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Procyanidins: a comprehensive review encompassing structure elucidation via mass spectrometry

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Abstract

Procyanidins are polyphenols abundant in dietary fruits, vegetables, nuts, legumes, and grains with a variety of chemopreventive biological effects. Rapid structure determination of these compounds is needed, notably for the more complex polymeric procyanidins. We review the recent developments in the structure elucidation of procyanidins with a focus on mass spectrometric approaches, especially liquid chromatography-tandem mass spectrometry (LC-MS/MS) and matrix-assisted laser desorption ionization (MALDI) MS/MS.

Keywords

Procyanidins; oligomeric; polymeric; structure elucidation

Introduction

Polyphenols are the largest group of secondary plant metabolites; their structural determination has been an intense area of investigation (Balas and Vercauteren 1994). Within polyphenols, procyanidins are derived from proanthocyanidins, also known as condensed tannins (Han et al. 2007). Procyanidins and proanthocyanidins naturally occur throughout the plant kingdom; commonly found with varying concentrations in commonly consumed foods such as fruits, vegetables, legumes, grains, and nuts (Table 1) (Gu et al. 2004; Han et al. 2007; Wang et al. 2011), as well as cosmetics (Kamimural et al. 2000) and pharmaceuticals (Fine 2003; Han et al. 2007; Wang et al. 2011) containing plant materials.

The study of proanthocyanidins began with Jacques Masquelier in the 1940s with the investigation of the pine bark that Native Americans brewed to heal scurvy (Rastogi et al. 2015). Masquelier identified monomeric proanthocyanidins within the pine bark preparation, noted their safety, and characterized some of their biological activities. Continuing to be used as natural and alternative medicines, products rich in proanthocyanidins entered the natural product market as dietary supplements during the 1980s (Fine 2003).

As natural antioxidants, proanthocyanidins are used to stabilize food colors and to prevent rancidity due to oxidation of unsaturated fats (Malien-Aubert et al. 2002; Li and Deinzer 2009; Hellenbrand et al. 2015) as well as for chemoprevention of a variety of degenerative

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diseases (Shi et al. 2003; Faria et al. 2006; Han et al. 2007). In addition to antioxidant properties, procyanidins have been reported to exhibit anticancer (Gossé et al. 2005; Martin et al. 2013; Li et al. 2015), anti-infectious (Chaves and Gianfagna 2007), anti-inflammatory (Gentile et al. 2012; Tatsuno et al. 2012; Vázquez-Agell et al. 2013), cardioprotective (de Pascual-Teresa et al. 2010; Arranz et al. 2013), antimicrobial (Benavente-García et al. 1997), antiviral (Kimmel et al. 2011), antimutagenic (Duan et al. 2010), wounding healing (Zhang et al. 2016), antihyperglycemic (Montagut et al. 2010) as well as anti-allergic (Akiyama et al. 2000) activities.

Plants metabolites such as carbohydrates, fats, and proteins can form complexes with procyanidins that interfere with their extraction and isolation (Jakobek 2015). Solvents that have been used are methanol, ethanol, water, dimethylsulfoxide, acetone, and other similar alcohols. Gel permeation chromatography may then be used to obtain fractions enriched in procyanidins (Sui et al. 2016). Different approaches to extraction and subsequent chromatographic isolation of these compounds have contributed to the uncertainty regarding procyanidin concentrations in plant and foods (Tsao 2010).

An area that needs attention is the quantitative analysis of procyanidins. Adamson et al. (1999) analyzed procyanidins up to decamers in size using gel permeation chromatography followed by preparative normal-phase HPLC. Similarly, quantitative analysis of polymeric procyanidins were performed on grape seed extracts with a reverse-phase HPLC method (Peng et al. 2001). Sultana et al. (2008) looked at three different extraction processes on tea leaves, in which microwave-assisted extraction gave the highest yield, and further analysis was performed using reverse-phase HPLC. Proanthocyanidins from crude plant extracts were quantified with respect to procyanidins and prodelphinidins utilizing a UPLC-MS/MS method (Engstrom et al. 2014).

Although procyanidins levels within plants and foods remain unclear, the application of nuclear magnetic resonance (NMR) and mass spectrometry (MS) has enabled the structure elucidation in these phenolic compounds. To date, the structures of monomeric units catechin and epicatechin as well as some of their lower order oligomers have been established, but the identification of polymeric procyanidins and the extent of their formation in many plants and foods remains limited (Tarascou et al. 2006; Bittner et al. 2013).

Chemical Structures of Procyanidins

Procyanidins are proanthocyanidins built from flavan-3-ols (+)-catechin and (−)-epicatechin (Bittner et al. 2013). Proanthocyanidins are classified based on their monomeric unit linkages and are present in homo- and hetero-polymers. The most common proanthocyanidins are procyanidins (Figure 1). Procyanidins are homo-oligomeric (epi)catechin with two B-ring hydroxyl groups (Ge et al. 2016). This review concerns the structure determination of procyanidins and not their related proanthocyanidins.

Procyanidins can be categorized into A-type and B-type depending on the stereo configuration and linkage between monomers. B-type procyanidins are characterized by a

single interflavan bond between carbon-4 of the B-ring and either carbon-8 or carbon-6 of the C-ring (Figure 1). B-type procyanidins are the most abundant, with procyanidins B1, B2, B3 and B4 occurring most frequently. A-type procyanidins have not only an interflavan bond but also a second ether linkage between the A-ring hydroxyl group and carbon-2 of the A-ring (Figure 1) (Bittner et al. 2013). The most common A-type compounds are A1 and A2.

Procyanidins can be categorized by their degree of polymerization (DP) (Gu et al. 2004); monomers form linkages leading to oligomers, further forming polymers. The most common monomeric unit is (−)-epicatechin, with B-type being the most prominent. Procyanidins containing 2–7 monomeric units are defined as oligoprocyanidins (Tsao and McCallum 2010).

Structure Elucidation

Nuclear Magnetic Resonance

NMR is a standard spectroscopic approach for the structural elucidation of a wide variety of natural products including procyanidins (Kind and Fiehn 2010). The structural elucidation of procyanidins by NMR has used a combination of homonuclear correlation (COSY) and heteronuclear one-bond (HSQC/HMQC) and multiple bond (HMBC) experiments (Morris 1986). Using long range correlation, the flavan junction carbon-4 and carbon-8 or carbon-4 and carbon-6 can be identified (Balas et al. 1995).

However, there has been considerable debate over the assignment of some protons and carbons in catechin and epicatechin (Balas et al. 1995). The position of these elements are solvent-dependent and are altered when derivatized (Balas and Vercauteren 1994). It has been suggested that rotation around the interfavan bond and ring interconversion is possible thereby confounding structure determination. To overcome this obstacle, the hydroxyl groups can be acetylated to impede the rotation, or alternatively, the spectra can be measured at varying temperatures with lower temperature slowing rotation and higher temperatures contributing to faster rotation (Khan et al. 1997).

Significant limitations of NMR analysis of procyanidins include sample isolation and large sample quantities. MS is generally carried out in the picogram range, while NMR is less sensitive requiring around 500 ng. Chromatographic separations of procyanidins are typically carried out prior to NMR analysis, but with the quantity requirements, purity problems often arise contributing to multiple interpretations of the data. Compared with mass spectrometry (MS), NMR is orders of magnitude less sensitive, orders of magnitude slower and to the best of our knowledge, unlike liquid chromatography-mass spectrometry (LC-MS), no LC-NMR analyses of procyanidins have yet been reported. Overcoming limitations of samples size, purity and speed, mass spectrometry and LC-MS have become effective tools for fast procyanidin structure elucidation (Silva Elipe 2003).

Liquid Chromatography and Mass Spectrometry (LC-MS)

Introduced in the 1960s, high performance liquid chromatography (HPLC) has become a standard tool for the rapid analysis and purification of nonvolatile compounds including natural products (Miller 2005). The recent commercial introduction of ultra-high pressure

liquid chromatography (UHPLC) enables even faster and higher resolution separations than HPLC. A variety of stationary phases are available for HPLC and UHPLC, however separations of polar procyanidins typically utilize reversed phase or normal phase columns (Churchwell et al. 2005). Mobile phases should have low ionic strength and contain only volatile additives when interfaced to mass spectrometry for on-line LC-MS analysis of procyanidins (Figure 2). Otherwise, ionization will be suppressed and the mass spectrometer inlet will become fouled by non-volatile deposits from the mobile phase (Hiraoka 2013).

As examples of reversed phase separations, Wollgast et al. (2001) studied procyanidins in crude chocolate extracts by reversed phase LC-MS, and Calderón et al. (2009) studied cocoa proanthocyanidins using C_{18} reversed phase LC-MS/MS and gradient elution from water to acetonitrile. More recently, Ortega et al. (2010) utilized reversed phase UHPLC-MS/MS to identify and quantify procyanidins up to nonamers in cocoa nib samples. UHPLC separations were carried out using gradient elution from water/acetic acid to acetonitrile.

As an example of normal phase LC-MS, Shoji et al. (2006) characterized procyanidins (dimers to octamers) in apple extracts according to the degree of polymerization using a combination of silica column fractionation followed by on-line normal phase LC-MS. The mobile phase consists of a hexane-methanol-ethyl acetate mixture. In another example, Karonen et al. (2004) utilized normal-phase and reversed phase LC-MS with negative ion electrospray ionization for the identification of procyanidins in pine bark. In general, proanthocyanidins from monomer to tetramers are optimally separated by reversed phase HPLC while polymers can be separated by their DP more efficiently using normal phase (Rigaud et al. 1993; Hammerstone et al. 1999; Gu et al. 2002).

Recently, several studies of procyanidins have utilized hydrophilic interaction chromatography (HILIC), a type of normal phase separation, in which the analyte is retained by partitioning between an aqueous layer on the hydrophilic stationary phase and the hydrophobic eluent (Hemström and Irgum 2006). Oligomeric and polymeric procyanidins from apples and cocoa eluted in order of increasing DP with individual peaks being obtained up to dodecamers for cocoa and apple extracts and up to tetradecamers for cacao seeds (Yanagida et al. 2007). Karonen et al. (2011) used LC-MS with HILIC and high resolution electrospray mass spectrometry to characterize oligomeric and polymeric procyanidins with degrees of polymerization up to 22 units that were obtained from silver birch bark.

Electrospray LC-MS (Figure 2) has become the most popular (Kind and Fiehn 2010) and is the only one to have been applied successfully to the analysis of procyanidins (Wollgast et al. 2001). Electrospray was first reported by Dole et al. (1968) as a technique to ionize high molecular weight synthetic polymers for mass spectrometric measurement. However, the application of electrospray mass spectrometry to the measurement of biopolymers such as proteins would not be realized for another 20 years (Fenn 2002), and its application to procyanidins would take even longer (Wollgast et al. 2001). Electrospray is one of the softest ionization techniques, which means that ions will form with the addition of little energy that might contribute to fragmentation in the ion source. Although the formation of molecular ions by the addition or loss of an electron can occur during electrospray, most analytes like

procyanidins ionize by losing or gaining a proton to form $[M-H]^-$ or $[M+H]^+$ ions, respectively (Hiraoka 2013).

During LC-MS, protonation/deprotonation of analytes containing heteroatoms can be facilitated by adjusting the pH of the mobile phase. As organic acids such as formic acid are often added to reversed phase mobile phases to facilitate HPLC separations (Miller 2005), positive ion electrospray mass spectrometry is used more often than negative ion mode for reversed phase LC-MS. However, electrospray LC-MS is not limited to reversed phase chromatography and may be used with normal phase columns as well (Nguyen and Schug 2008).

Once an intact procyanidin ion is formed during electrospray, it can be weighed in the mass spectrometer. The use of high-resolution accurate mass measurement analyzers such as time-of-flight (ToF), ion trap-ToF, quadrupole-ToF, as well as Fourier transform- ion cyclotron resonance and orbitrap mass spectrometers enable the elemental compositions of procyanidins to be determined. Additional structural information may be obtained by fragmenting the procyanidin ions using collision-induced dissociation and then weighing the product ions with high resolution tandem mass spectrometry (Kumar et al. 2016).

In studies of white birch bark procyanidins with DP up to 22 using negative ion electrospray LC-MS on a ToF analyzer, Karonen et al. (2011) found that high-resolution was essential to establish the charge state of each procyanidins based on their isotopic patterns. They also determined that only B-type procyanidins were present. As examples of ultrahigh resolution mass spectrometric analysis of procyanidins, Li et al. (2012) utilized FT-ion cyclotron resonance mass spectrometry with electrospray to identify oligomeric procyanidins in *Litchi chinensis*.

Unlike NMR, LC-MS/MS is so rapid that both accurate mass measurement of intact ions and their MS/MS product ions may be completed during single HPLC or UHPLC separations. The recent implementations of rapid polarity switching also enables on-line LC-MS/MS measurements of positive ions and negative ions during the sample chromatographic separation.

Two key components in the mass spectrometric analysis of complex mixtures such as procyanidins include resolution and mass accuracy. Mass accuracy is the degree of conformity of the measured value to the true value. Resolution is the ability to resolve two ions of similar mass-to-charge. Without high resolution accurate mass analysis, accurate charge state determination and accurate quantitation cannot be achieved (Mann and Kelleher 2008). The ability to perform structure elucidation of ions using high resolution accurate mass measurement and tandem mass spectrometry has been described by Kind and Fiehn (2010).

Another feature of electrospray that is less common with other ionization techniques is the ability to form not just singly charged ions, but also multiply charged species. Multiple charging is particularly valuable for large molecules, such as high order procyanidins polymers, that might be outside the mass range of a particular mass spectrometer (Hiraoka 2013). For example, mass spectrometers measure the *mass-to-charge* or *m/z* value of an ion,

so that if $z = 2$, an ion of m/z 3000 will appear as m/z 1500. Therefore, multiple charging would enable a mass spectrometer with a mass range of just m/z 2000 to measure procyanidins with masses of 3000 or more.

As in peptide sequencing using tandem mass spectrometry (Wysocki et al. 2005; Wang et al. 2016), multiple charging can facilitate the fragmentation of procyanidins into more structurally significant product ions that can be obtained from single charged precursor ions (Gu et al. 2003; Karonen et al. 2004; Sarnoski et al. 2012; Patras et al. 2014; Ge et al. 2016). For example, Wollgast et al. (2001) identified procyanidins in cocoa using collision-induced dissociation and tandem mass spectrometry of their doubly-deprotonated, $[M\text{-}2H]^{2-}$, pentamers, hexamers, and heptamers.

Positive electrospray mass spectrometry has been used successfully in measuring procyanidins up to pentamers in length, but larger procyanidins do not ionize efficiently in positive mode (Karonen et al. 2004). Due to the acidity of procyanidins, they are more readily measured as deprotonated molecules using negative ion electrospray mass spectrometry (Hellström et al. 2007). For example, oligomeric procyanidins have been detected using negative ion electrospray as singly charged deprotonated molecules of m/z 577, 865, 1153, 1441 and 1729 (Table 2) for dimeric, trimeric, tetrameric, pentameric, and hexameric procyanidins, respectively (Hammerstone et al. 2000; Ito et al. 2013). Multiply charged molecules formed using negative ion electrospray have been reported for higher order procyanidins such as doubly charged heptamers detected at m/z 1009 (Hellström et al. 2007).

As the DP increases, high mass procyanidins tend to form multiply charged species. However, as the DP increases, the ionization efficiency decreases. For example, Karonen et al. (2011) reported that singly-charged molecules and fragment ions are abundant for dimers and trimers, doubly-charged species $[M\text{-}2H]^{2-}$ are observed for octamers and nonamers, nonamers through octadecamers form triply charged species, $[M\text{-}3H]^{3-}$, and only $[M\text{-}4H]^{4-}$ are observed for procyanidins with DP higher than hexadecamers. In studies of apple procyanidins ranging from dimers to octamers, Shoji et al. (2006) reported similar results; they observed singly charged species for the lowest mass compounds and doubly and triply charged signals for the higher order procyanidins.

Note that B-type procyanidin oligomers are composed of multiple monomer subunits with interflavanoid C-C linkages that differ by multiples of 288, which corresponds to the mass of the monomeric subunit (Karonen et al. 2004). As expected, B-type deprotonated procyanidins have been reported to fragment between monomeric subunits forming a series of product ions of $[M\text{-}288n\text{-}1]^{-1}$ (Hellström et al. 2007). The m/z values of deprotonated procyanidin oligomers, their degrees of polymerization (DP), and the masses of their most abundant fragment ions are shown in Table 2.

The main fragmentation pathways of procyanidins include quinone methide (QM) cleavage of the interflavanoid bond, as well as heterocyclic ring fission (HRF) and retro Diels-Alder (RDA) fission of the heterocyclic ring system subunits which are distinctive of proanthocyanidins (Hellström et al. 2007). These pathways can be seen in Figure 3 for B-

type dimer procyanidins and in Figure 4 for an A-type dimer. The key component in understanding A- and B- type is recognizing that A-type dimers have been found to be 2 Da less than those of B-type, this difference accounts for the additional C-O-C linkage.

QM formation will result in fragmentation between two catechin or epicatechin subunits in a procyanidin polymer. During this process, procyanidins will fragment to form one of two different QM ions. In the case of A-type dimers (Figure 4), fragmentation can form monomeric fragment ions of *m/z* 289 and *m/z* 285, and this difference of 4 Da is characteristic of A-type linkages. B-type procyanidin dimers (Figure 3), fragment to form monomeric ions of *m/z* 287 or *m/z* 289. Note that this 2 Da difference between fragment ions distinguishes B-type linkages from A-type. Therefore, QM fragmentation of dimers leads to pairs of product ions differing by 4 Da or 2 Da, which can be used to distinguish between types of procyanidins (Hellström et al. 2007; Sui et al. 2016). A-type trimers undergo the QM cleavage producing ions of *m/z* 575 and *m/z* 287. B-type trimers undergo QM cleavage of the upper interflavanoid bond producing ions of *m/z* 287 and 577, whereas cleavage of the lower interflavanoid bond forms ions of *m/z* 289 and 575. For B-type tetrameric and higher order procyanidins, fragment ions for QM cleavage were observed at *m/z* 287, 289, 575, 577 and 865 (Karonen et al. 2004). Chen et al. (2014) also described the conversion of B- to A-type trimers by quinone methide reaction mechanisms.

Karonen et al. (2004) reported that fragmentation of B-type procyanidin dimers via HRF can take place on either monomeric unit (Figure 3). The fragment ion of the dimer at *m/z* 451 indicates B-type (Figure 3) whereas a fragment ion at *m/z* 449 indicates an A-type dimer (Figure 4), loss of a phloroglucinol molecule. The B-type procyanidin trimer fragment similarly to that of the dimer, in which a fragment ion of *m/z* 739 is produced.

RDA fragmentation, which was the most common fragmentation pathway of the B-type procyanidin dimer (Friedrich et al. 2000), produces a fragment ion of *m/z* 425 with subsequent water elimination giving rise to an ion of *m/z* 407 (Figure 3). Fragmentation on the upper unit is considered to be energetically more favorable than the lower unit because it produces fragment ions with a larger $\Pi - \Pi$ hyperconjugated system (Gu et al. 2003). A-type dimers produce fragment ions of *m/z* 423 (Figure 4), and A-type trimers produce fragment ions of *m/z* 711 (Sui et al. 2016). The ion at *m/z* 713 indicates RDA fragmentation of a B-type trimer (Karonen et al. 2004).

Matrix-Assisted Laser Desorption Ionization (MALDI)

Invented in the 1980s, MALDI is an ionization technique for mass spectrometry that enables the simultaneous desorption and ionization of solid-phase biopolymers (Karas et al. 1987; Tanaka et al. 1988). Originally used for polymers, MALDI is also suitable for the ionization of procyanidins. As seen in Figure 5, the sample is dissolved in a solvent containing a matrix that will absorb the laser light, usually a UV or IR laser. The mixture is dried, loaded onto a MALDI target, and short laser pulses are used to evaporate the matrix which results in desorption and ionization of the associated procyanidin. If using a non-scanning mass spectrometer such as a ToF analyzer, complete mass spectra may be obtained with each pulse of the laser, thereby making MALDI mass spectrometry highly efficient and suitable

for small procyanidin samples. Some ToF analyzers are also capable of high resolution and tandem mass spectrometry.

During MALDI, analytes typically form abundant singly-protonated or deprotonated molecules, although molecular ion radicals and multiply charged species are possible. Analyte ionization during MALDI has been described as a photo-ionization process during which analytes become charged by proton transfer during collisions with matrix ions (Ehring et al. 1992). Alternatively, a cluster ionization mechanism has been proposed for MALDI in which preformed analyte ions are released from clusters during evaporation of matrix ions (Karas 1996).

The first MALDI MS measurements of procyanidins were reported by Ohnishi-Kameyama et al. (1997) using a ToF analyzer. Looking only at monomeric and dimeric procyanidins, DHB, α-cyano-4-hydroxycinnamic acid, sinnapinic acid, and 9-nitroanthracene were effective matrices. DHB is an optimum UV matrix for procyanidin MALDI mass spectrometry (Hurst et al. 2009). While there might be advantages of using IR lasers for procyanidin analysis, most MALDI MS studies of procyanidins have utilized UV lasers (Niu et al. 1998).

Unlike electrospray mass spectrometric studies of procyanidins, which usually utilize negative ion mode, the majority of MALDI MS studies of procyanidins have been carried out using positive ion mode. In positive ion MALDI mass spectra, the ion current is divided among several cationized species including $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, and sometimes others. Dividing the procyanidin signals in this manner lowers the sensitivity of the analysis and complicates interpretation of the data, especially when measuring mixtures of compounds. To enhance the abundance of particular cationized procyanidins, cationization agents have been added to the MALDI matrix (Monagas et al. 2010). Addition of sodium chloride, sodium iodide, silver trifluoroacetate, and cesium trifluoroacete have all been used with varying degrees of success to detect procyanidins as $[M+Na]^+$, $[M+Ag]^+$ or $[M+Cs]^+$ ions (Ohnishi-Kameyama et al. 1997; Krueger et al. 2003; Vivas et al. 2004; Sivakumaran et al. 2006; Spencer et al. 2007). Unless there is a compelling reason to use positive ion MALDI, the detection of procyanidins can be improved significantly by utilizing negative mode. This is consistent with the electrospray studies discussed above as well as with related desorption ionization studies of procyanins using fast atom bombardment (Self et al. 1986).

MALDI MS/MS of Procyanidins

Although MALDI-ToF/ToF is frequently used for peptide sequencing, few applications have been reported for procyanidin analysis. The published MALDI MS/MS spectra of procyanidins to date have reported relatively few product ions and have high background noise levels (Mateos-Martín et al. 2012; Pérez-Jiménez and Torres 2012). Because MALDI MS is not compatible with on-line HPLC or UHPLC, samples must be prepared in advance and might degrade before analysis or contain impurities that can suppress ionization. In this respect, MALDI mass spectrometry shares this limitation with NMR analysis of procyanidins. However, an advantage of MALDI analysis is easy interpretation of data including complex mixtures of procyanidins. While it is difficult to interpret DP with NMR, it is easily obtained using MALDI mass spectrometry.

While it is possible to determine the type of procyanidin using MALDI-ToF mass spectrometry (MS^1) (Stringano et al. 2011; Feliciano et al. 2012), definitive typing can also be obtained based on fragmentation patterns during product ion tandem mass spectrometry. A-type procyanidins have different fragmentation patterns than B-type procyanidins that can be used to differentiate unknown procyanidins by the type of linkages between monomeric units. For example, in the tandem mass spectrum of a B-type procyandin trimer (Figure 6), 13 fragment ions are recognizable corresponding to the expected fragmentation pathways of procyanidins – quinone methide formation, HRF, and RDA fragmentation.

Fragmentation observed for MALDI is similar to that for electrospray. The advantages of MALDI over electrospray include speed, sensitivity, and the ability to obtain singly charged procyanidin ions for tandem mass spectrometric analysis, especially for the higher DP compounds. With the benefit of multiple charging, procyanidins of higher DP have been reported using electrospray, although product ion tandem mass spectrometric analysis of larger procyanidins have been reported using MALDI ToF/ToF mass spectrometry (Wang et al. 2000; Karonen et al. 2004; Shoji et al. 2006; Oliveira et al. 2015)

Using MALDI ToF/ToF mass spectrometry for product ion analysis of higher order procyanidins, Mateos-Martín et al. (2012) described the fragmentation pathway of a tetrameric proanthocyanidin. In tetrameric form, the proanthocyanidin ion follows the general fragmentation pathway of QM, RDA, and HRF. The tetrameric ion showed a loss of 126 Da during HRF, loss of 152 Da via RDA reaction followed by subsequent loss of water and a loss of 288 Da to form the trimeric form. The trimeric ion showed the same fragmentation pathways including HRF, RDA reaction and subsequent water elimination as well as QM fragmentation forming the dimeric form. The dimeric ion showed similar fragmentation and to produce two monomeric units. In Figure 7, a tetrameric proanthocyanidin, the fragmentation pathways facilitated identification of the mixed type containing two B-type linkages with a lower A-type linkage (Mateos-Martín et al. 2012).

In the negative ion MALDI product ion tandem mass spectrum of Procyanidin C1 in Figure 6, the dimer doublet ions of *m/z* 575 and 577 and monomer doublet ions of *m/z* 287 and 289 show the 2 u mass difference that is characteristic of quinone methide formation by B-type procyanidins. In the case of the tandem mass spectrum of Procyandin A1 in Figure 8, the pair of ions of *m/z* 285 and 289 shows a 4 u mass difference indicating the formation of quinone methides by an A-type procyanidin. Figure 6 shows a fragment ion at *m/z* 739 corresponding to HRF of the trimeric unit whereas the fragment ion of *m/z* 451 corresponds to HRF of the dimeric form. In Figure 8, the fragment ion of *m/z* 449 corresponds to HRF from procyanidin A1 which is a dimer. Figure 6 shows fragment ions at *m/z* 713 which corresponds to RDA and a subsequent loss of water at *m/z* 695. Fragment ions of *m/z* 425 and *m/z* 407 correspond to RDA and water loss of the dimeric form.

All of these patterns of fragmentation – QM, HRF and RDA – are useful in quickly identifying the type of procyanidin. While MALDI-ToF MS analyses of procyanidins has been reported frequently, more attention should be given to the abundant structural information that may be obtained when using negative ion MALDI-ToF/ToF mass spectrometry.

Conclusion

Procyanidins, oligomeric compounds composed of catechin and epicatechin monomers, are widespread in foods and can have significant medicinal properties. Although much is already known about their biological activities, research concerning their medicinal benefits is continuing. Essential to this effort is a more thorough understanding of the procyanidin structures (polymer chain length and composition of A-type versus B-type linkages) and content in plants, foods and research materials. The high molecular weight of the longer chain procyanidins has hindered their analysis, but advances in MALDI ToF/ToF mass spectrometry mass range and the ability to form multiply charged ions using electrospray are helping to overcome this limitation. To date, the highest DP for procyanidins yet reported has been 28 (Hayasaka et al. 2003), but this value is expected to increase.

As the capabilities of mass spectrometers improve, not only with respect to mass range but also improved sensitivity, resolving power, accuracy, and new functionalities, the identification of higher order procyanidins will become routine. An example of an emerging new functionality in the field of biomedical mass spectrometry that might be useful for procyanidin analysis is ion mobility. A fast gas-phase separation technique based on ion size and shape, ion mobility mass spectrometry is orders of magnitudie faster than HPLC and should be useful for procyanidin analysis. Another area that requires additional development is the quantitative analysis of higher order DP procyanidins using mass spectrometry. Altogether, the application of state-of-the-art biomedical mass spectrometry is facilitating the structural analysis of procyandins, and the quantitative analysis of these important botanical natural products will follow.

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Abbreviations

PC	procyanidins
DP	degree of polymerization
MS	mass spectrometry
MALDI	matrix-assisted laser desorption ionization
QM	quinone methide
RDA	retro Diels-Alder

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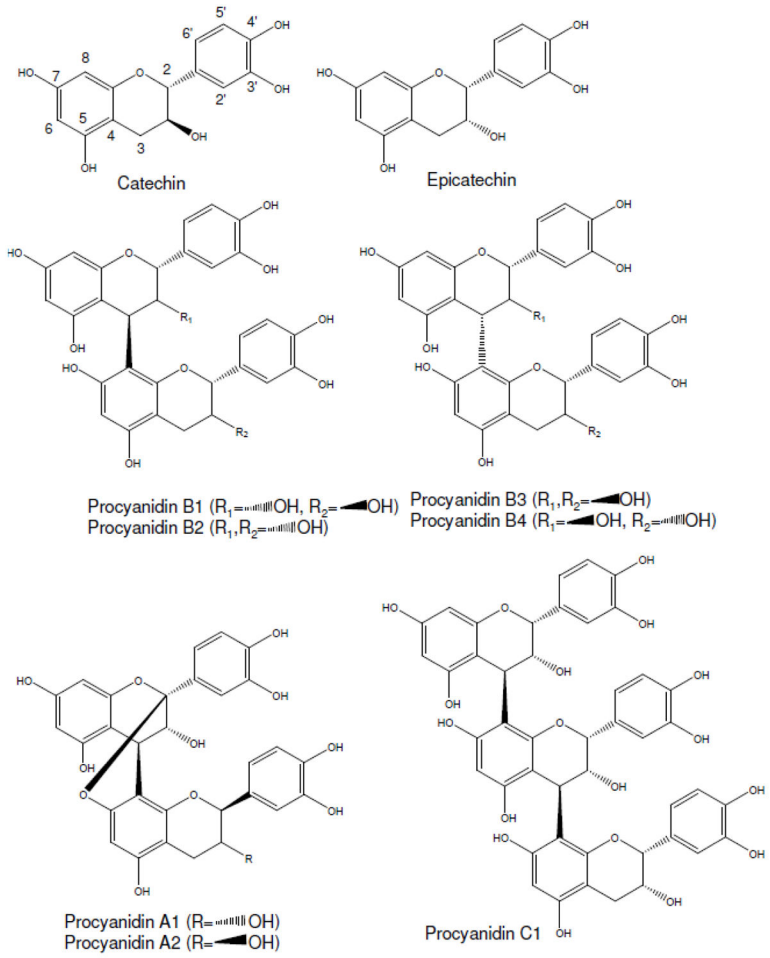

Figure 1.

Chemical structures of monomeric and polymeric procyanidins (Xie and Dixon 2005; Tsao 2010)

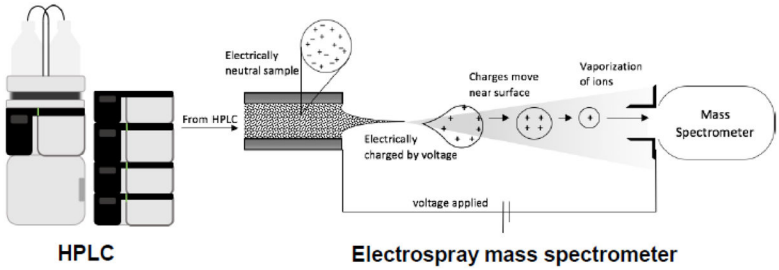

Figure 2.

Electrospray serves as an atmospheric pressure interface between the HPLC and the mass spectrometer while simultaneously facilitating the formation of gas-phase ions of non-volatile and thermally labile solutes in the mobile phase such as procyanidins

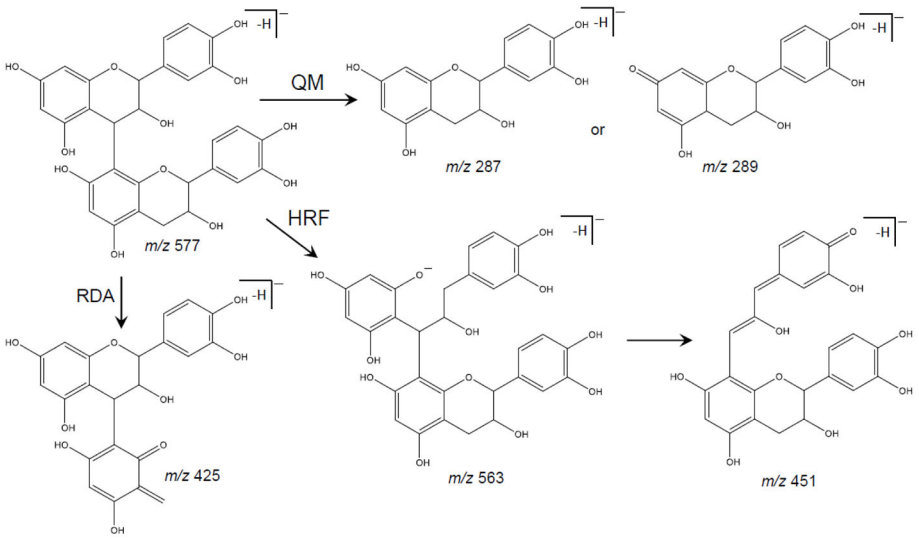

Figure 3.

Fragmentation pathway of B-type procyanidin dimer showing the products formed by quinone methide (QM), heterocyclic ring fussion (HRF), and retro-Diels Alder (RDA) reactions (Hellström et al. 2007)

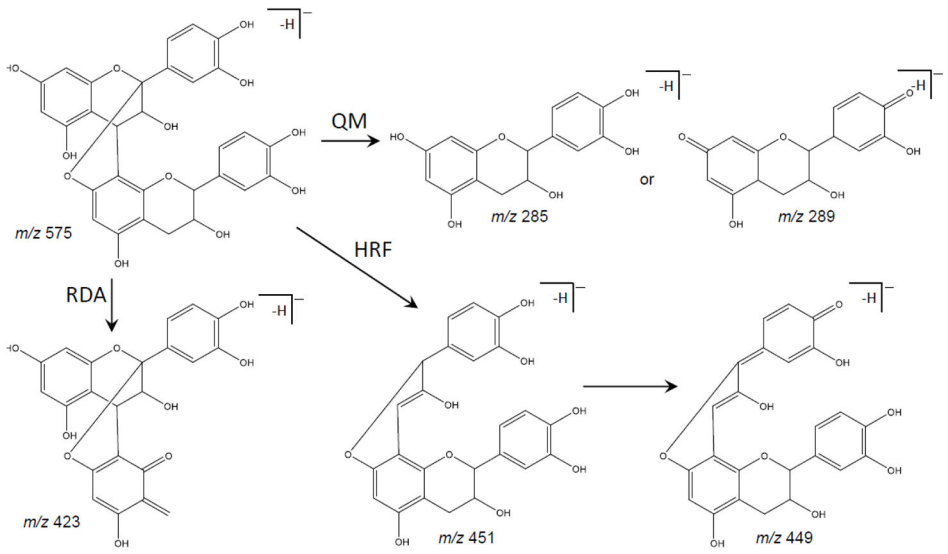

Figure 4.

Fragmentation pathway of A-type procyanidin dimer showing the products formed by quinone methide (QM), heterocyclic ring fussion (HRF), and retro-Diels Alder (RDA) reactions (Sui et al. 2016)

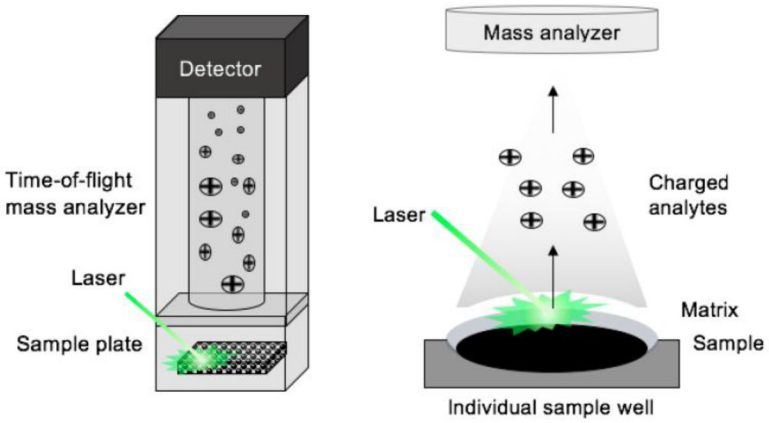

Figure 5.

Matrix-assisted laser desorption ionization utilizing a laser for desorption of a sample in a matrix material facilitating the protonation/deprotonation of samples such as procyanidins

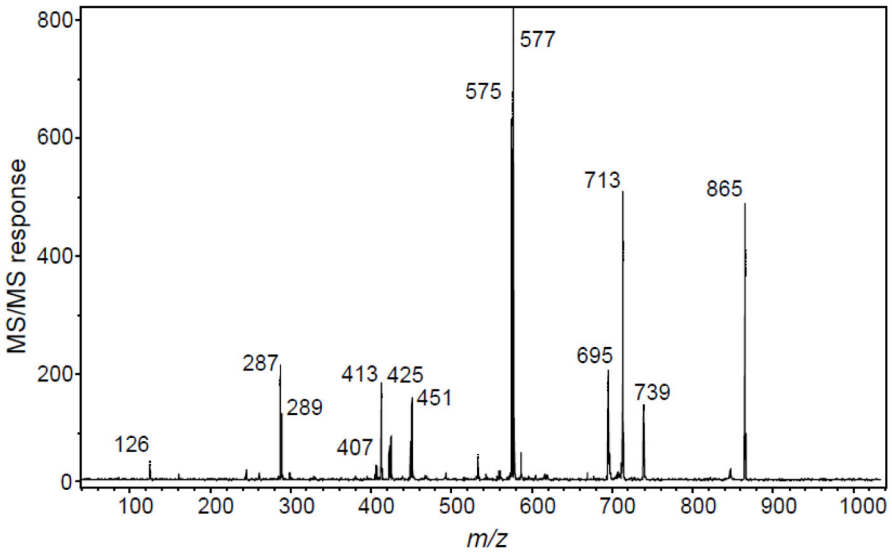

Figure 6.

MALDI-ToF/ToF product ion mass spectrum of deprotonated procyanidin C1. This procyanidin B-type trimer was provided by Jan Glinski of Planta Analytica (New Milford, CT), and the tandem mass spectrum was obtained by Paul Kowalski using a Bruker Daltonics UltrafleXtreme (Billerica, MA) MALDI-ToF/ToF mass spectrometer.

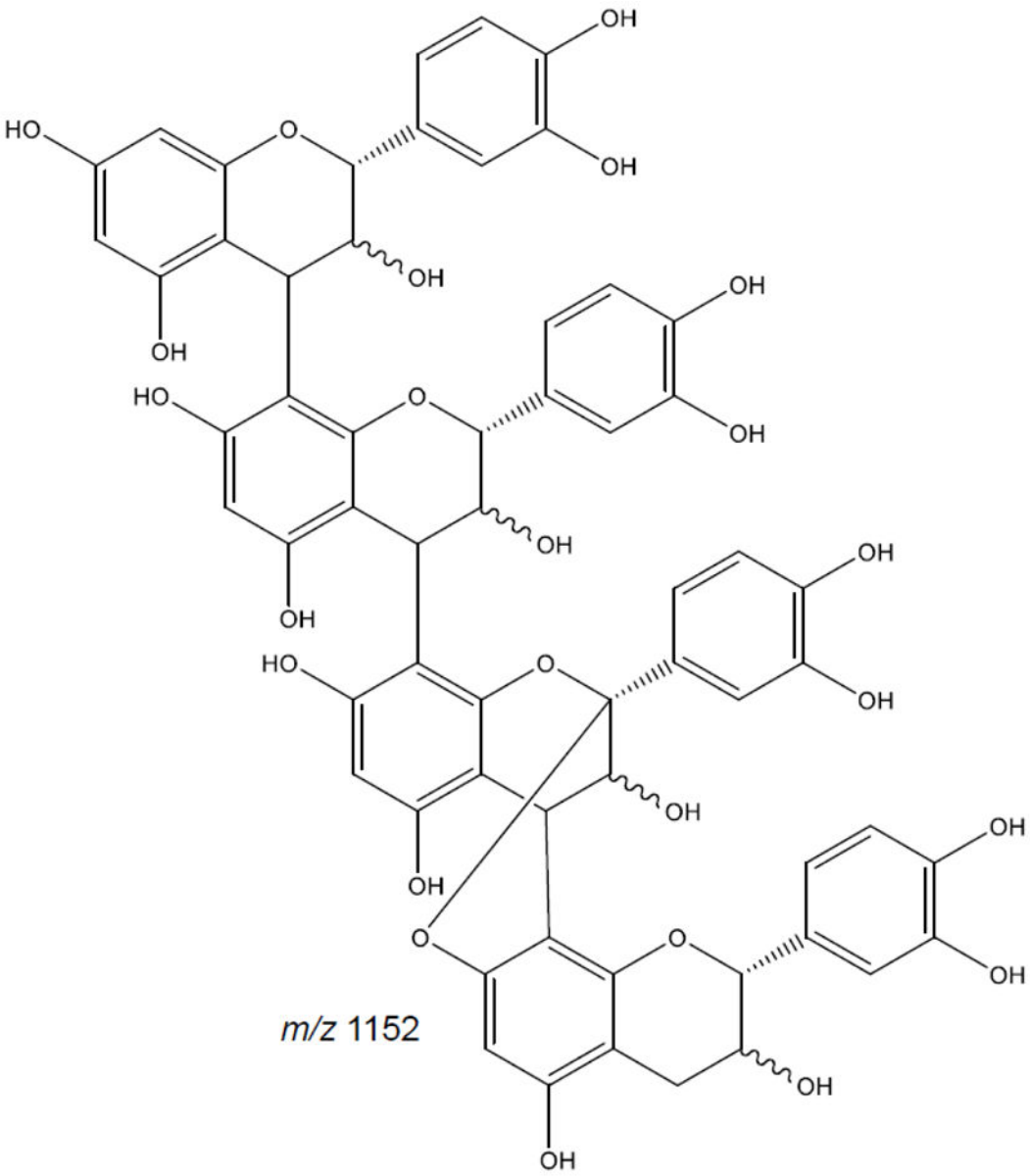

Figure 7.
Tetramer proanthocyanidin displaying mixed type configuration (based on Mateos-Martín et al. 2012)

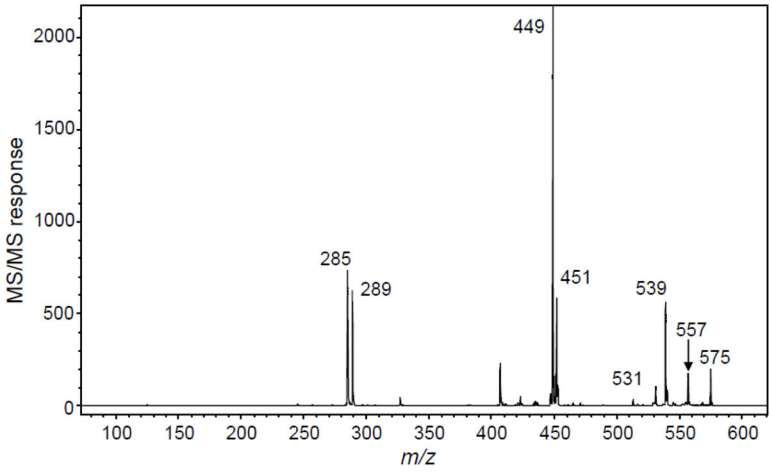

Figure 8.

MALDI-ToF/ToF product ion mass spectrum of deprotonated procyanidin A1. This A-type procyanidin dimer was provided by Jan Glinski (Planta Analytica), and the tandem mass spectrum was obtained by Paul Kowalski (Bruker Daltonics).

Table 1
Commonly consumed Foods Containing Procyanidins

Commonly consumed food that have been found to contain procyanidins. (Gu et al. 2004; U.S. Department of 2004; Hellström et al. 2009)

Fruits		
apple	grape	quinces
apricot	kiwi fruit	raspberry
avocado	lingonberry	red currant
banana	lychee	rhubarb
bilberry	mango	rose hip
black currant	marionberry	rowanberry
blackberry	nectarine	Saskatoon berry
blueberry	orange	sea buckthorn
cherry	peach	strawberry
chokeberry	pear	sweet rowanberry
cloudberry	persimmons	tangerine
cranberry	pineapple	tomatoes
crowberry	plum	watermelon
dates, deglet noor	pomegranate	whortleberry
gooseberry		
Vegetables		
carrots	indian squash	pepper
eggplant	lettuce	potato
figs	onion	zucchini
Nuts, Legumes, Grains, Miscellaneous		
almonds	chickpeas	sorghum
cashews	faba beans	wheat flours
hazelnut	french beans	cinnamon
peanuts	kidney beans	dark/milk chocolate
pecan	lentils	hops
pistachio	pinto beans	tea beverage
walnuts	white beans	red wine
black beans	barley flour	white wine
black eye peas	buckwheat grits/flour	rose wine
cacao beans	rice	sherry wine

Table 2
Molecular ions and Characteristic fragments in negative mode

Deprotonated molecules and characteristic fragment ions reported for procyanidins using negative ion electrospray. (Hammerstone et al. 1999; Karonen et al. 2004; Hellström et al. 2007; Li et al. 2012; Sui et al. 2016)

Degree of Polymerization		Characteristic Fragments	Type
1	289	245, 205	(+)-catechin, (-)-epicatechin
2	577	559, 451, 425, 407, 289,287	B
	575	449, 423, 289, 285	A
3	865	739, 713, 695, 577, 575, 451, 407, 289, 286	B
	863	711, 693, 575, 573, 559, 451, 449, 423, 411, 289	A
4	1153	1027, 1001, 983, 865, 863, 739, 577, 576*, 575, 289, 287	B
	1151	863, 861, 711, 577, 573, 451, 449	A
5	1441	1315, 1289, 1271, 1153, 1151, 1027, 865, 863, 720*, 575	B
	1439		A
6	1730	739, 575	Mixed
7	2017	1729, 1441, 1151, 1009*, 863, 673*, 575	Mixed
8	1152*, 769*		
9	1297*, 865*		
10	1441*, 960*		

*multi charged species

HRF, RDA, QM, water elimination