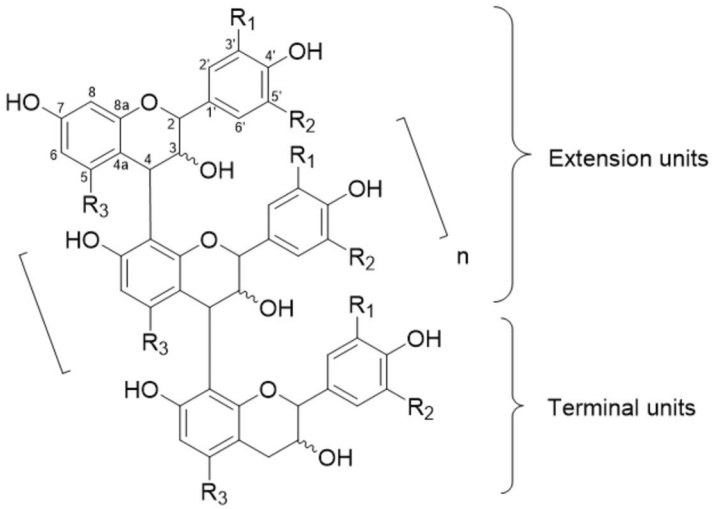
The tannin composition of a single plant species can be complex (Salminen and Karonen 2011). Even the organs of the same plant species may produce very variable tannin structures (Tuominen et al. 2013). On the basis of the major differences in their structures, terrestrial tannins are divided into two groups: proanthocyanidins (PAs, syn. condensed tannins) and hydrolysable tannins (HTs). PAs are oligomers (two to ten monomeric units) and polymers (> 10 monomeric units) of flavan-3-ol units which have variation in their hydroxylation patterns, stereochemistry at C2 and C3, degree of polymerization, sequential order of the flavan-3-ol units and location and stereochemistry of interflavanoid bonds (Figure 1, Hemingway 1989a). Monomeric units can be linked by C4 → C8 and/or C4 → C6 (B-type PAs) or doubly linked with an additional C2 → O → C7 ether bond (A-type PAs). The most common PAs are procyanidins containing (+)-catechin with 2*R*,3*S* stereochemistry and (-)-epicatechin with 2*R*,3*R* stereochemistry as monomeric units and prodelphinidins containing (+)-gallocatechin and (-)-epigallocatechin. In addition to the six main classes, some less common PA classes exist, such as proapigenidins and proluteolinidins (Hemingway 1989a; Krueger et al. 2003; L. J. Porter 1989a; L. J. Porter 1989b). In plants, individual PA structures often contain various types of subunits; for instance, pure procyanidin or prodelphinidin oligomers or polymers are less common than their mixtures.

HTs are typically divided into three major subclasses, that is, simple gallic acid derivatives, gallotannins and ellagitannins. Simple gallic acid derivatives most commonly have either glucose or quinic acid as the central polyol galloylated with monogalloyl groups (Figure 2A), whereas gallotannins also contain digalloyl or trigalloyl groups in their structures. Ellagitannins can be further classified into multiple subgroups, these seven being the most common groups for ellagitannin monomers: hexahydroxydiphenoyl esters (Figure 2B), dehydrohexahydroxydiphenoyl esters and their modifications (Figure 2C,D), gallagyl esters (Figure 2E), nonahydroxytriphenoyl esters (Figure 2F), and flavanoellagitannins (Figure 2G). The diversity of ellagitannin structures is multiplied by their various types of oligomerization, a feature typically not found with simple gallic acid derivatives or gallotannins.

2 | Different Ionization Techniques for Tannin Analysis

In the mass spectrometric analysis of tannins, two important choices must be made. The first one is related to the ionization, that is, the selection of the ionization technique used, and the second is related to the actual mass analysis, that is, the selection of mass analyzer used. There are several suitable ionization techniques for tannins. The early ionization techniques, such as electron ionization (EI), are poorly suited for tannin analysis (Cheynier and Fulcrand 2003). EI requires volatile analytes, but tannins are thermosensitive and nonvolatile in their native form. However, this limitation can be overcome by derivatization, for example by using methylation by dimethyl sulfite (Nonaka et al. 1983). In addition, EI is a hard ionization technique and with typical ionization energy of 70 eV induces extensive fragmentation in which case the detection of molecular ions can be challenging. Later ionization techniques, such as fast atom bombardment and liquid secondary ion mass spectrometry, which generate the secondary ions when the sample is irradiated with energetic neutral atoms in fast atom bombardment or primary beam of ions in liquid secondary ion mass spectrometry, have been mainly used for the analysis of purified tannins as the sample must be dissolved in a matrix. For example, PAs have been analyzed using glycerol (Freitas et al. 1998), thioglycerol (Vivas et al. 1996), or eutectic mixture of dithiothreitol and dithioerythritol (Barofsky 1989; Karchesy et al. 1986) as matrices.

Matrix also plays an important role in matrix-assisted laser desorption-ionization (MALDI) of tannins. The use of MALDI techniques involves the optimization of many factors, such as



R_1	R_2	R_3	Monomeric unit
OH	H	OH	Procyanidin
OH	OH	OH	Prodelphinidin
H	H	OH	Propelargonidin
OH	H	H	Profisetinidin
H	H	H	Proguibourtinidin
OH	OH	H	Prorobinetinidin

FIGURE 1 | Model structure and numbering of a B-type proanthocyanidin with C4 → C8 linkages.

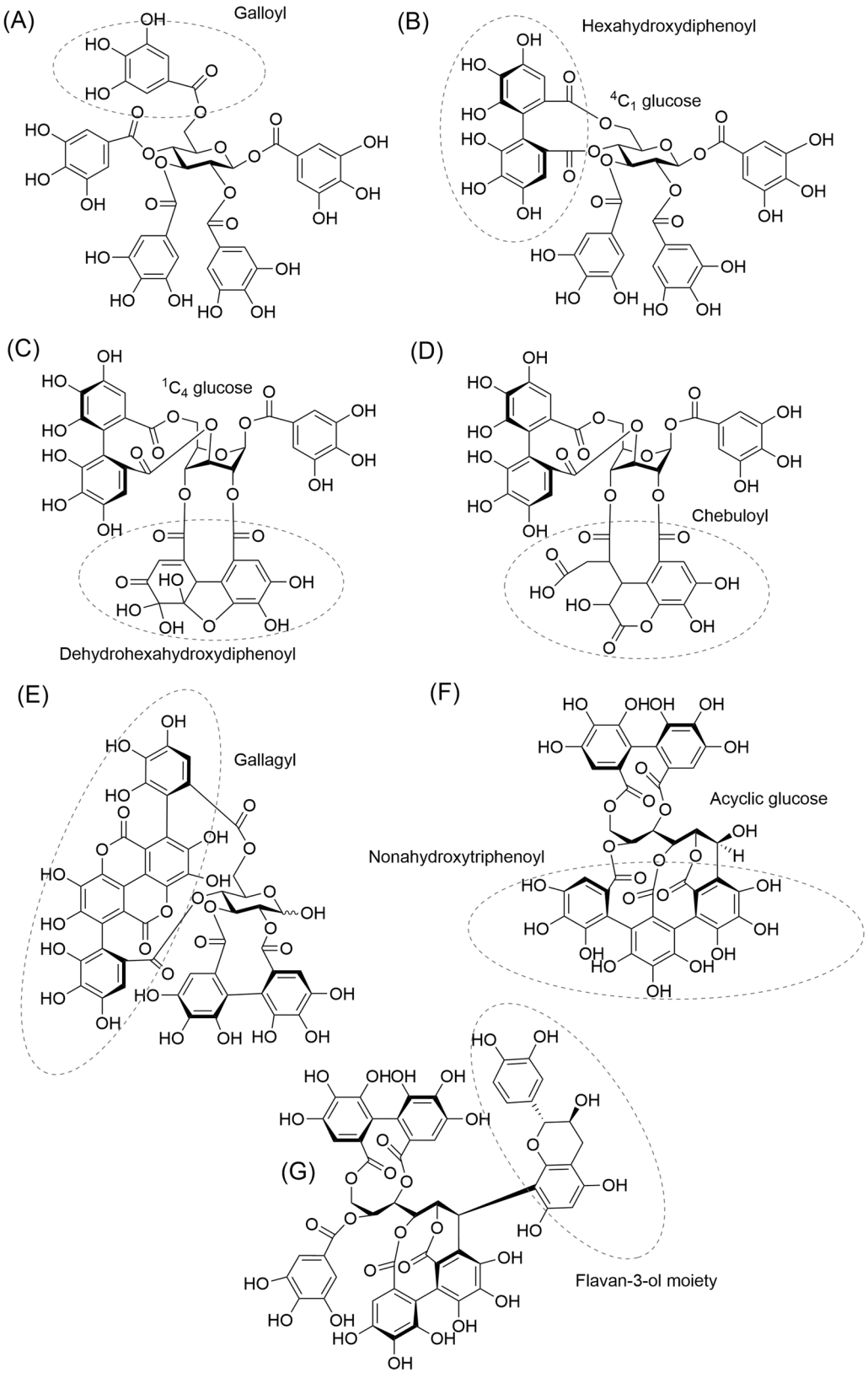


FIGURE 2 | Model structures of monomeric hydrolysable tannins: (A) pentagalloylglucose, (B) tellimagrandin I, (C) geraniin, (D) chebulagic acid, (E) punicalagin, (F) vescalagin and (G) stenophyllanin A.

the selection of an appropriate matrix, optimal sample preparation including the mixing and drying or crystallization of matrix and sample, the actual ionization parameters related, for example, to the laser power and behavior of the matrix during laser irradiation, and the selection of calibration standards (Behrens et al. 2003; Es-Safi et al. 2006; Reed et al. 2005). The advantages of MALDI are its sensitivity, a single ionization event and the infinitesimal fragmentation of tannins when

main target is the determination of molecular formula (Krueger et al. 2000; Yang and Chien 2000). If controlled fragmentation is desired, this can be obtained by using postsource decay fragmentation, which allows the detailed characterization and sequencing of individual PA chains, that is, a sequential loss of one monomeric unit after the other (Behrens et al. 2003). This technique allows the selection of a precursor ion, for example an $[M+Na]^+$ ion for a procyanidin tetramer at m/z 1177, in a distinct mass window and the subsequent analysis of its fragment ions, in the case of procyanidin tetramer signals at m/z 889 and 887 and at m/z 601 and 599 corresponding to trimeric and dimeric fragments, as presented in Figure 3, respectively (Behrens et al. 2003). MALDI techniques have their own benefits and challenges related to direct tannin analyses as previously reviewed by Flamini (2003), Reed et al. (2005) and Monagas et al. (2010).

Atmospheric pressure ionization techniques, such as atmospheric pressure chemical ionization (APCI), atmospheric pressure photo ionization (APPI), and electrospray ionization (ESI), can be easily combined with liquid chromatographic techniques. APCI is rarely used for tannins as it is most suitable for thermally stable compounds with molecular weights less than 1500 Da as it produces mainly singly charged ions (de Hoffmann and Stroobant 2001; Holčapek et al. 2010). APCI has been used, for example, for the analysis of flavan-3-ols, PA dimers, trimers and their gallates, modified tannins in wines and dimeric and trimeric ellagitannins (de Pascual-Teresa et al. 2000; Franceschi et al. 2011; Maillard et al. 1996; Mazerolles et al. 2010; Weber et al. 2007). Based on literature search, APPI is even less used and to our knowledge it has been applied only for tannins in soil samples to complement ESI (Vinci et al. 2022). However, based on our experience ESI is a superior ionization technique regarding tannin analyses. Its ability to produce multiply charged ions folds up the m/z scale by the number of charges, shifting the ions into an m/z range below 3000, which is accessible by most mass analyzers (Gross 2017). In ESI, the analyte solution is supplied through a thin spray capillary, nebulizer needle, which is kept at high potential of 3–6 kV (de Hoffmann and Stroobant 2001) and sheathed with nebulizer gas to form an electrically charged aerosol. When the analyte solution comes out from the capillary, it breaks up to form highly charged droplets. The ions present in the charged droplets are liberated into gas phase, via several evaporation (droplet shrinking) and droplet disintegration steps, either according to charge-residue model or ion evaporation model (Gross 2017), yielding multiply charged ions for larger molecules. In general, ESI is a soft, rapid, sensitive technique which can be used either by direct infusion, as nicely outlined previously (Hayasaka et al. 2003; Roux et al. 1998), or in combination with HPLC or UHPLC techniques (Baert et al. 2015; Hammerstone et al. 1999; Karonen et al. 2011, 2010, 2004; Lazarus et al. 1999; Rauha et al. 2001; Salminen et al. 1999). Multiply charged ions obtained by ESI-MS enables the detection and characterization of high-molecular weight tannins, including PAs with a degree of polymerization of up to 22 (Es-Safi et al. 2006; Karonen et al. 2011) and undecameric ellagitannins with a molecular weight of 8624 Da (Salminen et al. 2011). Although ESI is a soft ionization technique, it produces some fragment ions for tannins. Therefore, the different monomeric units in oligomeric tannins can be detected, see for example Karonen et al. (2010) for HT oligomers, and Karonen et al. (2011) for PA oligomers. For PAs, ESI-MS allows the determination of the nature and

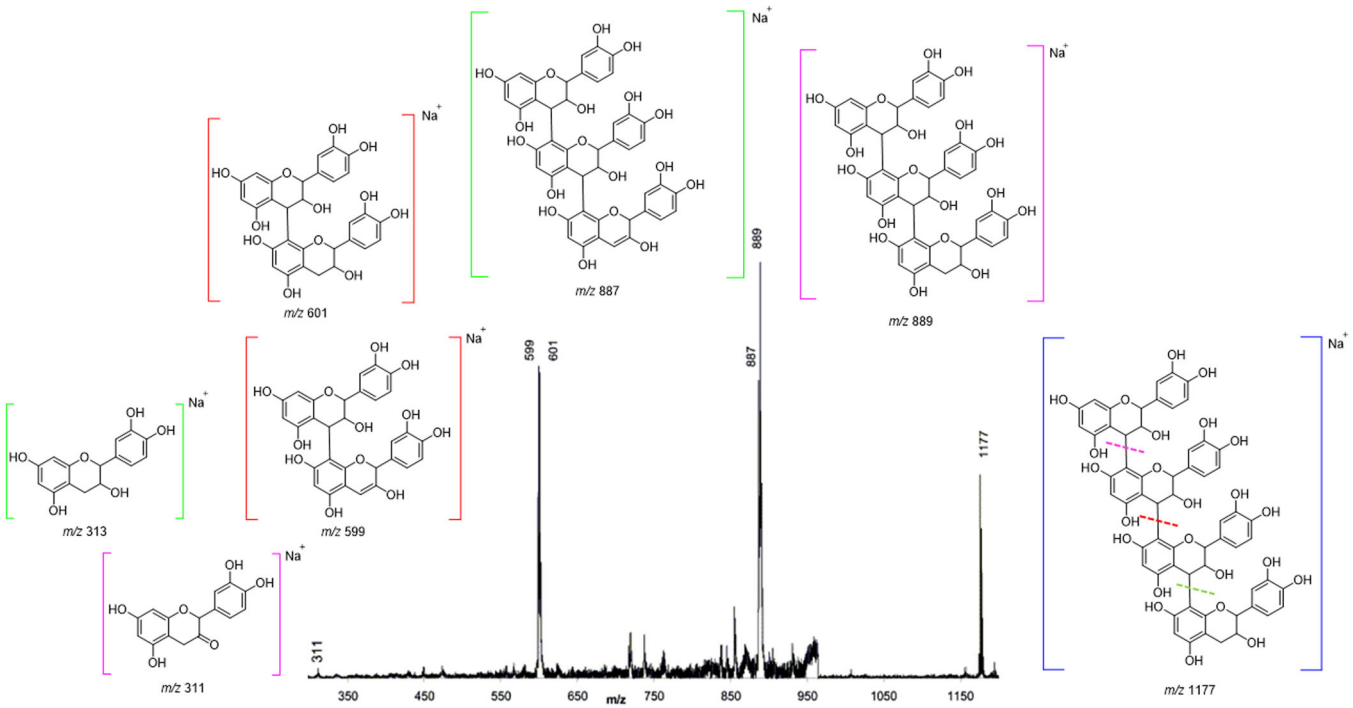


FIGURE 3 | Postsource decay matrix assisted laser desorption/ionization—time-of-flight mass spectrum of a procyanidin tetramer from lime (*Tilia cordata*) together with the possible fragmentation pattern in positive ion mode. The colors of the brackets refer to the corresponding fragmentation site drawn with a dashed line of the same color. The mass spectrum is reprinted from Behrens et al. (2003) with permission from Elsevier. [Color figure can be viewed at wileyonlinelibrary.com]

proportions of constitutive units, degree of polymerization, type of the interflavanoid linkages (A- or B-type), and the nature and proportion of substituents as discussed by Cheynier and Fulcrand (2003). A-type PAs can be recognized by their molecular ions corresponding to the loss of two hydrogens during the biosynthetic formation of the additional ether bond in comparison with B-type PAs. Therefore, the *m/z* values of A-type PAs differ by 2 Da from the corresponding B-type ones. The substituents in PA structures identified by ESI can be, for example, xylose (Liimatainen et al. 2012), glucose (Salminen et al. 2005), or galloyl group (Karonen et al. 2021). In general, tannins are better ionized in the negative mode in comparison to positive mode due to the acidity of phenolic hydroxyl groups. For example, PA pentamers seem to be the limit of PA detection when positive ESI is used (Hammerstone et al. 1999). We have also experimentally observed that the use of positive ESI produces more in-source fragment ions in comparison to negative ESI which might hinder the correct identification of the molecular ion. The negative ionization gives also simpler mass spectra due to the absence of intensive adduct ions (de Hoffmann and Stroobant 2001; Hayasaka et al. 2003). Typical adducts in positive ion mode are formed with metal ions (e.g., Na^+, K^+), solvent molecules (e.g., H_2O), and counter ions (e.g., Cl^-, AcO^-) which can complicate mass spectra by generating additional peaks.

Desorption electrospray ionization (DESI) introduced in 2004 and direct analysis in real time (DART) introduced in 2005 have been less used so far, but their applications for rapid visualization or imaging of monomers and small oligomers seem promising. For example, tannins among other metabolites have been imaged via imprint, followed by DESI/postphotoionization (imprint DESI/PI) mass spectrometry imaging (Wu et al. 2022). The technique also allows the comprehensive mapping of the metabolomic network of catechin in fresh tea leaves. DESI has also been used for rapid visualized characterization of tannins together with other phenolics in tea extracts after their separation by high-performance thin-layer chromatography (Tang et al. 2021). DART is a soft ionization technique suitable for the fast detection of flavan-3-ols. For example, eucalyptus leaf and stem tissues were placed directly in the gap between the skimmer of DART ion source and the capillary inlet of time-of-flight (TOF) mass analyzer which enabled the ionization and analysis of catechin among other compounds (Maleknia et al. 2009). Similarly, epiafzelechin, the monomeric building block of propelargonidin, was successfully ionized from a root sample of *Cassia sieberiana* placed in the open ion source (Kpegba et al. 2011). Ambient desorption/ionization methods are superior in many ways as they are directly applicable to solids, liquids, frozen solutions or complex biological materials, but they have a limited potential for quantification as the detection of the compound heavily depends on the matrix (Gross 2017).

3 | Importance of High-Resolution in Qualitative Tannin Analysis

Different mass analyzers have different roles in tannin analysis. Due to their low-resolution, quadrupole instruments are not suitable for accurate mass analysis but give superior qualitative and quantitative results when tandem mass spectrometric methods, such as multiple reaction monitoring, are applied. These applications are discussed in detail in Section 6, here the focus is on high-resolution mass analyzers that excel at tannin characterization due to their ability to provide detailed isotopic patterns (Gross 2017). Most commonly orthogonal-acceleration TOF or orbitrap mass analyzers are used as they can be conveniently combined with LC, see for example, Karonen et al. (2011), Lin et al. (2014), Navarro-Hoyos et al. (2017) and Leppä et al. (2018). The main advantages of TOF instruments for tannins are the same as for all TOF analysis: the instrument construction is straightforward and allows accurate mass measurements, the *m/z* range of a TOF analyzer is in theory unlimited; in addition, TOF analyzers have high sensitivity through high ion transmission and the mass spectrum acquisition rate of TOF is very high (Gross 2017). Similarly, the advantages of using an orbitrap for tannin analyses are the same as for other compounds: ultrahigh-resolution, high mass accuracy, and increased speed and sensitivity, especially when combined with a bent RF-only quadrupole C-trap (Gross 2017). Typically, the resolving power of TOF and orbitrap varies widely based on their design, mode of operation and intended use. Generally, TOF can achieve the resolving power to $R > 30{,}000$ and orbitrap the resolving power of $R = 20{,}000–200{,}000$ (Gross 2017). When a TOF or an orbitrap is combined with a quadrupole, tandem MS experiments can be used with high-resolution and mass accuracy. In addition, a few studies have utilized Fourier transformation ion cyclotron resonance (FT-ICR) mass spectrometers for tannins or tannin-like compounds (Cooper and Marshall 2001; Ferreira et al. 2014; He et al. 2019). The operating principle of FT-ICR is similar to orbitrap as they both employ image current detection of ion oscillations and Fourier transformation for the conversion of the transient into the frequency domain. However FT-ICR operates with a magnetic field, resulting in higher costs and more stringent requirements (installation room, power consumption, air conditioning load, personnel qualification, and maintenance) compared to orbitrap which utilizes an electrostatic field (Gross 2017).

The isotopic patterns obtained by high-resolution mass spectrometers enable the accurate mass calculations for each singly or multiply charged ion and thereby provides the exact mass of the corresponding tannin. The charge state of the ion is determined by the mass difference of adjacent isotopic peaks, and the multiply charged ions and their charge states can be easily distinguished from the singly charged ones. Tannins contain H, C and O which are all isotopic elements, but only the abundance of ^{13}C (1.08%) is significant from the isotopic pattern point of view. The isotopes ^{2}H (0.01%) and ^{17}O (0.04%) and ^{18}O (0.21%) have so low abundances that they do not affect the isotopic pattern significantly. In the mass spectrum, the information of the analyte ions is displayed at the *m/z* scale, and the mass difference between the isotopic peaks can be described as $\Delta\ (m/z) = 1/z$ and thereby, the charge state calculated based on the observed mass difference as $z = 1/\Delta\ (m/z)$. As an example, two oligomeric ellagitannins both with molecular ions at *m/z* 934.07 (sanguiin H-6 and lambertianin C) are shown in Figure 4A,B. When the isotopic distributions of the ions are zoomed, different isotopic patterns can be detected (Figure 4C,D). By accurate mass data, the charge states of the ions are first determined: for sanguiin H-6 (Figure 4C), $\Delta\ (m/z)$ is $0.5 = 1/2$

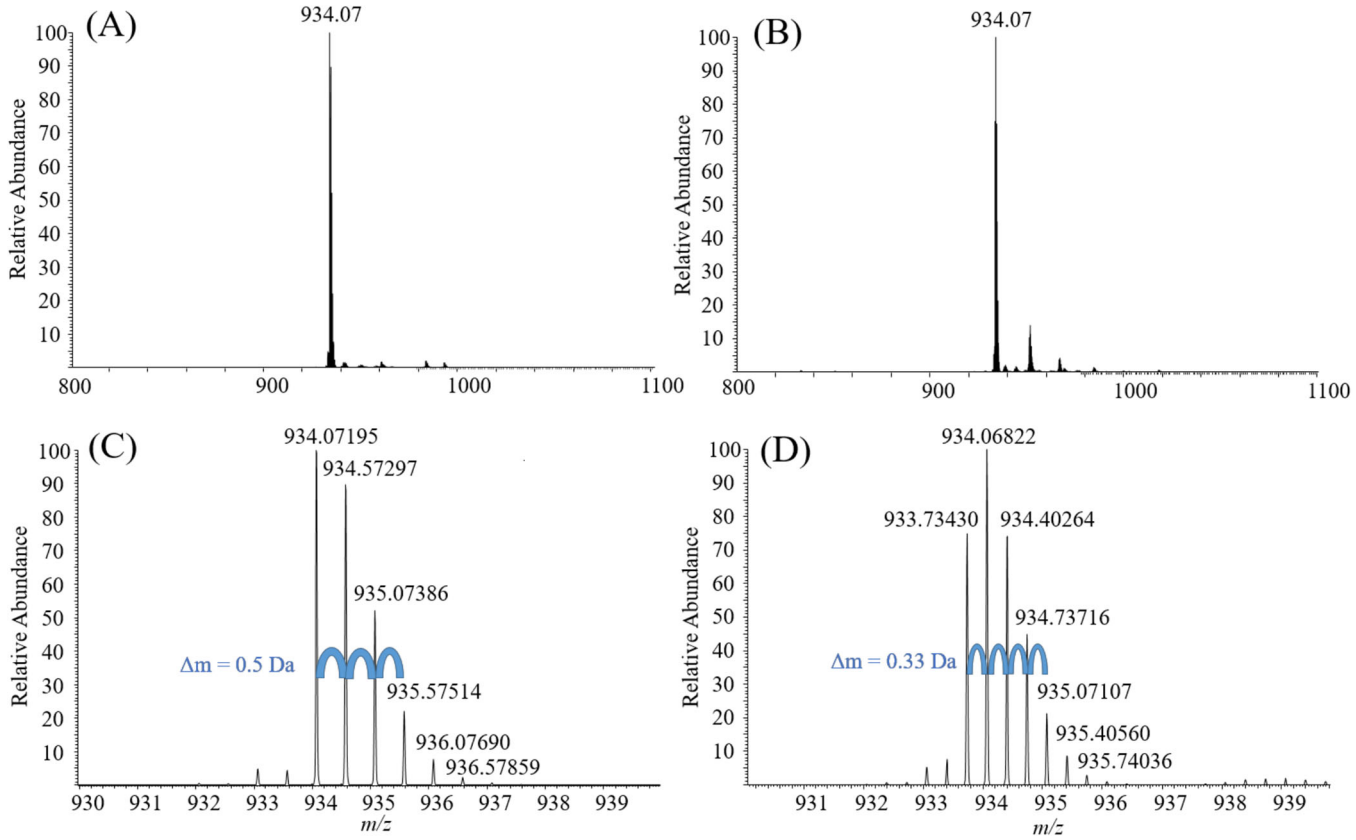


FIGURE 4 | Molecular ions of (A) sanguiin H-6 and (B) lambertianin C with their isotopic distributions (C and D), respectively, highlighting the mass differences between the isotopic peaks in negative ion mode ESI-MS. When the data is examined with a precision of two decimal places (A and B), the mass spectra looks the same, but when the isotopic distributions are zoomed and more precise numerical values are viewed (C and D), a clear difference between the mass spectra is noticed. [Color figure can be viewed at wileyonlinelibrary.com]

corresponding to $z = 2$, i.e., the doubly charged $[M–2H]^{2–}$ ion, and for lambertianin C (Figure 4D), Δ (m/z) is $0.33 = 1/3$ corresponding to $z = 3$, i.e., the triply charged $[M–3H]^{3–}$ ion. Then, the corresponding exact masses and molecular formulae based on the monoisotopic peak are calculated.

As the size of the tannin (and the number of carbon atoms) increases, the probability that an ion contains at least one ^{13}C becomes larger. Therefore, the monoisotopic peak is smaller for the trimeric lambertianin C ($C_{123}H_{80}O_{78}$; Figure 4D) in comparison to the one of dimeric sanguiin H-6 ($C_{82}H_{54}O_{52}$; Figure 4C). When the molecular size is large enough, as illustrated in Figure 5 with an example of *Lysimachia vulgaris* PAs exhibiting the degree of polymerization of 17 (Leppä et al. 2018), it might be challenging to detect the monoisotopic peak. In such a case, it is helpful to use the simulation tools of mass spectrometric software to simulate the isotopic distribution and compare it with the measured one. In the example shown, the experimental data contain many isotopic PA patterns in addition to background ions (Figure 5A). However, with the isotope simulation the correct monoisotopic peaks can be delineated and found to consist of 3 procyanidin and 14 prodelphinidin units (Figure 5B) and of 4 procyanidin and 13 prodelphinidin units (Figure 5C).

It must also be noted that polyphenols can be oxidized into quinone forms during the mass spectrometric analysis and, therefore, there can be minor signals in the mass spectra of polyphenols having the m/z values 2 Da smaller than the corresponding polyphenol (Karonen et al. 2021). However, these minor peaks caused by the oxidation of tannins should not be mixed with A-type PAs having an additional ether bond and a mass difference of 2 Da in comparison to B-type PAs. Figure 6A shows an example of procyanidin dimer having only one B-type linkage (578 Da): the mass spectrum exhibits the deprotonated molecule at m/z 577 and the minor signal corresponding to the oxidation product formed during the analysis at m/z 575. In Figure 6B, the corresponding procyanidin dimer with both A- and B-type linkages (576 Da), exhibiting a deprotonated molecule at m/z 575, can be seen along with minor signals corresponding to the oxidation product at m/z 573.

Despite these few things to be considered in the interpretation of mass spectra, it is still important to remember that multiply charged ions are an advantage in the tannin analysis, as they significantly expand the available mass range. For example, a previous work showed detection of procyanidin polymers up to the degree of polymerization of 22 by using ESI-QTOF-MS: the singly charged ions were dominant for dimers and trimers (578 and 866 Da), doubly charged ions for tetra- to octamers (1154–2306 Da), triply charged ions for nona- to octadecamers (2594–5187 Da) and fourfold charged ions were detected from the hexadecamers onwards (> 4611 Da, (Karonen et al. 2011). When the multiply charged ions are analyzed with ultrahigh-resolution, very complex patterns can be accurately characterized for oligo- and polymeric PAs isolated from *Lysimachia vulgaris* extract as

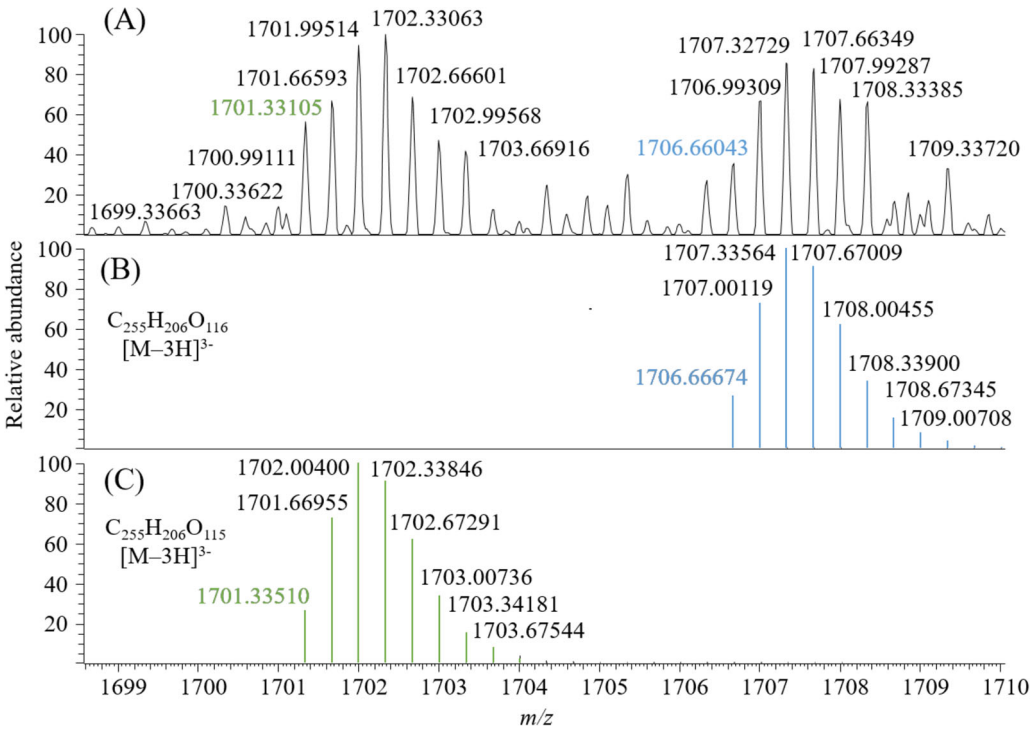


FIGURE 5 | (A) Experimental ESI-MS data in negative ion mode of heptadecameric proanthocyanidins of *Lysimachia vulgaris* with the simulated isotopic patterns of heptadecamers consisting of (B) 3 procyanidin and 14 prodelphinidin units and (C) 4 procyanidin and 13 prodelphinidin units. [Color figure can be viewed at wileyonlinelibrary.com]

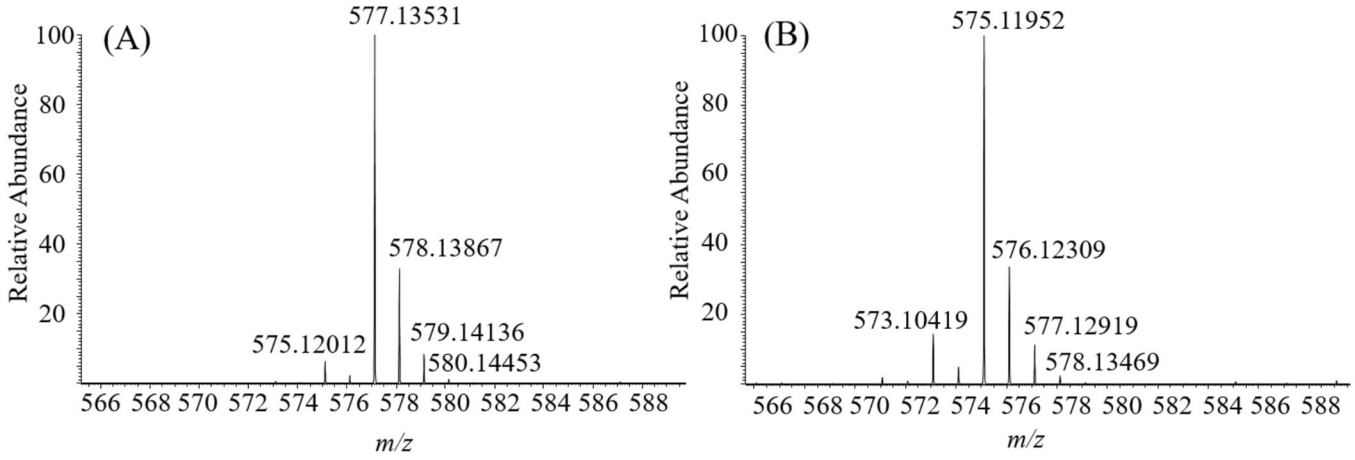


FIGURE 6 | Mass spectra of procyanidin dimers having (A) only one B-type linkage and (B) both A- and B-type linkages obtained by negative ion mode ESI-MS.

shown in Figure 7 (Leppä et al. 2018). The sample contained a diverse mixture of B-type PAs consisting of both procyanidin and prodelphinidin units. Procyanidin oligomers can be observed at m/z $577.13 + 288.06 \times n$ (where n is the number of additional monomeric units attached to the dimer) when they are present as singly charged ions, at m/z $576.13 + 144.03 \times n$ when doubly charged and at m/z $959.87 + 96.02 \times n$ when triply charged and, similarly, prodelphinidin oligomers at m/z $609.12 + 304.06 \times n$ when singly charged, at m/z $608.12 + 152.03 \times n$ when doubly charged and at m/z $1013.19 + 101.35 \times n$ when triply charged. The same systematic approach can be applied when a procyanidin unit is replaced with a prodelphinidin unit (having an additional hydroxyl group) in the PA oligomer. In this case, there is an increment in the m/z value of the ion depending on the charge state of the ions: this increment is +16.00 Da for singly charged ions, +8.00 Da for doubly charged ions, +5.33 Da for triply charged ions and +4.00 Da for fourfold charged ions (Leppä et al. 2018).

The exact masses are needed for correct structural identification and molecular formulae. The differences between the molecular weights of tannins can be minor although the tannin structures are totally different. This phenomenon can be illustrated by comparing a galloylated dimeric PA structure consisting of two procyanidin units and two galloyl groups (molecular formula $C_{44}H_{34}O_{20}$, $M_{calculated}$ 882.16435) with a trimeric PA consisting

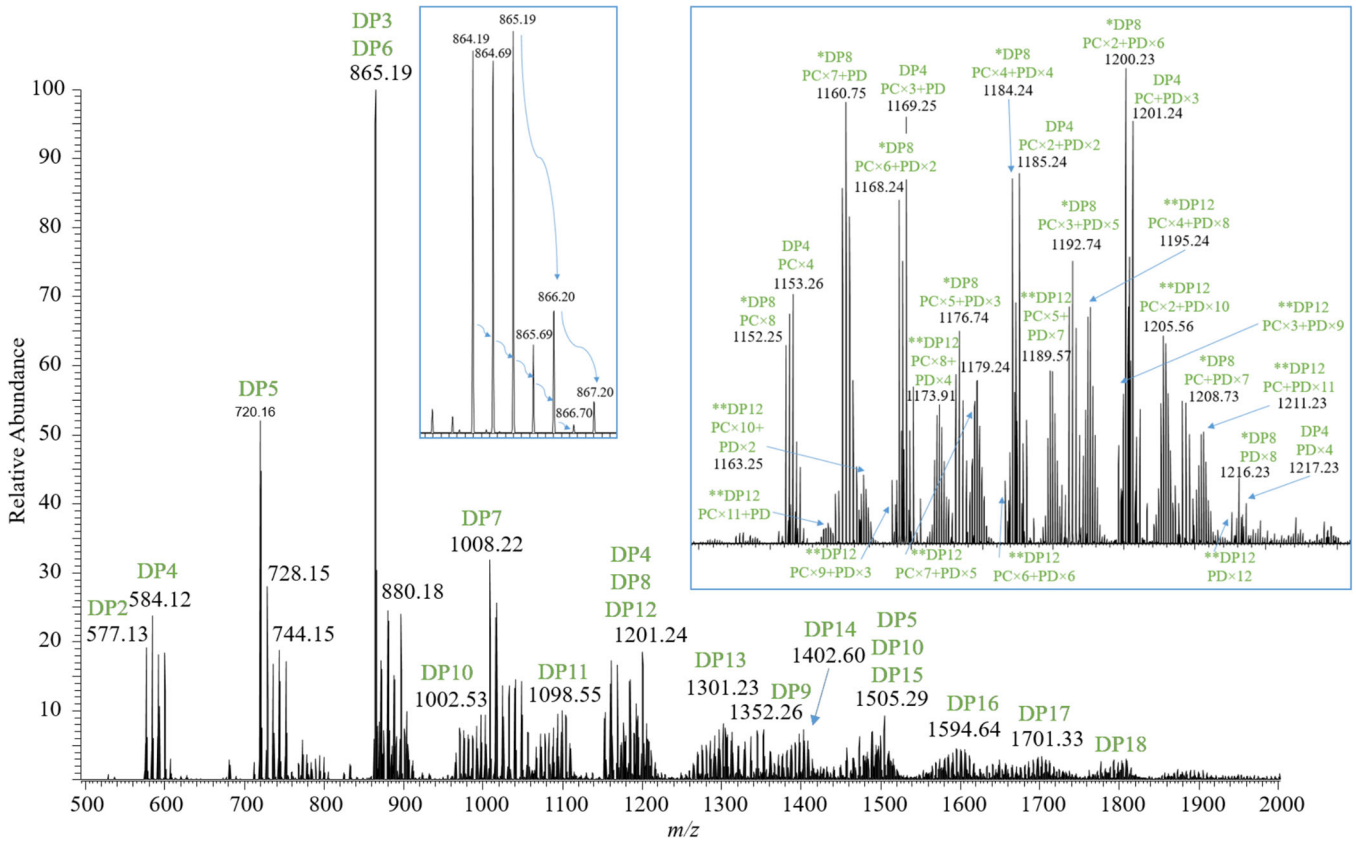


FIGURE 7 | High-resolution mass data of *Lysimachia vulgaris* proanthocyanidins (Leppä et al. 2018) obtained by negative ion mode ESI-MS. DP refers to degree of polymerization, PC to procyanidin unit and PD to prodelphinidin unit. The isotopic patterns of the ions at *m/z* 865 and 1201 are zoomed to highlight the detailed information available. *The ions are $[M–2H]^{2–}$ ions. **The ions are $[M–3H]^{3–}$ ions and the *m/z* values on the top of the peaks refer to the most intensive isotopic peaks, not to the monoisotopic masses. [Color figure can be viewed at wileyonlinelibrary.com]

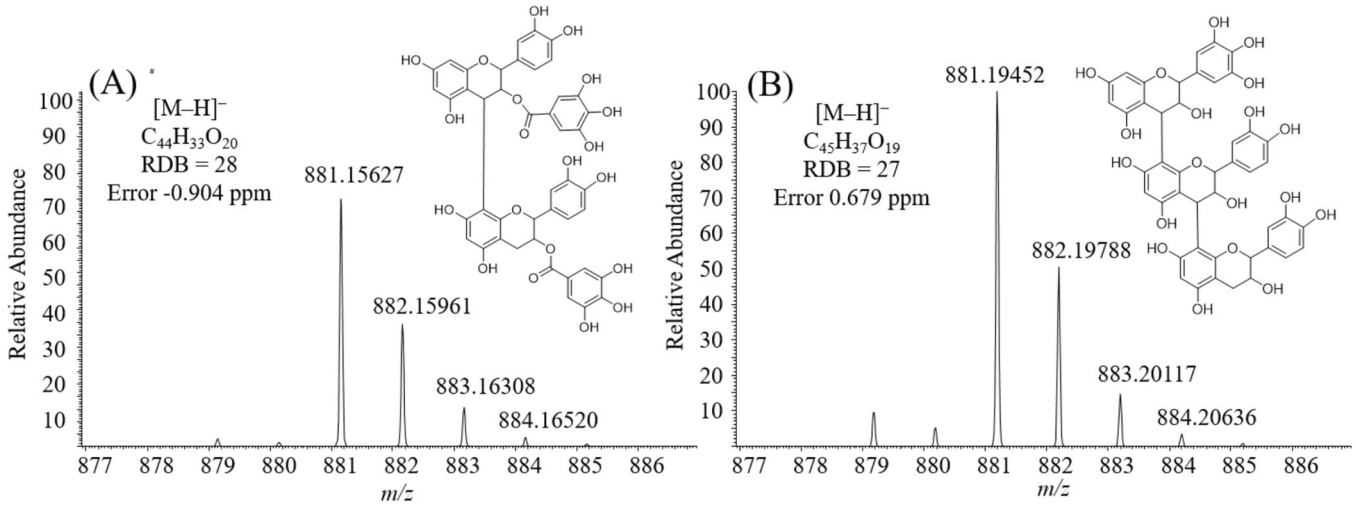


FIGURE 8 | Ultrahigh-resolution mass spectra of (A) a galloylated procyanidin dimer and (B) a proanthocyanidin trimer consisting of two procyanidin and one prodelphinidin units exhibiting almost similar molecular ions in negative ion mode ESI-MS.

of two procyanidin and one prodelphinidin units (molecular formula $C_{45}H_{38}O_{19}$, $M_{calculated}$ 882.20074) (Karonen et al. 2021). The dimer has a deprotonated molecule at *m/z* 881.15267 and the trimer at *m/z* 881.19452 (Figure 8) and these *m/z* values cannot be separated from each other by low-resolution mass analyzers. In addition, cluster and fragment ions can be observed. The fragmentation of tannins is discussed in detail in Chapter 4 (PAs) and Chapter 5 (HTs).

4 | Characteristic Fragmentations of Proanthocyanidins

PAs have very characteristic and well-known fragmentation patterns in negative ion mode ESI-MS analysis (Friedrich et al. 2000; Gu et al. 2003a; Karonen et al. 2004). The fragmentation of heterocyclic ring occurs via retro-Diels–Alder fragmentation or heterocyclic ring fission (Figure 9). The retro-

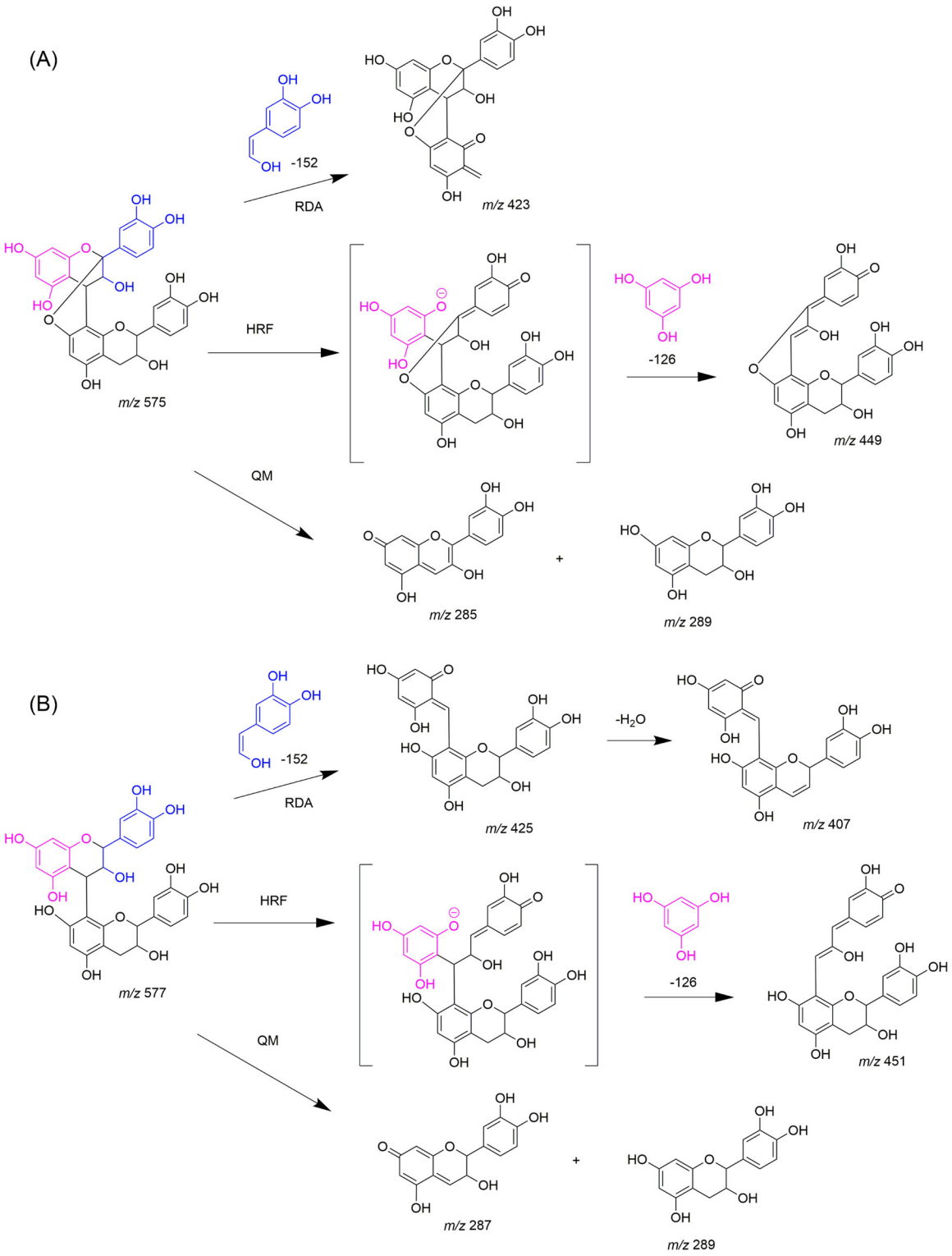


FIGURE 9 | Fragmentation pathways of (A) A-type and (B) B-type procyanidin dimers in negative ion mode ESI-MS. The fragmentation mechanisms are retro-Diels–Alder fragmentation (RDA), heterocyclic ring fission (HRF) and quinone methide cleavage (QM). The unstable intermediate ions are marked with brackets. Structures shown are neutral molecules; the *m/z* values below correspond to the detected ions. [Color figure can be viewed at wileyonlinelibrary.com]

Diels–Alder fragmentation has been considered to be the most important fragmentation reaction as it allows the reliable characterization of PA dimers. The retro-Diels–Alder fragmentation of the extension unit produces fragment ions with larger π–π hyperconjugated systems than the fragmentation of the terminal unit and is therefore considered to be energetically more favorable (Friedrich et al. 2000; Gu et al. 2003a; Karonen et al. 2004). Typically, the retro-Diels–Alder fragmentation is followed by subsequent elimination of water (−18 Da). Sometimes, the abundance of the water loss product is higher than

that of the retro-Diels–Alder product ion (Friedrich et al. 2000). The interflavanoid bond cleaves via quinone methide cleavage or direct cleavage which does not include charge or radical driven rearrangement. The fragmentation patterns are similar for both A-type and B-type PAs as presented for procyanidin dimers in Figure 9 (Friedrich et al. 2000; Gu et al. 2003a; Karonen et al. 2004; Rue et al. 2018; Sui et al. 2016). In retro-Diels–Alder fragmentation, the catechol unit is cleaved producing the ions with *m/z* 423 (A-type) and *m/z* 425 followed by the water elimination producing the ion at *m/z* 407 (B-type). In heterocyclic ring fission, a phloroglucinol unit is cleaved resulting in the ions with *m/z* 449 (A-type) and *m/z* 451 (B-type). The quinone methide cleavage produces the ions with *m/z* 285 (A-type) and *m/z* 287 (B-type) for the extension units and the ion with *m/z* 289 for the terminal units.

The fragmentation patterns are similar for oligomers and polymers but they tend to yield numerous smaller fragment ions rather than a few larger ones (Gu et al. 2003b; Karonen et al. 2004). This also makes the interpretation of their mass spectra challenging as it may be difficult to know with certainty whether an ion is an intact deprotonated molecule or whether it is a fragment ion. Therefore, a good chromatographic separation can support the interpretation of MS data and characterization of PAs; for this purpose diol columns and hydrophilic interaction chromatography are beneficial (Kalili and de Villiers 2010, 2009; Karonen et al. 2011; Kelm et al. 2006). In hydrophilic interaction chromatography-ESI-MS analysis, PAs elute according to increasing degree of polymerization and the sensitivity is enhanced in ESI due to the high proportion of organic solvent used in the gradient (Kalili and de Villiers 2010, 2009; Karonen et al. 2011). In reversed phase liquid chromatographic analysis, the elution order is not directly related to the degree of polymerization. The following order has been generally observed for flavan-3-ols, procyanidin dimers and procyanidin trimers (de Pascual-Teresa et al. 1998; Escribano-Bailon et al. 1992; Zhao et al. 1999):

$$\mathbf{C2} < \mathbf{B3} < \mathbf{B1} < (+) - \mathbf{catechin} < \mathbf{B6} < \mathbf{B4} < \mathbf{B2} < (-) - \mathbf{epicatechin} < \mathbf{B7} < \mathbf{C1} < \mathbf{B5},$$

where C2=catechin-(4α→8)-catechin-(4α→8)-catechin, B3=catechin-(4α→8)-catechin, B1=epicatechin-(4β→8)-catechin, B6=catechin-(4α→6)-catechin, B4=catechin-(4α→8)-epicatechin, B2=epicatechin-(4β→8)-epicatechin, B7=epicatechin-(4β→6)-catechin, C1=epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin, and B5=epicatechin-(4β→6)-epicatechin.

Only shorter PA oligomers can be separated and defined as sharp peaks by reversed phase liquid chromatography. Polymeric PAs are detected by the formation of characteristic large unresolved "humps" in the chromatograms (Adamson et al. 1999; Guyot et al. 1998, 1997; Roux et al. 1998). On the other hand, the challenges of poor separation can be overcome by single and multiple reaction monitoring (SRM and MRM, respectively) methods utilizing a specific PA fragmentation as discussed in Section 3. Even though PAs can be characterized by mass spectrometric techniques, the position and stereochemistry of the interflavanoid linkages (C4 → C8 or C4 → C6) cannot be identified (Friedrich et al. 2000). Moreover, the differentiation of stereoisomers needs additional information, such as the retention order in LC-MS analysis. For example, monomeric flavan-3-ols are known to elute in the following order in reversed phase liquid chromatographic analysis (Koupai-Abyazani et al. 1992):

$$(+) - \mathbf{gallocatechin} < (-) - \mathbf{epigallocatechin} < (+) - \mathbf{catechin} < (-) - \mathbf{epicatechin}.$$

Monomeric units also have their own characteristic product ions, that is, *m/z* 245 for procyanidin unit or *m/z* 261 for prodelphinidin unit, resulting from the cleavage of the heterocyclic ring by the loss of the $-CH_2-CHOH-$ group (Gariboldi et al. 1998; Pérez-Magariño et al. 1999).

5 | Characteristic Fragmentations of Hydrolysable Tannins

The fragmentation patterns of HTs are well studied and documented. However, there are some variations in the reported fragment ions, which mainly stem from the different instrumentation and analysis conditions used (Mena et al. 2012). The fragmentation patterns of negatively charged galloylglucoses and gallotanninss have been studied by utilizing full scan MS (e.g., Barry et al. 2001; Mämmelä et al. 2000; Salminen et al. 1999) but tandem mass spectrometry has enabled more detailed studies on their fragmentation patterns. Galloylglucoses and gallotannins contain two types of acyl moieties that can be differentiated upon fragmentation. The current knowledge of fragmentation patterns of galloylglucoses is summarized in Figure 10. The most characteristic fragment ion for galloylglucoses is a gallate anion at *m/z* 169 which stems from the fragmentation of the galloyl groups attached to the core polyol by ester bonds as gallate ions (Figure 10). Alternatively, the galloyl group may be eliminated as a neutral fragment (152 Da). For example, pentagalloylglucose yields repetitive loss of galloyl moieties (152 Da, Figure 10) resulting in tetra-, tri-, di-, and monogalloylglucose ions (Barry et al. 2001; Salminen et al. 1999; Singh et al. 2016; Hooi Poay et al. 2011). Other fragmentations include the loss of water (−18 Da) and decarboxylation (−44 Da). For the smaller galloylglucoses, di- and monogalloylglucoses, the additional losses of 60 Da (Barry et al. 2001; Soong and Barlow 2005; Zywicki et al. 2002) is caused by the cross-ring fragmentation of a glucose unit (Meyers et al. 2006; Sobeh et al. 2019), for example via removal of formaldehyde moieties (Hooi Poay et al. 2011; Taylor et al. 2005) as presented in Figure 10.

In gallotannins, the depside bonds between the core galloyls and additional galloyl groups are less stable than the ester bonds between the core polyol and therefore undergo fragmentation before the core galloyl groups, leading to the loss of 152 Da (Berardini et al. 2004; Luo et al. 2014; Regazzoni et al. 2013; Salminen et al. 1999). After the removal of these additional galloyl groups, the fragmentation of the remaining galloylglucose structure follows the patterns presented above.

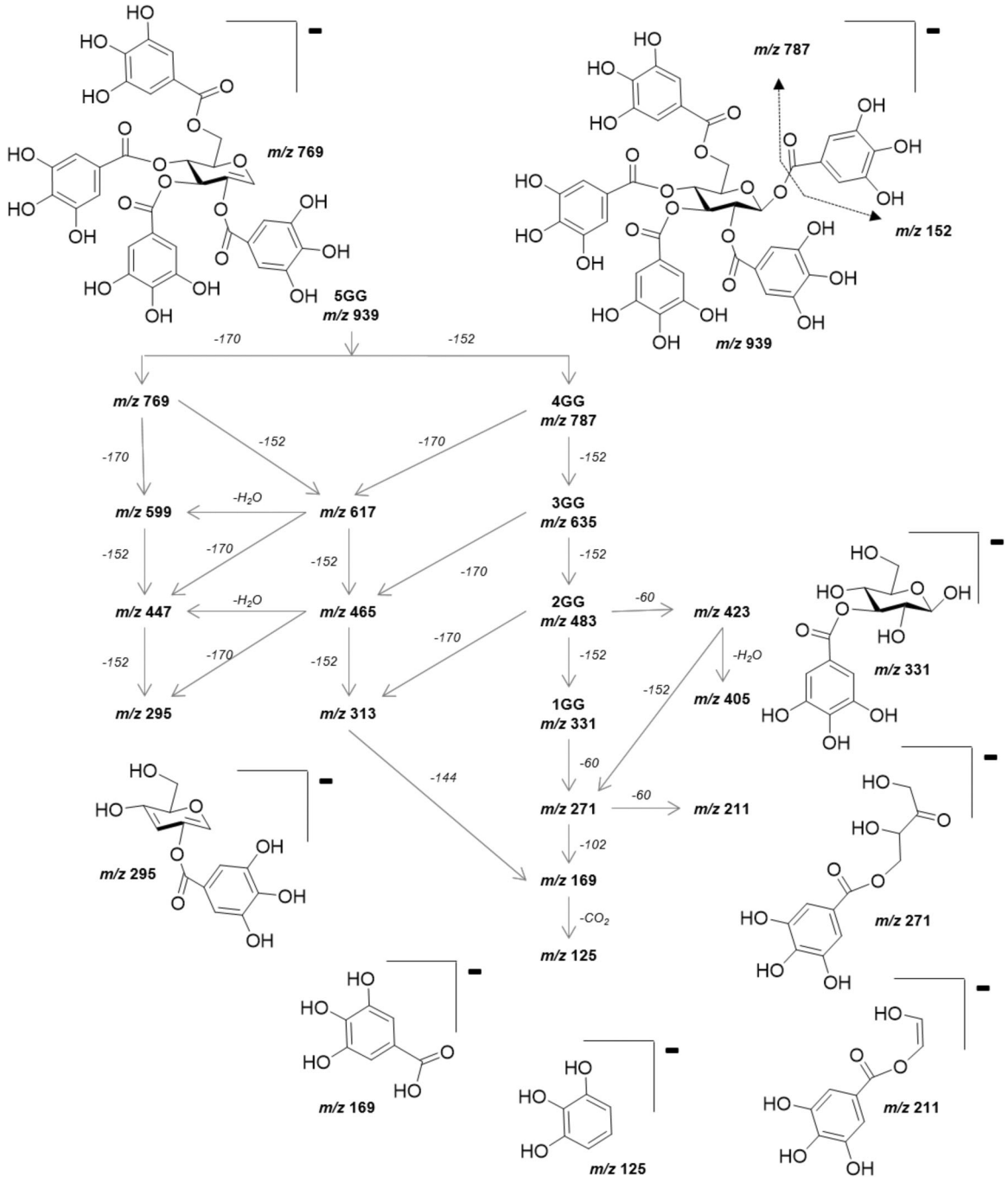


FIGURE 10 | The fragmentation pathways of galloylglucoses in negative ion mode ESI-MS. GG, galloylglucose.

In comparison to galloylglucosess and gallotanninss, ellagitannins are structurally more diverse and correspondingly, their fragmentation patterns in negative ESI-MS are more complex. For primary hexahydroxydiphenoyl esters, such as tellimagrandin II (Figure 2B), the main fragment ions are often related to the galloyl group and the hexahydroxydiphenoyl group, yielding fragment ions of *m/z* 169 (Figure 10) and m/z 301 (Figure 11), respectively (Fracassetti et al. 2013; Moilanen et al. 2013; Mullen et al. 2003; Regueiro et al. 2014; Zywicki et al. 2002). For the further fragmentation, fragment ions at *m/z* 275, *m/z* 257, *m/z* 229 and *m/z* 145 have been reported (Aguilar-Zárate et al. 2017; Bowers et al. 2018; Del Bubba et al. 2012; Duckstein et al. 2013; Engström et al. 2015; Mullen et al. 2003; Singh et al. 2016; Ventura et al. 2024; Yang et al. 2012). Other functional groups also experience characteristic fragmentations, which is a great benefit for structural determination. For example, punicalagin (Figure 2E) with an hexahydroxydiphenoyl group and a gallagyl group, yields fragment ions at *m/z* 301 (hexahydroxydiphenoyl), *m/z* 601 (gallagyl) and *m/z* 781 (gallagyl-glucose) as presented in Figure 12A (Mena et al. 2012; Pfundstein et al. 2010; Seeram et al. 2005). For dehydroellagitannins that contain dehydrohexahydroxydiphenoyl group, such as geraniin (Figure 2C), loss of water from the deprotonated molecule at *m/z* 951 is characteristic, giving a fragment ion at *m/z* 933 for geraniin (Palanisamy et al. 2011). If the ET contains a chebuloyl group instead (Figure 2D), diagnostic fragment ion at *m/z* 337 $[\text{Chebulic acid–H-H}_2\text{O}]^-$ is formed. In addition, fragment ions at *m/z* 319, *m/z* 293 and *m/z*

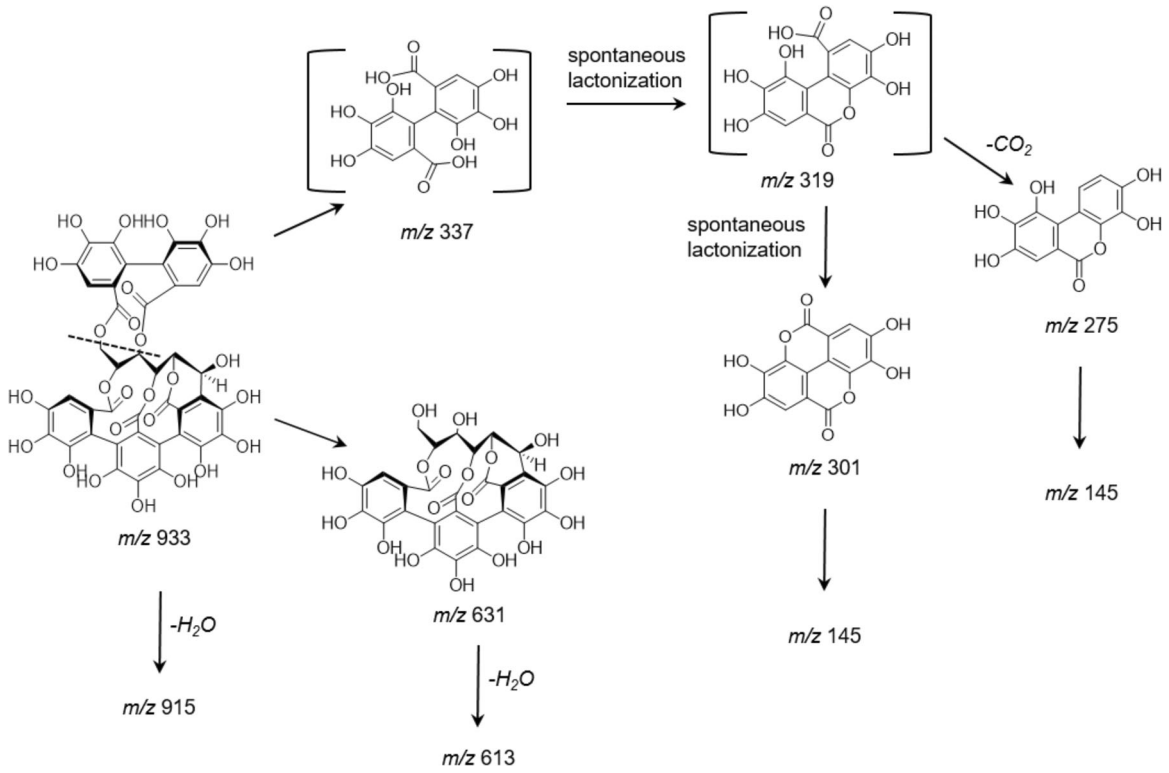


FIGURE 11 | Fragmentation pathway of vescalagin in negative ion mode ESI-MS. The unstable intermediate ions are marked with brackets. Structures shown are neutral molecules; the *m/z* values below correspond to the detected ions.

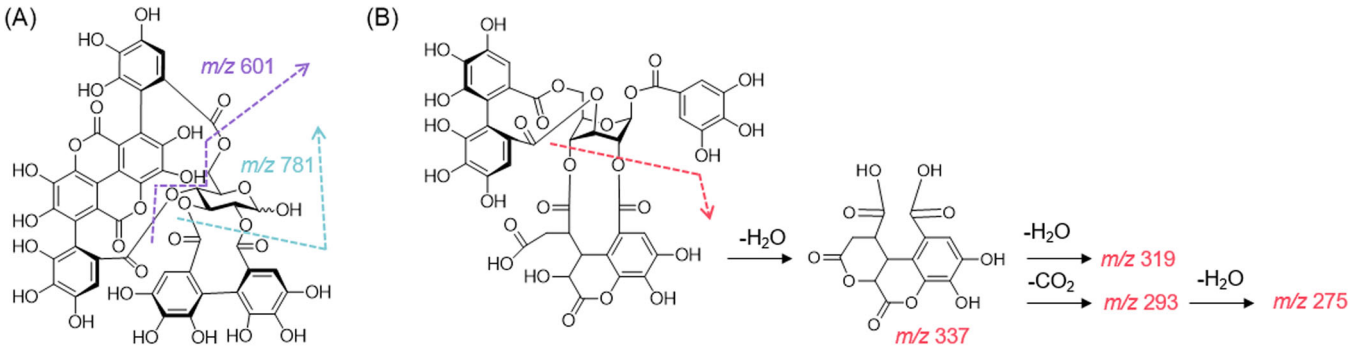


FIGURE 12 | (A) Fragmentation pathway of punicalagin and (B) tentative fragmentation pathway of chebulagic acid in negative ion mode MS. The *m/z* values of deprotonated ions corresponding to neutral structures are presented. [Color figure can be viewed at wileyonlinelibrary.com]

275 are formed due to consequent losses of H_2O or CO_2 as shown in Figure 12B (Pfundstein et al. 2010; Yang et al. 2012). The loss of water is a characteristic fragmentation also for some other ellagitannins and this feature can be used for example to differentiate between the different orientations of the hydroxyl group at the C1 position of the *C*-glycosidic ellagitannin isomers (e.g., vescalagin vs. castalagin, see Figure 2F). The loss of water is seen for vescalagin type β-OH (Figure 11) but not castalagin type α-OH *C*-glycosidic ellagitannins due to steric and intramolecular stabilization effects (Moilanen et al. 2013; Quideau et al. 2005, 2003; Yoshida et al. 1991). Regarding other fragmentations, the nonahydroxytriphenoyl group is rather stable, and therefore vescalagin yields characteristic fragment ions at *m/z* 631 (vescalin) and *m/z* 613 (vescalin-H_2O) (Figure 11). If the carboxylic acid group is free in the ET structure, as it is, for example, in vescavaloninic acid and castavaloninic acid, the cleavage of –COOH (−44 Da) can be detected (Moilanen et al. 2013). In addition, flavanoellagitannins have the characteristic cleavage of the flavan-3-ol moiety (Figure 2G) exhibiting a fragment ion at *m/z* 289.

For oligomeric ellagitannins, the individual monomeric units can be detected (Baert et al. 2015; Del Bubba et al. 2012; Hager et al. 2008; Mullen et al. 2003). In Figure 13, we show an example of a tetrameric ellagitannin having DOG type linkages (Karonen et al. 2010)). In the lower part of the structure is a macrocyclic dimer, oenothein B (1568 Da) which consists of two tellimagrandin I monomers attached via two *m*-DOG type linkages (2DOG, the blue part of the structure in Figure 13). If one additional monomer is linked to oenothein B via one DOG linkage, trimeric oenothein A is formed (MW 2352 Da, the blue and green parts in Figure 13), and if one more monomer is linked via one DOG, a tetramer (MW 3136 Da) is formed (the whole structure in Figure 13). The structure of the tetramer can

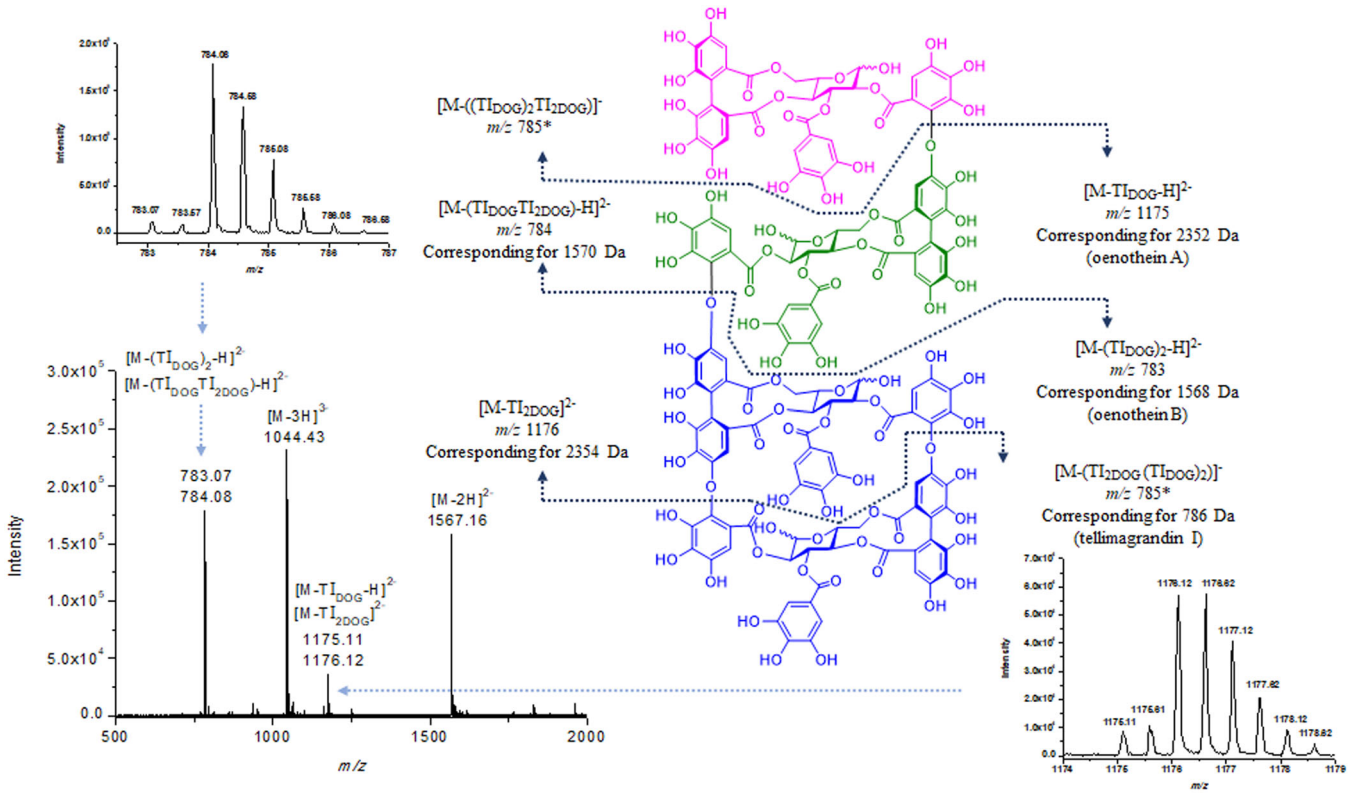


FIGURE 13 | The fragmentation of an ellagitannin tetramer in negative ion mode ESI-MS. On the upper part of the structure, the monomeric units, that is, tellimagrandin I, are linked to the oligomer via one DOG-type linkage (TI_{DOG}) and on the lower part of the structure, via two DOG-type linkages (TI_{2DOG}). The figure is adapted from Karonen et al. (2010). *The signal is obscured by the signal of the $[M–(TI_{DOG}TI_{2DOG})–H]^{2–}$ ion. [Color figure can be viewed at wileyonlinelibrary.com]

be concluded based on its fragment ions: $[M–TI_{DOG}–H]^{2–}$ at *m/z* 1175.11, $[M–TI_{2DOG}]^{2–}$ at *m/z* 1176.12, $[M–(TI_{DOG})_2–H]^{2–}$ at *m/z* 783.07 and $[M–(TI_{DOG}TI_{2DOG})–H]^{2–}$ at *m/z* 784.08 (Karonen et al. 2010). To minimize isobaric interferences, high-resolution MS is needed for the correct identification of these ions (*m/z* 783, 784 and 785, and *m/z* 1175 and 1176).

6 | Quantitative Analysis of Tannins

In general, many instrument and sample related aspects need to be considered in quantitative tannin analysis by mass spectrometry. These include sample preparation, optimization of the ionization and the transmission efficiency of ions, testing of the effects of sample matrix, and optimization of the calibration curves taking into account the standardization either by internal or external standards (Chen et al. 2016). There can be long-term signal variations which affect the repeatability and reproducibility. Quantitative capabilities of mass spectrometry are improved when chromatographic techniques are used as ion suppression and contamination effects are reduced and the effect of sample matrix variability minimized (Chen et al. 2016). It is also practical to combine MS to UV or diode array detector as they are nondestructive, reliable and have wide linear range. In addition, the signal response is not affected by changes in the mobile phase as long as the eluents are UV transparent at the wavelengths used. For example, when oligomeric ellagitannins from willowherb extracts were analyzed with reversed-phase UHPLC-DAD-ESI-QqQ-MS/MS using negative ionization and the MRM mode, relatively high concentrations of the most abundant oligomer, that is, dimeric oenothein B, saturated the detector whereas the scarcer hexamers and heptamers did not (Baert et al. 2015). Therefore, all extracts were diluted to a range where oligomers ranging in size from trimers to hexamers were within the linear range when quantified by MRM and the dimeric oenothein B was quantified from the UV trace at 280 nm (Baert et al. 2015). This method was selective, sensitive, and stable with good reproducibility.

For targeted analysis of tannins, triple quadrupoles (QqQ) and hybrid instruments combining the features of a triple quadrupole and a linear ion trap (Q-Traps) are currently the most used instruments. In comparison to traditional LC-MS, LC–MS/MS provides considerable noise reduction and sensitivity improvements due to enhanced specificity of the SRM/MRM scan modes. QqQ and Q-Trap instruments are categorized as low-resolution mass spectrometers. Practically, they can measure the *m/z* ratio of an ion to the nearest integer value (Adrey 2003). However, different modes of data collection can be utilized, such as full scan, product ion scan, precursor ion scan, neutral loss scan, and selected/multiple reaction monitoring. In hybrid Q-Trap instruments, the final quadrupole can function as an ion trap providing MS(n) capabilities and slightly better mass resolution when using enhanced MS scan or enhanced product ion scanning (Sargent 2013).

The main benefit of QqQ and Q-Trap instruments in tannin analysis is that several hundred mass transitions can be

measured during one run when utilizing SRM/MRM methodology. In general, in the SRM/MRM analysis of an analyte, the precursor ion and its most abundant fragment ion are selected for the quantitative SRM/MRM transition and the second most abundant fragment ion is used for the qualitative MRM transition (Baert et al. 2015; Saha and Imran 2020). When the molecular weight of the tannin exceeds the mass range of the analyzer, multiply charged ions are utilized (Baert et al. 2015). To measure hundreds of transitions in one run, the MS/MS method must be divided into several time-scheduled windows with different SRM/MRM channels and time segments (Guillarme et al. 2010). By focusing a limited number of transitions within each MRM window, the instrument can allocate longer dwell times for each transition. For example, Baert et al. developed an MRM method for the analysis of seven oligomeric ellagitannins in willow herb leaves: in the method, dwell times between 16 and 25 ms were used in UHPLC-DAD-ESI-QqQ-MS/MS to secure at least 12 data points per UHPLC peak for quantitative work (Baert et al. 2015). On the other hand, when Lambert et al. analyzed 154 polyphenols in wine, including flavan-3-ols and dimeric PAs, the dwell time of overlapping analyte peaks were reduced to 5 ms (Lambert et al. 2015). By this, the full capacity of the mass spectrometer was harnessed and 10–15 data points per analyte peak obtained. The short dwell time did not yet cause problems but it is worth noticing that shorter dwell times can lead to lower measurement precision and sensitivity as the instrument has less time to collect data for each ion transition. Importantly, to avoid problems related to retention time variations with UHPLC analyses, one should always have a proper width in the MRM window (retention time segmentation), for example, three times the MRM peak width. If the time windows become very narrow, small fluctuations outside the original time-windows might cause partial loss of detection (Guillarme et al. 2010). Nevertheless, the modern MS/MS instruments can monitor up to 150 simultaneous transitions with enough data points per peak for quantification purposes (Ehrhardt et al. 2014; Lambert et al. 2015; Liebler and Zimmerman 2013).

Quantitative studies of tannins using MS/MS are scarce compared to the numerous investigations focused on identification. At least two challenges may explain this. From a practical point of view, the absolute tannin quantitation with SRM/MRM is constrained by the diversity of tannins. Thousands of tannin structures are found in the plant kingdom while the number of commercial standards is limited. Compound-specific SRMs/MRMs are optimized separately for individual analytes, at least, by optimizing the cone voltage for the precursor ion and collision energies for the generation of fragment ions for both quantitative and qualitative MRM transitions (Tsakalof et al. 2024). For quantitation, typically the most abundant fragment ion is selected, while the second most abundant fragment ion is used to qualitatively ensure accurate identification. The ratio between the peak areas corresponding to the quantitative and qualitative MRM transitions should remain consistent across different samples and matrices. For method development, pure compounds should be used whenever possible. In practice, this requires the isolation and purification of the reference tannin materials. Although monomers and small oligomers can be purified and isolated to provide reference standards (Moilanen et al. 2013; Moilanen and Salminen 2008; Pfundstein et al. 2010; Saha and Imran 2020; Salminen and Karonen 2011; Tuominen and Sundman 2013), the purification of larger oligomers and polymers is difficult due to the number of isomers and consequent chromatographic behavior of the larger tannins discussed in Section 4. While method optimization can be performed using small amounts of reference standards, accurate quantification requires purified reference materials in larger quantities; milligrams are needed for each tannin. Therefore, studies often utilize relative quantification and express quantities as equivalents of selected tannins (Lambert et al. 2015; Pfundstein et al. 2010; Tuominen and Salminen 2017). Table 1 includes examples of SRM and MRM transitions used in the literature for tannin quantitation.

For mere screening purposes, the traditional compound-specific SRM/MRM methodology faces certain constrains due to two challenges—individual optimization of MRM transitions and lack of reference standards, as mentioned above. When screening thousands of plant samples for their bioactive polyphenol content, the primary interest is often in the relative abundance of polyphenol subgroups rather than the precise quantification of individual compounds. These kind of data can be acquired in tannin analysis via qualitative parent ion scan and neutral loss scan modes (Bai et al. 2022; Robbins et al. 2014), group-specific MRMs (Engström et al. 2015, 2014; Salminen 2018) or more commonly, via chemical depolymerization or hydrolysis of the tannins before their UHPLC-MS/MS analysis (Deshaies et al. 2022; Giribaldi et al. 2020; Mouls et al. 2011; Singh et al. 2016).

The parent ion and neutral loss scanning methods suffer from the time needed for data generation and are not suitable for the simultaneous detection or quantitation of many types of polyphenol groups (Engström et al. 2015, 2014; Salminen 2018). In contrast, the SRM/MRM method has the advantage of short scanning times as one MRM transition can be measured as fast as in 5 ms, making it especially suitable for the group-specific analysis. Traditionally, the SRM/MRM methods of triple quadrupoles utilize Q1 for precursor selection, Q2 for fragmentation, and Q3 for product ion selection, however, many instruments enable also the use of collision-induced in-source fragmentation by increasing the cone voltage difference between the sample cone and the extraction cone above a certain limit (Adrey 2003; de Hoffmann and Stroobant 2001; Salminen 2018). This in-source fragmentation technique allowed the development of group-specific UHPLC-MS/MS methods for tannin analyses (Engström et al. 2015, 2014; Salminen 2018). Using the methods developed, MRM chromatograms serving as fingerprints of HTs and PAs, and other compounds, such as gallic and quinic acid derivatives, and quercetin, kaempferol and myricetin glycosides, were recorded alongside with UV and mass spectra. Thus, an overview of fingerprints of eight common polyphenol classes was achieved in a single run. Furthermore, when reference standards are available, the information can be transformed into quantitative data (Engström et al. 2015, 2014; Salminen 2018).

The so-called Engström method relies on the traditional grouping of tannins based on their chemical structures (Engström et al. 2014). Compounds in the same polyphenol group typically share similar or identical building blocks (see

TABLE 1 | Examples of transitions used in single/multiple reaction monitoring methods in negative ion mode in literature.

Compound	Nominal mass	Precursor ion (m/z)[a]	Product ion (quant./qual.) (m/z)[a]	References
Monogalloylglucose	332	331	169/271	Zywicki et al. (2002); Kårlund et al. (2014); Tuominen and Salminen (2017); Saha and Imran (2020)
Digalloylglucose	484	483	169/211	Zywicki et al. (2002); Tuominen and Salminen (2017); Saha and Imran (2020)
Trigalloylglucose	636	635	169	Zywicki et al. (2002)
		635	465/169	Kårlund et al. (2014); Tuominen and Salminen (2017); Saha and Imran (2020)
Tetragalloylglucose	788	787	169	Tuominen and Salminen (2017)
		787	483/634	Saha and Imran (2020)
Pentagalloylglucose	940	939	169	Tuominen and Salminen (2017)
		939	769/617	Lin et al. (2015); Bae et al. (2017); Saha and Imran (2020)
Hexagalloylglucose	1092	1091	787	Tuominen and Salminen (2017)
		1091	939/769	Saha and Imran (2020)
Heptagalloylglucose	1244	621	169	Tuominen and Salminen (2017)
		1243	939/1091	Saha and Imran (2020)
Octagalloylglucose	1396	1395	939/769	Saha and Imran (2020)
Tellimagrandin I	786	785	301/275	Kårlund et al. (2014); Saha and Imran (2020)
Tellimagrandin II	938	937	301	Tuominen and Salminen (2017)
Pedunculagin	784	783	301/275	Kårlund et al. (2014); Saha and Imran (2020)
2,3-(*S*)-HHDP-glucose	482	481	301	Kårlund et al. (2014)
Strictinin	634	633	301	Saha and Imran (2020)
Galloyl-HHDP-glucose	634	633	301	Kårlund et al. (2014)
Casuarictin	936	935	633/301	Gasperotti et al. (2015)
Vescalagin	934	933	301	García-Estévez et al. (2012); Engström et al. (2015)
		933	915/301	Saha and Imran (2020)
		466	301	Stark et al. (2010)
Castalagin	934	933	301/425	Saha and Imran (2020)
		933	631	García-Estévez et al. (2012)
		466	301	Stark et al. (2010)
Grandinin/roburin E	1066	1065	249	García-Estévez et al. (2012)
Vescavaloninic acid	1102	1101	1083/569	Saha and Imran (2020)
Castavaloninic acid	1102	1101	1057/425	Saha and Imran (2020)
Acutissimin A/B	1206	602	457	Stark et al. (2010)
Epiacutissimin A/B	1206	602	457	Stark et al. (2010)
Geraniin	952	951	933	Tuominen and Salminen (2017)
		951	301/933	Saha and Imran (2020)
Carpinusin	952	951	933	Tuominen and Salminen (2017)

(Continues)

TABLE 1 | (Continued)

Compound	Nominal mass	Precursor ion (m/z)[a]	Product ion (quant./qual.) (m/z)[a]	References
Geraniic acid	952	907	291	Tuominen and Salminen (2017)
Ascorgeraniin	1110	1109	933	Tuominen and Salminen (2017)
		1109	301/973	Saha and Imran (2020)
Sylvatiin A	1144	571	169	Tuominen and Salminen (2017)
Sylvatiin B	992	495	169	Tuominen and Salminen (2017)
Sylvatiin C	1348	673	169	Tuominen and Salminen (2017)
Sylvatiin D	1160	579	169	Tuominen and Salminen (2017)
Sylvatiin E	840	839	373	Tuominen and Salminen (2017)
Chebulagic acid	954	953	301/275	Kumar et al. (2017); Tuominen and Salminen (2017); Saha and Imran (2020)
Chebulanin	652	651	169/481	Saha and Imran (2020)
Chebulinic acid	956	955	169/205	Saha and Imran (2020)
		955	337/617	Kumar et al. (2017)
Oenothein B	1568	783	765/301	Baert et al. (2015); Engström et al. (2015)
Oenothein A	2352	1176	301/275	Baert et al. (2015)
Tetrameric ET	3136	1045	301/275	Baert et al. (2015)
Pentameric ET	3920	1306	301/275	Baert et al. (2015)
Hexameric ET	4704	1568	301/275	Baert et al. (2015)
Heptameric ET	5488	1829	275/301	Baert et al. (2015)
Sanguiin H6	1870	934	633/301	Gasperotti et al. (2015)
		934	301/275	
Gemin A	1872	935	301/275	Saha and Imran (2020)
Agrimoniin	1870	934	633/301	Gasperotti et al. (2015)
		934	301	Kårlund et al. (2014)
Lambertianin C	2748	935	301/275	Saha and Imran (2020)
Cocciferin D2	1868	933	301/275	Saha and Imran (2020)
Rubusuaviin C	2804	934	301/275	Saha and Imran (2020
Salicarinin A	1868	933	249/301	Saha and Imran (2020)
Salicarinin B	1868	933	301/249	Saha and Imran (2020)
Rugosin D	1874	935	301/275	Saha and Imran (2020)
Rugosin E	1722	860	301/275	Saha and Imran (2020)
Proanthocyanidin B1	578	577	289/407/125	Delgado de la Torre et al. (2013); Ehrhardt et al. (2014)
		577	425/407	Margalef et al. (2014)
Proanthocyanidin B2	578	577	289/407/125	Delgado de la Torre et al. (2013); Ehrhardt et al. (2014)
		577	289/425	Ortega et al. (2010)
		577	425/407	Margalef et al. (2014)
Proanthocyanidin B3	578	577	289/407	Ehrhardt et al. (2014)
		577	425/407	Margalef et al. (2014)
Proanthocyanidin A2	576	575	285/539/449	Delgado de la Torre et al. (2013)

(Continues)

TABLE 1 | (Continued)

Compound	Nominal mass	Precursor ion (m/z)[a]	Product ion (quant./qual.) (m/z)[a]	References
A-type procyanidin dimer	576	575	285	Pérez-Jiménez and Torres (2012)
B-type prodelphinidin dimer	610	609	125	Tuominen and Salminen (2017)
A-type prodelphinidin dimer (PD-PC)	592	591	465	Pérez-Jiménez and Torres (2012)
B-type propelargonidin dimer (PP-PC)	562	561	289	Pérez-Jiménez and Torres (2012)
B-type procyanidin trimer	866	865	289	Kårlund et al. (2014)
		865	577/695	Ortega et al. (2010); Pérez-Jiménez and Torres (2012)
B-type propelargonidin trimer	850	849	577	Pérez-Jiménez and Torres (2012)
PA tetramer	1154	1153	865/575	Ortega et al. (2010)
PA pentamer	1442	1441	1028/1151	Ortega et al. (2010)
PA hexamer	1730	1729	1153/863	Ortega et al. (2010)
PA heptamer	2018	1008	865/575	Ortega et al. (2010)
PA octamer	2306	1152	875/983	Ortega et al. (2010)
PA nonamer	2594	1296	577/1152	Ortega et al. (2010)

Abbreviations: ET = ellagitannin, HHDP = hexahydroxydiphenoyl, PA = proantocyanidin, PC = procyanidin, PD = prodelphinidin, PP = propelargonidin
[a]Values with decimals were rounded to unit mass.

Figure 1). Regarding tannins, all galloylglucoses and gallotannins yield a gallic acid fragment ion at m/z 169, see Figure 10) that could be detected by the parent ion scan of m/z 169 in negative ion mode (Engström et al. 2015; Salminen 2018). Similarly, all hexahydroxydiphenoyl-containing ellagitannins yielded a fragment ion at m/z 301 (see Figure 11). When these ions were selected in Q1 as precursor ions, followed by fragmentation in Q2 and subsequent qualifier and quantifier selection in Q3, group-specific MRM methods were produced for HTs (Engström et al. 2015).

The need for rapid screening methods for PAs is even more urgent due to the chromatographic challenges and poor ionization of the larger PAs in their analysis via LC-MS (see Section 4). To facilitate the rapid analysis of PAs, a method was developed to separately measure the procyanidin and prodelphinidin units in the PA structure (Engström et al. 2014). Conceptually, the method is similar to the widely adopted thiolysis and phloroglucinolysis methods (Thompson et al. 1972; Hemingway 1989b; Matthews et al. 1997; Fulcrand et al. 1999; Guyot et al. 2001; Kennedy and Jones 2001), which are used to depolymerize PAs into smaller units for analysis. However, instead of using chemical reactions for the depolymerization of PAs, this method fragments them in the ESI interface of a mass spectrometer. The quinone-methide fragmentation in the ion-source allows determining the procyanidin/prodelphinidin ratios and the mean degree of polymerization for the PAs present in any studied sample. The most powerful feature of the method is that the degradation of the PAs is done after the chromatographic step. This is beneficial as the original compound profile is retained in the form of chromatographic fingerprints (Engström et al. 2015, 2014; Salminen 2018). Consequently, it allows not only qualitative but also quantitative comparison via sample-specific fingerprints of the PA composition, procyanidin/prodelphinidin ratio and mean degree of polymerization throughout the chromatographic hump produced by the larger PA oligomers and polymers (Engström et al. 2014; Salminen 2018). The group-specific methodology can be applied also for other compound groups if they share a common structural unity. For example, Laitila et al. developed a group-specific method for the analysis of anthocyanins and anthocyanin adducts in red wines, which utilizes the same in-source fragmentation approach (Laitila et al. 2019).

7 | Bioactivities of Tannins by Mass Spectrometric Tools

In addition to the qualitative and quantitative analysis of tannins as such, MS can be used as a tool for bioactivity studies. When a studied plant species expresses certain bioactivity, the next step is to understand the underlying mechanisms and what type of compounds are the most active and why. In plants, the protective role of tannins against herbivory and many pathogens actualizes through two main mechanisms, protein binding and oxidation reactions (Appel 1993; Salminen and Karonen 2011). In addition to the plant–herbivore interplay, tannin–protein interactions are important in numerous other plant related domains and the interest in studying the complexes between tannins and proteins has increased steadily during the past few decades. Mass spectrometry has been widely used to study tannin–protein complexes and to understand the exact roles of tannins and proteins in these

interactions (Baron et al. 2019; Canon et al. 2015, 2011, 2009; Di et al. 2024; Dias et al. 2016; Engström, Sun et al. 2016; Ghahri et al. 2021; Manjón et al. 2024; Mau et al. 2011; Sarni-Manchado and Cheynier 2002; Shraberg et al. 2015; Simon et al. 2003). To explain the mechanisms behind tannin-protein interactions and to understand what types of tannins are the most active, pure compounds must be utilized in the activity tests.

While the protein precipitation capacity of tannins, that is, ability to precipitate proteins and other macromolecules out of aqueous solution, has traditionally been recognized as the source of defensive function against herbivory, oxidation and the following reactions are more prevalent at high pH (Appel 1993; Gross et al. 2008; Johnson and V. Barbehenn 2000; Kim et al. 2018; Salminen and Karonen 2011). Tannins can be oxidized even at low to neutral pH by plant polyphenol oxidases and peroxidases (Appel 1993; Kim et al. 2018; Salminen and Karonen 2011). LC-MS has been used to study the oxidation of both purified tannins and tannins present in plant extracts to explore the structure–activity relationships (Kim et al. 2020, 2018; Tuominen and Sundman 2013; Vihakas et al. 2014). Moreover, the in vitro oxidation of plant tannins in insect herbivores has been examined by LC-MS (Salminen and Lempa 2002; Vihakas et al. 2015). In these applications, the sensitivity of LC-MS and LC-MS/MS is needed due to the small amounts of sample materials and low tannin concentrations, where less sensitive methods, such as LC-DAD, are inadequate for quantifying all compounds present in the sample.

The structural changes of HTs caused by oxidation on a molecular level can be revealed by NMR spectroscopy (Hatano et al. 2004; Tanaka et al. 1990) but its use is limited to stable oxidation products that can be isolated in large quantities (mg scale). LC-MS and especially LC-DAD-MS, are effective tools to study the oxidative activity and kinetics of oxidation of purified HTs (Tuominen and Sundman 2013). These approaches also allow the tentative identification of the main degradation products based on UV and MS spectra without isolating the compounds. Regarding PAs, their oxidation reactions are generally well-known but site of modification has been reported mainly for individual smaller PAs (Burger et al. 1990; Ferreira et al. 1992; Guyot et al. 1996; Hibi and Yanase 2019; Laks et al. 1987). Earlier, the definitive structural studies of PAs and their oxidation products often relied on the use of NMR (Burger et al. 1990; Ferreira et al. 1992; Laks et al. 1987). Lately, MS has been successfully applied for the analysis of PA oxidation (Chen et al. 2014; Imran et al. 2021; Karonen et al. 2021; Kondo et al. 2000; Mouls and Fulcrand 2012). These studies showed different reactivities for procyanidins and prodelphinidins, suggesting that the presence of prodelphinidin units enhanced the probability of modification reactions. By high-resolution MS and by measuring qualitative changes in the procyanidin/prodelphinidin ratio and mean degree of polymerization by MRM methods, the main reaction routes for both A- and B-type PAs were concluded to be intramolecular (Imran et al. 2021; Karonen et al. 2021). For prodelphinidin-rich and galloylated PAs, also intermolecular reactions were indicated (Imran et al. 2021; Karonen et al. 2021).

One special field where MS is often used to study tannin oxidation is the studies related to wine production. During wine aging, the controlled oxygen levels allow the wine to evolve, particularly via the chemical modification of the tannins, for example, via oxidation of tannins and the following inter- and intramolecular reactions (Arapitsas et al. 2014; Petit et al. 2019). Tannins and their oxidation products are important for the sensory quality of the wine; they are thus responsible for astringency, bitterness and contribute to its color. The ability to measure the oxidative evolution of the wine tannins enables to predict the optimal aging times when linked to the modulation of astringency feeling (Deshaies et al. 2022). Often untargeted approaches are used to evaluate the overall changes of tannins in the wines (Arapitsas et al. 2020; Arbulu et al. 2015; Castro et al. 2014) but alternatively targeted analysis utilizing marker ions for the quantification of specific changes can be used (Deshaies et al. 2022; Mouls and Fulcrand 2012; Ontañón et al. 2020). In addition, the changes can be measured indirectly by determining the disappearance of the original PAs. All these approaches face the same constrains as PA analysis in general: the chromatographic co-elution of the numerous isomers creates the characteristic chromatographic hump and the efficiency of ionization is reduced by the saturation of more abundant PAs (Mouls et al. 2011). Moreover, in comparison to intact tannins, their oxidation further increases the chemical diversity and structural complexity making their identification challenging (Imran et al. 2021; Karonen et al. 2021; Mouls and Fulcrand 2012).

Chemical depolymerization of the PAs to their monomeric units are often applied to measure the changes in their concentration and mean degree of polymerization. This is especially beneficial when searching for specific oxidation markers. The new covalent bonds formed in oxidized PA structures are not depolymerized efficiently, as oxidation makes the initial PAs resistant to the depolymerization conditions (Mouls and Fulcrand 2015, 2012). Thus, dimeric and trimeric oxidation markers are observed after chemical depolymerization of the tannins (Deshaies et al. 2022) and these oxidation markers can be used to evaluate the oxidation state of the wine, for example, for quality control purposes. However, these chemical depolymerization methods do not enable characterization of the changes in the overall profiles of the oligomeric and polymeric PAs as the information on individual PA oligomers or polymers and their oxidized products are lost. To avoid this, Imran et al. utilized a group-specific approach to study the oxidation and stability of different types of natural PAs via nonspecific alkaline oxidation (Imran et al. 2021). This approach allowed quantifying the decrease in PA concentration and changes in PA composition as mentioned above. However, there is still a need for new analytical method developments in the identification and characterization of the oxidation products of PAs by MS. Many of the oxidation products are not detected under the standard ESI-MS conditions even though a hump indicating PAs is detected by UV at 280 nm (Deshaies et al. 2022; Karonen et al. 2021).

The ability of tannins to interact with proteins in biological systems is important for many plant related domains, including the most basic functions in plant physiology and ecology, but also increasingly in applications related to agriculture, foods/feeds and medicine. Due to the increasing interest in these interactions, their consequences, and their utilization in various

applications, numerous physicochemical methods have been developed over the years to study, for example, complex formation, protein precipitation, binding affinities, binding stoichiometry, conformational changes, and kinetics (Baron et al. 2019; Canon et al. 2015, 2011, 2009; Di et al. 2024; Dias et al. 2016; Engström et al. 2022, 2019; Engström, Sun et al. 2016; Ghahri et al. 2021; Mau et al. 2011; Sarni-Manchado and Cheynier 2002; Shraberg et al. 2015; Simon et al. 2003).

MS has many benefits when studying tannin–protein complexes. The underlying reasons are the same as those mentioned above for other applications: it is a sensitive technique, and therefore, only small sample volumes are required. In addition, it allows the determination of the stoichiometry of the formed complexes, the tentative mechanism of complex formation and the structures of the complexes formed (Baron et al. 2019; Canon et al. 2015, 2011, 2009; Di et al. 2024; Dias et al. 2016; Engström, Sun et al. 2016; Ghahri et al. 2021; Mau et al. 2011; Nguyen et al. 2021; Sarni-Manchado and Cheynier 2002; Shraberg et al. 2015; Simon et al. 2003). The main challenge in MS analysis is the stability of the noncovalent tannin–protein complexes. Partially due to this, soft ionization techniques MALDI (Engström, Sun et al. 2016; Ghahri et al. 2021; Mau et al. 2011; Simon et al. 2003) and ESI (Baron et al. 2019; Canon et al. 2015, 2011; Dias et al. 2016; Sarni-Manchado and Cheynier 2002; Shraberg et al. 2015) are the most used ionization techniques. The simplicity of MALDI benefits data interpretation as only singly charged ions are present in the mass spectra of the tannin–protein complexes as shown in Figure 14 (Mané et al. 2007). However, MALDI may suffer from low sample-to-sample and scan-to-scan reproducibility and the signal level strongly depends on the laser beam homogeneity and irradiance, sample preparation (i.e., the choice of a suitable matrix and the solvents), the analyte properties, sample purity, as well as the crystallization process of the sample (Gusev et al. 1995). In addition, neither ESI nor MALDI can unambiguously describe the tannin–protein complexes as noncovalent or covalent, which is often a primary interest (Engström, Sun et al. 2016). In ESI, tentative identification of complex types and evaluation of complex strengths can be achieved by increasing the ionization voltage, which promotes dissociation of noncovalent complexes in the gas phase through ion–molecule collisions at the atmospheric–vacuum interface. However, this is also a source of misinterpretation, as detecting complexes requires optimizing ionization to increase sensitivity while minimizing the dissociation of noncovalent complexes (Canon et al. 2009; Sarni-Manchado and Cheynier 2002). Also, as the complexes are analyzed in the gas phase, the results cannot be directly reflected to the reactions in solution (Loo 1997; Sarni-Manchado and Cheynier 2002).

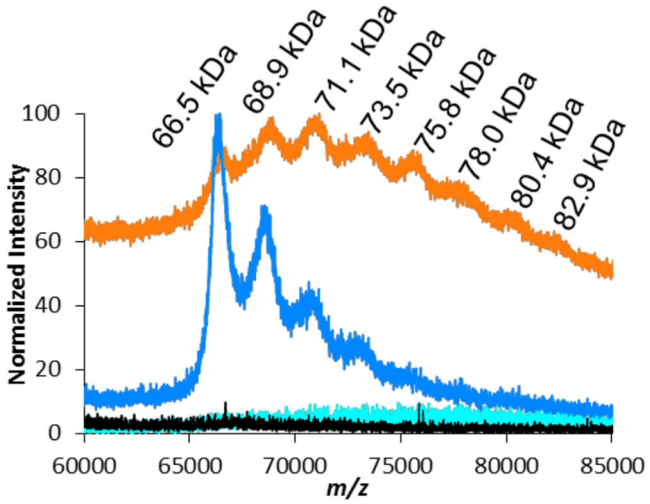


FIGURE 14 | Example of data produced with MALDI-TOF in positive ion mode when bovine serum albumin (average MW 66.5 kDa) and oligomeric ellagitannin oenothein A (MW 2353.6 Da) were incubated at pH 5 (black color) or pH 6.7 (orange color), and after removal of unstable complexes by ultrafiltration (pH 5 turquoise, pH 6.7 blue). Figure modified from (Engström, Sun et al. 2016). [Color figure can be viewed at wileyonlinelibrary.com]

The analyzers used for detection of tannin–protein complexes are variable including quadrupoles (Q and QqQ), TOFs, quadrupole ion traps, orbitraps, FT-ICR instruments, and different hybrid mass analyzers (Baron et al. 2019; Canon et al. 2015; Dias et al. 2016; Engström, Sun et al. 2016; Perez-Gregorio et al. 2014; Sarni-Manchado and Cheynier 2002; Shraberg et al. 2015; Vergé et al. 2002). Also, ion mobility spectrometry has been successfully used to study the interactions between human salivary proline-rich proteins and the galloylated flavan-3-ol epigallocatechin gallate (Canon et al. 2011). It offers the significant advantage of enabling the determination of complex distributions and the structural isomers of each species (Bowers et al. 1993; Niu et al. 2013). While other mass spectrometric methods evaluate tannin–protein complexation and the resulting complexes as a static process, ion mobility spectrometry enables the assessment of complexation as a dynamic process, in which conformational changes in tannin and protein structures play a key role in the observed function or activity (Canon et al. 2011).

8 | Conclusions

Various mass spectrometric techniques provide powerful tools for the characterization and quantification of tannins. In the qualitative analysis of diverse oligo- and polymeric tannins, ESI combined with high-resolution mass analyzers is particularly effective, as ESI enables the ionization of high-molecular-weight tannins in the form of multiply charged ions, thereby significantly expanding the accessible mass range. For quantitative tannin analysis, tandem MS techniques and MRM stand out due to their superior specificity and sensitivity. These methods can also provide structural information, such as the procyanidin/prodelphinidin ratio and mean degree of polymerization for PAs, as well as the presence of galloyl and hexahydroxy groups in HT structures. Mass spectrometry is also well-suited for investigating the bioactivities of tannins, particularly in elucidating tannin–protein interactions. Future advancements in MS technologies, including the integration of conventional ionization methods with novel ionization processes, may offer enhanced capabilities for tannin imaging and direct analysis in biological matrices. These developments could also lead to improved tools for screening the bioactivities and other beneficial effects of tannins. Moreover, automated analytical platforms may streamline practical tannin analysis, while advanced data processing approaches, such as molecular

networking and machine learning, can assist in the laborious and time-consuming interpretation of complex MS data.

Author Contributions

Marica T. Engström: conceptualization, data curation, investigation, resources, software, visualization, writing – original draft, writing – review and editing. **Maarit Karonen:** conceptualization, data curation, investigation, resources, software, visualization, writing – original draft, writing – review and editing.

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