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NMR, ESI/MS, and MALDI-TOF/MS Analysis of Pear Juice Polymeric Proanthocyanidins with Potent Free Radical Scavenging Activity

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The structure of a polymeric proanthocyanidin fraction isolated from pear juice was characterized by NMR, ESI/MS, and MALDI-TOF/MS analyses, and its antioxidant activity was investigated using the DPPH free radical scavenging method. The results obtained from ^{13}C NMR analysis showed the predominance of signals representative of procyanidins. Typical signals in the chemical shift region between 70 and 90 ppm demonstrated the exclusive presence of epicatechin units. The results obtained through negative ESI/MS analysis showed singly and doubly charged ions corresponding to the molecular mass of procyanidins with a degree of polymerization up to 22. The spectra obtained through MALDI-TOF/MS analysis revealed the presence of two series of tannin oligomers. Supporting the observations from NMR spectroscopy, the first series consists of well-resolved tannin identified as procyanidin polymers units with chain lengths of up to 25. A second series of monogalloyl flavan-3-ols polymers with polymerization degree up to 25 were also detected. This is the first mass spectrometric evidence confirming the existence of galloylated procyanidin oligomers in pear fruits. Within each of these oligomers, various signals exist suggesting the presence of several oligomeric tannins. The antioxidant properties of the polymeric fraction were investigated through reduction of the DPPH free radical, and the results obtained showed that the polymeric fraction exhibited a higher antioxidant power compared to those of (+)-catechin and B_3 procyanidin dimer.

KEYWORDS: Procyanidin; juice; pear; condensed tannins; degree of polymerization; NMR; mass spectrometry; ESI; MALDI-TOF; free radical scavenging

INTRODUCTION

Procyanidins (condensed tannins) are a class of oligomeric flavan-3-ol units with pronounced biological activities found in many plants, foods, and beverages. They are known to have powerful free radical scavenging activity, antioxidant activity, and anti-tumor-promoting effect (*1*−*3*). Recently, increasing evidence for oligomers in a wide range of active assays has attracted attention to their structural elucidation. However, condensed tannins are diverse compounds with great variation in structure and concentration within and among plant species. Owing to the complexity of the oligomers, most studies have been focused on the dimeric procyanidins. Therefore, biomedical research on the health benefits and risks of increased tannin consumption is severely limited by lack of methods for the rapid characterization and standardization of more polymerized proanthocyanidins. Many studies dealing with the biological activities of proanthocyanidins have suggested, however, that the antioxidant, antifungal, antienzymic, antisecretory, and antitumor properties (*4*) may be correlated to their polymerization degree.

The structural elucidation of polymeric proanthocyanidins is difficult because of their heterogeneous character. Proanthocyanidins are formed of flavan-3-ol monomers, which are linked through C4−C8 or C4−C6 linkages. The diversity of condensed tannins is given by the structural variability of the monomer units (different hydroxylation patterns of the aromatic rings A and B and different configurations at the chiral centers C2 and C3) and the distinct regio- and stereochemistries of the interflavanoid linkage (**Figure 1**). Due to this complexity and diversity, the characterization of highly polymerized proanthocyanidins thus remains very challenging, and less is known regarding structure−activity relationships.

Various techniques including NMR and mass spectroscopy (MS) have been used to characterize proanthocyanidins. It was thus demonstrated that the determination of the ratio of the 2,3-*cis* to 2,3-*trans* stereochemistries could be achieved through ^{13}C NMR by virtue of the distinct differences in their respective

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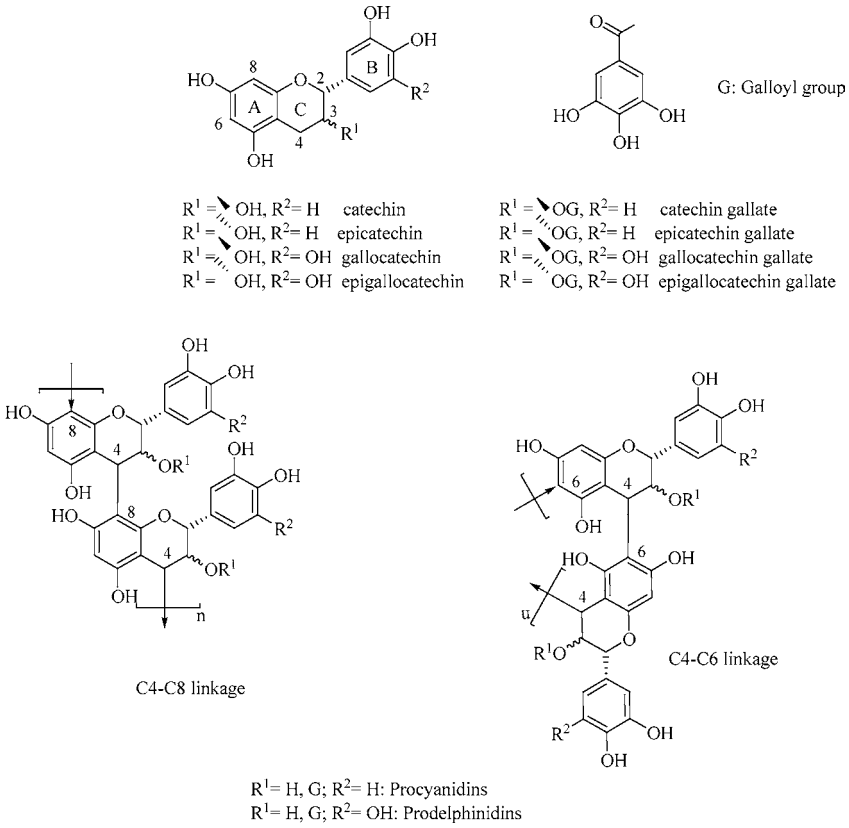

Figure 1. Chemical structures of flavan-3-ol monomers and polymers.

C2 chemical shifts. It was also noted that the areas of the C3 resonances for the extender and terminal flavanol could be integrated to obtain the number-average molecular weight (*5*, *6*). In recent work ^{1}H NMR was also used to estimate the polymerization degree by integrating the A-ring proton signals between 5.8 and 6.5 ppm and comparing them to the intensity of the H4 signals of the terminal units between 2.4 and 3.0 ppm (*7*).

In addition to NMR analysis, mass spectrometry has also been used for the characterization of condensed tannins. This method has been applied for the determination of molecular masses of proanthocyanidins from various plant sources using fast atom bombardment (FAB) MS (*8*, *9*), liquid secondary ion (LSI) MS (*10*, *11*), electrospray (ES) MS (*12*−*16*), and matrix-assisted laser desorption time-of-flight (MALDI-TOF) MS (*6*, *9*, *17*−*19*). In particular, electrospray and MALDI-TOF were used to characterize the degree of polymerization (DP) and structure of proanthocyanidins. Although both techniques are capable of detecting intact molecular ions, ESI is best suited for the analysis of monodispersed biopolymers (*20*). Alternatively, MALDI-TOF/MS is ideally suited for characterizing polydispersed oligomers (*21*) and is considered as the mass spectrometric method of choice for the analysis of proanthocyanidins. MALDI-TOF/MS produces only a singly charged molecular ion for each parent molecule and allows detection of high mass with precision (*20*).

In this study we undertook the structural characterization and the antioxidative activity of a complex mixture of a pear juice polymeric proanthocyanidins extract using a combination of NMR, ESI/MS, and MALDI-TOF/MS analysis. These techniques are used for the first time with pear juice condensed tannins to help the elucidation of sequences of monomer units. This would contribute to a better understanding of the chemical composition of pear juice and the biochemical changes that occur during their storage and would also further increase the applicability of ESI/MS and MALDI-TOF/MS in the analysis of food polymeric proanthocyanidins. Indeed, the native polymeric proanthocyanidins from pear juice has not been previously investigated, because the previous studies on the phenolic composition of pears (*22*−*28*), pear juices (*29*, *30*), and pear purees (*31*) have been conducted through HPLC/DAD analysis or through depolymerization using thioacidolysis prior to HPLC (*32*).

MATERIALS AND METHODS

Extraction and Purification of Pear Procyanidins. Extraction and purification of pear procyanidins followed previously reported methods (*33*, *34*). Ten kilograms of pear fruits (*Pirus communis* var. *Fausset*) were washed and then crushed with a Record type 1 C crusher (Blaumeyer, Bouzonville, France). One hundred milliliters of a 20 g/L solution of NaF, corresponding to 0.2 g/kg of fruit, was atomized at the exit of the crusher gradually during the process. The pear slices were pressed on a hydraulic press (Pressoir Colin, Seine, France) for 5 min at low pressure followed by 5 min at maximum pressure. The juice was filtered through a 250 μm porosity sieve to eliminate coarse particles. After sedimentation, the juice was racked and frozen.

Thawed juice (1 L) was centrifuged at 2000*g* for 15 min and then diluted to 4 L with water acidified with acetic acid (25 mL/L). The diluted juice was filtered successively through an 8 μm porosity filter (Millipore) and then through a Whatman 4-7 GF/D filter prior to the injection of 500 mL on a 20 × 5 cm i.d., 12 μm column of LiChrospher 100 RP-18 (Merck) for purification as previously described (*34*). This was repeated eight times. All procyanidin fractions were pooled prior to evaporation of the solvents on a rotary vacuum evaporator followed by freeze-drying.

Thioacidolysis. Acid-catalyzed cleavage of the obtained proanthocyanidin fraction was carried out in the presence of phenylmethanethiol, and the resultant reaction mixture was analyzed by HPLC as previously described (*32*).

UV−**Vis and NMR Analysis.** UV−vis spectra were recorded using a Kontron Uvikon 930 spectrophotometer fitted with a quartz cell. ^{1}H and ^{13}C NMR spectra were recorded in CD_3OD with a Varian Mercury-300 spectrometer at 300 and 75 MHz, respectively (proton decoupling mode for carbon).

ESI/MS Analysis. Positive- and negative-ion mode ESI/MS spectra were recorded on a Quattro LC MS/MS triple-quadrupole mass spectrometer (Micromass, Manchester, U.K.). LC-MS analyses were performed with an Alliance chromatographic system consisting of a 2695 separations module equipped with an autosampler and a 2487 dual lambda absorbance detector (Waters, Milford, MA). The column was a 150 × 2.1 mm i.d., 5 μm, Interchrom Uptisphere ODB with a 10 × 2.1 mm i.d. precolumn from Interchim (Montluçon, France). The HPLC system was coupled directly to the Quattro LC MS/MS mass spectrometer, which was equipped with a pneumatically assisted electrospray ionization source (ESI). Data acquisition and processing were performed using a MassLynx NT 3.5 data system. The electrospray source parameters were fixed as follows: electrospray capillary voltage, 3.25 kV in positive mode and 3 kV in negative mode; source block temperature, 120 °C; desolvation gas temperature, 400 °C. Nitrogen was used as drying gas and nebulizing gas at flow rates of approximately 50 and 450 L/h, respectively.

For direct injection, the solution was introduced into the electrospray source at a constant flow rate of 10 μL/min with a model 22 medical syringe infusion pump (Harvard Apparatus, South Natick, MA) in combination with a 100 μL syringe.

MALDI-TOF/MS. MALDI-TOF mass spectra were collected on a Bruker Reflex III-TOF mass spectrometer equipped with delayed extraction and a N_2 laser set at 337 nm. Positive-ion mode spectra in the linear and reflectron mode were used with an accelerating voltage of 19.0 kV. Spectra were the sum of 100−500 shots.

Following previously developed methods (*35*) dihydroxybenzoic acid was chosen as the matrix. The freeze-dried samples were reconstituted in acetonitrile/water (1:1, v/v) to give sample concentrations of 1.8 mg/100 mL. The sample was loaded onto the target plate (Bruker) by mixing 0.5 μL of each solution with 0.5 μL of the matrix solution and was left to dry at room temperature. Deflection of the low-mass ions was used to enhance the target signal. External calibration was performed with standard calibrants for each measurement.

Reduction of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical. In a TLC autographic assay, methanolic solutions of the extracts were deposited on a silica gel plate. After drying, TLC plates were sprayed with a 0.2% DPPH solution in MeOH. Compounds showing yellow on purple spots were regarded as antioxidant (*36*). In the spectrophotometric assay, the free radical scavenging activity was measured using the method of Brand-Williams et al. (*37*). A 0.1 mM solution of DPPH in methanol was prepared, and to 2 mL of this solution was added 0.1 mL of an antioxidant solution in methanol at different concentrations. The studied compounds were tested with MeOH as negative control and BHT and quercetin as positive control, and absorbance at 517 nm was determined after 30 min. The absorbance (*A*) of the control and samples was measured, and the DPPH scavenging activity in percentage was determined as follows:

$$\text{DPPH scavenging activity (\%)} = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$$

The data are presented as means of triplicates, and the amount required for a 50% reduction (IC_{50}) of DPPH radical was determined graphically. A smaller IC_{50} value corresponds to a higher antioxidant activity.

RESULTS AND DISCUSSION

NMR Analysis. The characterization of the pear juice polymeric proanthocyanidins fraction was initiated through NMR analysis. After extraction, the purified condensed tannin fraction was analyzed by ^{13}C NMR spectroscopy, and the signal assignment was performed according to the method of Czochanska et al. (*5*). As indicated above, ^{1}H and ^{13}C NMR spectroscopy techniques were used to estimate the degree of polymerization. In the absence of doubly linked A-type bonds, the average molecular weight can be determined from the spectra by comparing the areas of the C3 resonances of the terminal and extender flavan-3-ol units (*5*, *6*, *38*).

The obtained ^{13}C NMR spectrum showed distinct signals at 145.3 and 145.6 ppm, which are assignable to C3′ and C4′ in procyanidin units (catechin/epicatechin). The dominance of the procyanidin unit of the polymeric sample was further corroborated by the presence of strong peaks at 115−116.5 and 119.0−122.0 ppm, consistent with the C2′, C5′, and C6′ chemical shifts, respectively, of the catechol B-ring, with the almost complete exclusion of carbon chemical shifts, which could be attributed to the B-ring of another hydroxylation pattern. Indeed, prodelphinidin units (gallocatechin/epigallocatechin) generally showed a typical resonance at 146 ppm (*5*, *6*). The absence of a clear signal with such a chemical shift in the spectra of the condensed tannins from pear juice revealed that they are mostly composed of procyanidin units.

The region between 70 and 90 ppm is sensitive to the stereochemistry of the C-ring. The determination of the ratio of the 2,3-*cis* to 2,3-*trans* stereochemistries could thus be achieved through the distinct differences in their respective C2 chemical shifts (*5*). Whereas C3 of both *cis* and *trans* isomers occurs at 73 ppm, C2 gives a resonance at 76 ppm for the *cis* form and at 84 ppm for the *trans* form. The absence of the latter signal peak in the spectrum of the studied condensed tannin fraction indicated the presence of only epicatechin subunits. These results thus showed that the polymeric proanthocyanidin fraction of the studied pear juice is predominantly constituted of procyanidins with (−)-epicatechin as main constitutive monomer. This is in agreement with previously reported data concerning pome fruits such as apples (*39*−*41*) and pears (*22*, *32*, 42) for which the proanthocyanidins were reported to be essentially procyanidins composed mainly of (−)-epicatechin units.

The remaining outstanding question that needed to be resolved was related to the molecular size of the proanthocyanidins. It was noted earlier that the areas of the C3 resonances for the extender and terminal flavanol units could be integrated to obtain the number-average molecular weight. The validity of taking advantage of the signal intensity of the C3 signals of the terminating flavanol unit around 67−68 and the extending unit around 72−73 to obtain the number-average molecular weight from the ^{13}C NMR pectrum had been demonstrated (*5*). Theoretically, the intensity of the C3 signal in terminal units relative to that of the signal of the C3 in extension monomer units at 73 ppm could be used for elucidating the polymer chain length. However, the application of this technique for quantification of the molecular weight suffered from inaccuracy due to the low signal-to-noise ratio of the ^{13}C spectra.

ESI/MS Analysis. To overcome the problem related to the determination of polymer chain length, further characterization was continued by means of ESI/MS spectrometry. Analysis was performed in the negative ion mode as proanthocyanidin molecules are thereby better detected than in the positive ion mode due to the acidity of the phenolic protons. They are also more negatively charged as the chain length increases. Continuous flow injection was used as the studied polymeric proanthocyanidins were not properly separated and gave an unresolved hump at the end of the chromatograms due to the large number of isomers. Consequently, using the continuous flow injection technique, isomers add up in a single signal, resulting in a gain of sensitivity.

An example of the obtained results is shown in **Figure 2**. Among the observed peak signals, **Figure 2A** shows molecular ion species consistent with procyanidin oligomers containing singly linked units. A first series of abundant ions separated by 288 Da were observed from *m*/*z* 865 to 2306, corresponding to

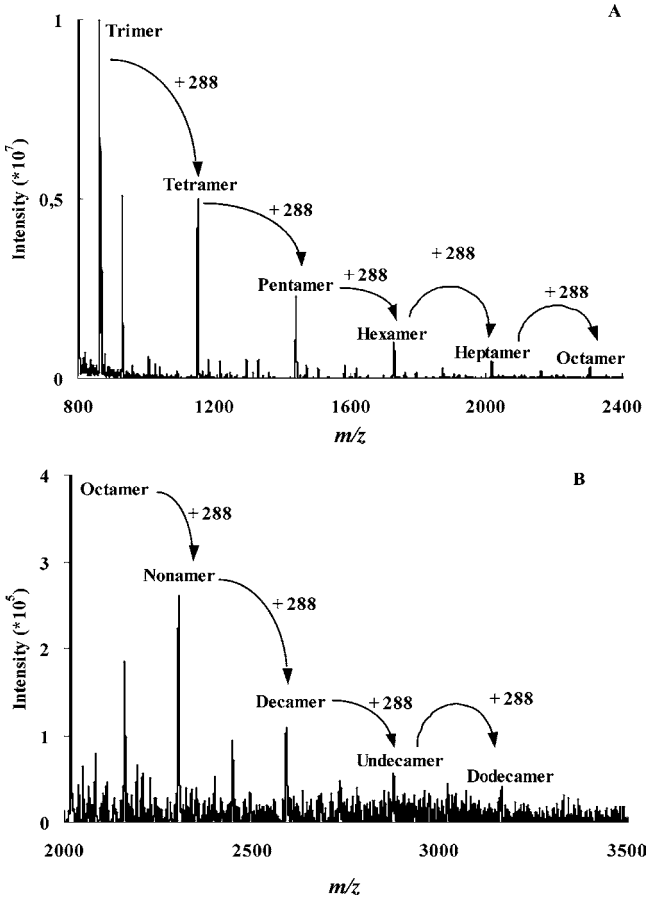

Figure 2. ESI/MS spectra recorded in the negative ion mode showing monocharged ions ranging from trimeric to dodecameric oligomers.

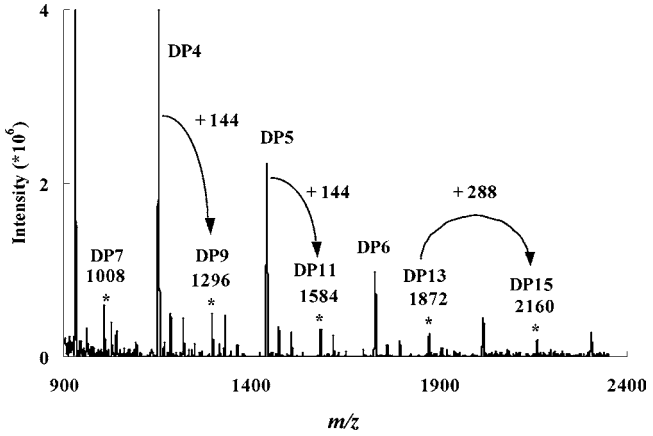

Figure 3. ESI/MS spectum showing monocharged and doubly charged (∗) procyanidin ions.

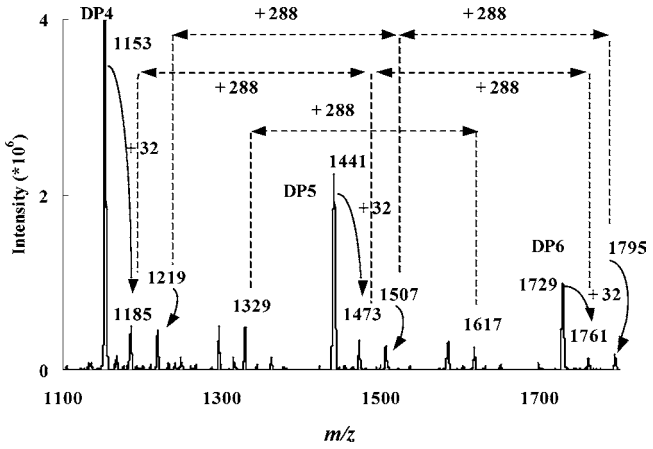

Figure 4. ESI/MS spectra showing the presence of various ion signals separated by 288 Da and probably corresponding to oxidized reaction products involving flavan-3-ols.

the molecular masses of procyanidins with DP 3−8. Indeed, these signals could be interpreted as $[M - H]^-$ ion peaks of trimeric (*m*/*z* 865), tetrameric (*m*/*z* 1153), pentameric (*m*/*z* 1441), hexameric (*m*/*z* 1729), heptameric (*m*/*z* 2017), and octameric (*m*/*z* 2305) procyanidins, respectively. In addition to the signals indicated above, other less intense signals were also observed in the higher *m*/*z* values (**Figure 2B**). These peaks could correspond to nonameric (*m*/*z* 2593), decameric (*m*/*z* 2881), and undecameric (*m*/*z* 3169) proanthocyanidins or to doubly charged ions $[M - 2H]^{2-}$ of DP 6, DP 8, DP 10, DP 12, DP 14, DP 16, DP 18, DP 20, and D P22 species.

Mass spectra also provided evidence for a series of compounds that are 144 mass units higher than those described above. Compounds of this series were separated by 288 mass units, with the most intense signals at *m*/*z* 1008, 1296, 1584, 1872, and 2160 (**Figure 3**). These signals were attributed to doubly charged ions due to their narrower signal width compared to the singly charged species. Existence of the doubly charged ions was proven by the presence of additional signals that can be unambiguously attributed to the doubly charged ions $[M - 2H]^{2-}$ of odd polymerization degree, starting from DP 7. Such multiply charged species are reported to be more frequently observed in ES/MS (*43*) and became more intense as the molecular weight increased, probably as a result of longer chain length, which allows a better charge separation, thus minimizing the electrostatic repulsive forces. The peaks at *m*/*z* 1008, 1296, 1584, 1872, and 2160 were attributed to the doubly charged species of heptameric, nonameric, undecameric, tridecameric, and pentadecameric procyanidins, respectively. No clear multiply charged species beyond the doubly charged ones were detected, presumably because of the lower concentration of larger tannin molecules. However, the apparent decrease of polymer concentration as the molecular weight increases may also be due to an increased dispersion of signal among variously charged ions, including large ones that cannot be detected.

In addition to these nongalloylated procyanidin polymers, monogalloylated derivatives were also observed, but they were detected as relatively weak signals. Thus, peak signals corresponding to dimeric, trimeric, tetrameric, and pentameric monogalloylated procyanidins were observed at *m*/*z* 729, 1017, 1594, and 1885, respectively. Other series of peaks separated by 288 Da were observed at *m*/*z* 1185, 1473, and 1761 (**Figure 4**). The 32 mass unit difference between these compounds and procyanidins is in agreement with the hypothetical presence of two (epi)gallocatechin units in tetrameric, pentameric, and hexameric structures. Signals corresponding to tetrameric (*m*/*z* 1169) and pentameric (*m*/*z* 1457) oligomers with only one epigallocatechin units (+ 16 Da) were also observed but were less abundant. However, as no clear signal corresponding to a trihydroxylated B-ring flavanol unit was observed in the ^{13}C NMR spectrum, we cannot rule out the hypothesis of such signals corresponding to oxidation reaction products involving oxygen addition.

Finally, some minor ions were also detected that did not match with any of the known usual proanthocyanidins. The partial mass spectrum in the range *m*/*z* 1153−1873 (**Figure 4**) exhibits some of these ions. This is the case of peaks observed at *m*/*z* 1328 and 1616, which should probably be attributed to oxidation or degradation reaction products. The fact that these

peak signals were separated by 288 Da, however, suggested the possible implication of flavanol units in these compounds. Finally, the signals separated by 288 Da and observed at *m*/*z* 931, 1219, 1507, and 1795 correspond probably to stacking of trimeric, tetrameric, pentameric, and hexameric procyanidins and chlorogenic acid (+ 354 Da), the presence of which has been detected through HPLC analysis.

This study shows the importance of ESI/MS analysis in determining the molecular weight of condensed tanins, revealing the presence of various oligomeric proanthocyanidins detected as singly and doubly charged ions. However, although polymeric species up to DP 22 were detected, the limited range imposed by the quadrupole analyzer as well as the easy generation of multiple ions for the larger molecules, inducing peak dispersion and frequent overlapping, results in an increased difficulty of interpretation and quantification of the signals due to higher DP procyanidins. Higher molecular weight proanthocyanidins are, for example, difficult to detect with a good precision as their singly charged ions are often observed with a weak intensity, as indicated in **Figure 2B**. MALDI was chosen as a complementary spectroscopic technique to limit the production of multiply charged species.

MALDI-TOF/MS Analysis. Since its introduction (*44*) the MALDI-TOF technique has revealed itself as a powerful method for the characterization of synthetic and natural polymers and has been recently introduced for the analysis of condensed tannins in food science (*9*, *18*, *35*, *45*, *46*). Several factors must be optimized to develop MALDI-TOF/MS techniques. These factors include the selection of an appropriate matrix, optimal mixing and drying of matrix and sample, optimal selection of alkali metal complex, adjustment of laser strength, selection of calibration standards, and correct interpretation of the spectra. During our study, several attempts showed us that the mixture acetonitrile/water (1:1) for solubilizing the condensed tannins and the matrix allowed a homogeneous sample preparation of our polymeric fraction. This resulted in the best conditions for their MALDI-TOF analysis and resulted in mass spectra with low signal-to-noise ratios. The included magnification demonstrates the good resolution of the spectra.

Figures 5 and **6** show the MALDI-TOF mass spectra of the studied polymeric mixture, recorded as sodium adducts in the positive reflectron ion mode and showing a series of repeating procyanidin polymers. The polymeric character is reflected by the periodic occurrence of peak series representing different chain lengths. The results obtained indicated that pear juice condensed tannins are characterized by mass spectra with a series of peaks with distances of 288 Da corresponding to a mass difference of one catechin/epicatechin between each polymer. Therefore, prolongation of condensed tannins is due to the addition of catechin/epicatechin monomers. Given the absolute masses corresponding to each peak, it further suggested that they contain only procyanidins, as was already indicated in the respective ^{13}C NMR spectrum.

Following previously reported studies (*19*), the formula $290 + 288(n - 1) + 152g + 23$ was used to calculate the oligomeric proanthocaynidin molecular weight. In this equation 290 represents the molecular weight of the terminal catechin/epicatechin unit, n is the degree of polymerization of catechin/epicatechin units, g is the number of galloyl esters, and 23 is the weight of sodium. Application of this equation to the experimentally obtained data revealed the presence of a series of condensed tannin consisting of well-resolved oligomers (**Figure 5**). The broad peaks in these spectra indicate, however, that there is large structural heterogeneity within each DP.

Higher molecular weight ions but with significantly less signal intensity were also observed and were attributed to procyanidin consisting of 20−25 flavanol units (**Figure 6**). These observations fully corroborated the interpretation accorded to the ESI/MS data and demonstrated that both techniques were comparable in usefulness for the analysis of low to moderate size proanthocyanidin polymers.

For the condensed tannins indicated above, each peak was always followed by mass signals at a distance of 152 Da (**Figures 5** and **6**) corresponding to the addition of one galloyl group at the heterocyclic C-ring. Thus, peak signals corresponding to monogalloylated derivatives of various procyanidin oligomers were easily attributed. No procyanidin containing more than one galloyl group was detected. Therefore, MALDI-TOF/MS indicates the simultaneous occurrence of pure procyanidin polymers and monogalloylated polymers. This showed that only monogalloylation occurs in pear juice procyanidin oligomers. To our knowledge, this is the first mass spectrometric evidence confirming the existence of galloylated procyanidin oligomers in pear fruits.

Antioxidant Activity. This proanthocyanidins fraction was further tested in an *in vitro* free radical scavenging assay as well as monomeric catechin, and its dimer B3 and their antioxidant power were compared to BHT and quercetin used as positive control. The results obtained with an amount of 20 μg and expressed as the percentage reduction of the initial DPPH absorption by the tested compounds showed that all of the examined compounds possess good DPPH scavenging activity. The obtained values indicated that the DPPH scavenging activity decreased in the following order: polymeric procyanidins (76.4%) > procyanidin B3 (60.8%) > quercetin (60.1%) > catechin (49.2%) > BHT (31.5%). It thus appears that the increasing DP enhances the effectiveness of proanthocyanidin radical scavenging. It was reported that the anti-radical activity of grape seed procyanidin fractions with different average molecular weights was not statistically different (*47*).

Figure 7 shows the DPPH free radical scavenging activity of the studied fractions at different concentrations and demonstrated that all of the tested fractions showed dose-dependent activity. The free radical scavenging activity is usually expressed as percentage of DPPH inhibition but can also be expressed by the antioxidant concentration required for a 50% DPPH reduction (IC_{50}). The obtained results showed that the polymeric procyanidin was the most potent radical scavenging fraction ($IC_{50} = 9.4$) followed by the dimer B3 ($IC_{50} = 15.7$) and catechin ($IC_{50} = 20.4$). The polymeric procyanidin fraction and the dimer B3 are as effective as quercetin ($IC_{50} = 12.6$) and significantly better than BHT ($IC_{50} = 33.0$). **Figure 8** illustrates the evolution of the remaining DPPH with time of the studied fractions. Noteworthy, the polymeric procyanidins fraction bleached the DPPH immediately, suggesting that they could be classified as kinetic antioxidants (*37*).

In the present study we have investigated the structural characterization and antioxidant activity of a polymeric proanthocyanidin fraction isolated from pear juice in order to correlate the observed activity to the polymerization degree of the proanthocyanidin. Different kinds of information were obtained from NMR and MS analysis. NMR measurements give structural information about the molecules and their functional groups in this fraction. The results obtained showed that the proanthocyanidin consisted of predominantly procyanidins with 2,3-*cis* stereochemistry. However, the low intensity of the signal for terminal C3 in our NMR spectra prevented the determination of the polymerization degree of the studied tannins.

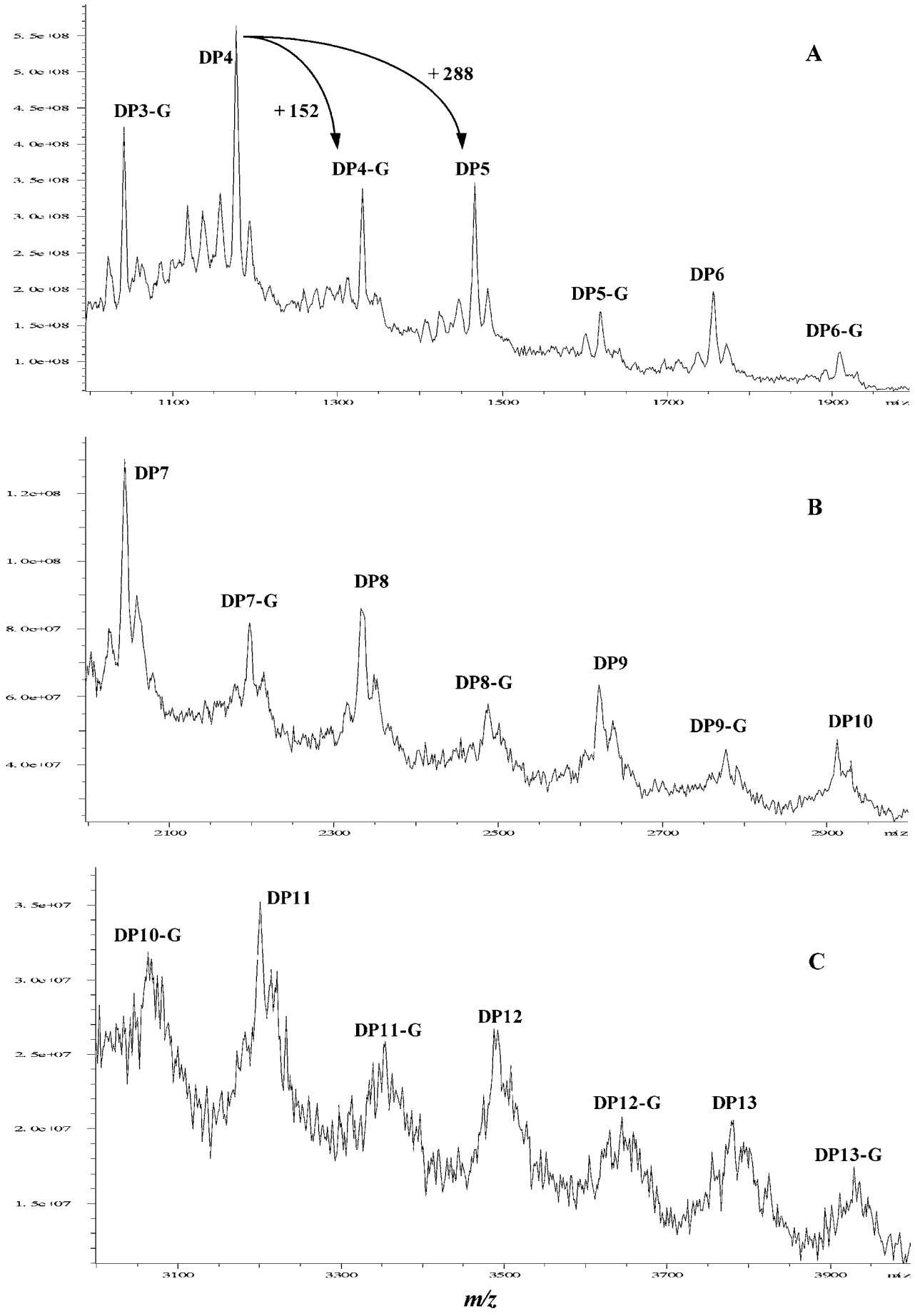

Figure 5. MALDI-TOF/MS spectra of the polymeric procyanidin fraction showing well-resolved oligomers for mass range (**A**) from *m*/*z* 1000 to 2000 Da, (**B**) from *m*/*z* 2000 to 3000, and (**C**) from *m*/*z* 3000 to 4000 Da.

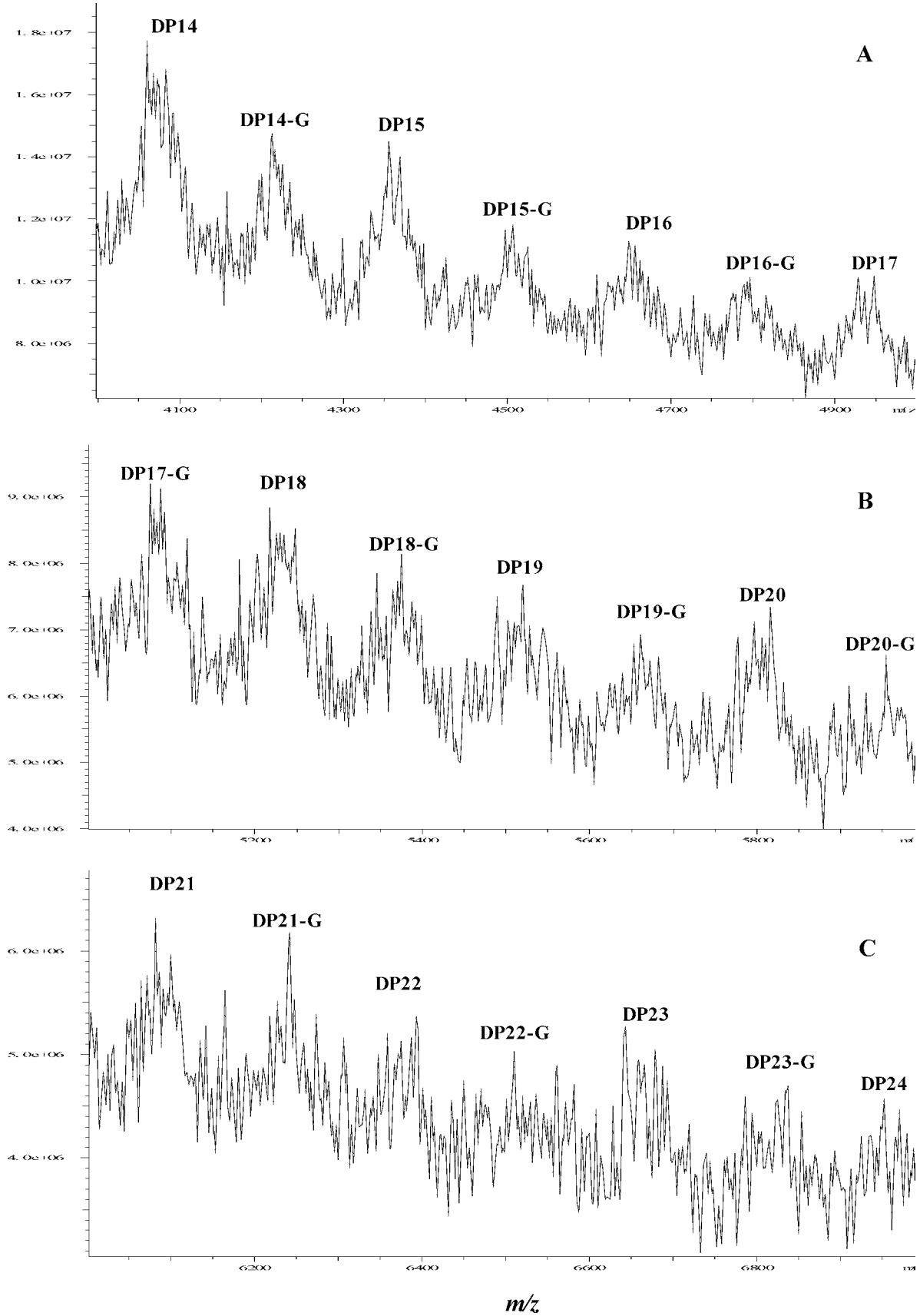

Figure 6. MALDI-TOF/MS spectra of the polymeric procyanidin fraction of higher procyanidins for mass range (**A**) from *m*/*z* 4000 to 5000 Da, (**B**) from *m*/*z* 5000 to 6000, and (**C**) from *m*/*z* 6000 to 7000 Da.

In contrast, polymer chain length distribution was elucidated through ESI/MS and MALDI-TOF/MS. Both techniques showed peak signals representative of masses that correspond to oligomeric series of catechin/epicatechin unit procyanidins. As the molecular weight of the oligomers increases, the signal intensity decreases. This is not surprising, as both MALDI and

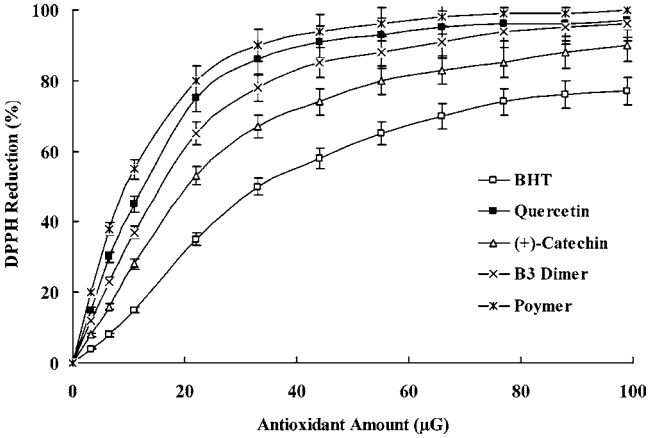

Figure 7. DPPH disappearance as a function of concentration of (+)-catechin, dimer B3, and the pear juice polymeric proanthocyanidin fraction.

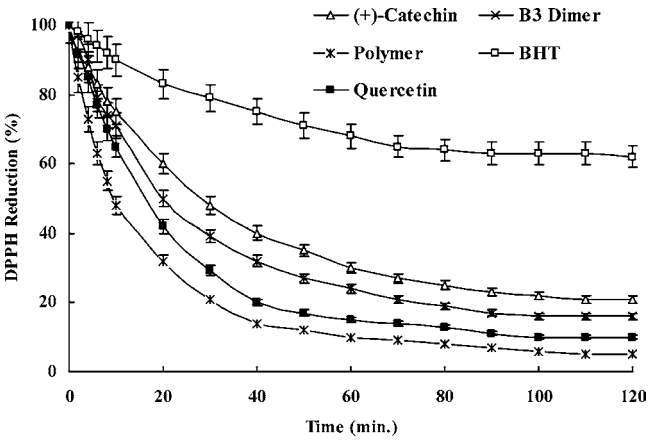

Figure 8. Remaining DPPH as a function of reaction time for (+)-catechin, dimer B3, and the pear juice polymeric proanthocyanidin fraction.

ESI mass spectroscopy discriminate in favor of the lower molecular weight ions, reducing the apparent abundance of the larger oligomers. The results obtained through ESI/MS analysis in the negative ion mode showed singly and doubly charged ions corresponding to the molecular mass of procyanidins with a degree of polymerization up to 22. To confirm the results obtained through ESI/MS, MALDI mass spectra were recorded in the positive-ion reflectron and linear mode. The mass spectra obtained showed a series of repeating procyanidin polymers that were observed as their sodium adducts. Additionally masses corresponding to a series of monogalloyl polyflavans were also detected.

It may be noted that the mean degree of polymerization determined through thioacidolysis followed by HPLC analysis was 28, which is higher than the DP observed through ESI/MS and MALDI-TOF/MS analyses. This result is in agreement with those previously reported by Taylor et al. (*48*) concerning the proanthocyanidin of hops and where the results determined by depolymerization method were higher than the ions detected through mass spectroscopic methods. This may be caused by the limitations imposed by the quadrupole analyzer used in the ESI method or by a weak or incomplete desorption of the matrix through the MALDI method. This may also be due to an overestimation of the mean polymerization degree when using the thioacidolysis depolymerization method. This overestimation could be due to the presence of galloylated units if they are involved as terminal units. Their presence was indeed not observed through thioacidolysis due to their weak occurrence compared to the ungalloylated derivatives.

The antioxidant properties of the polymeric fraction were investigated through reduction of the DPPH free radical, and the obtained results showed that the polymeric fraction exhibited a potent antioxidant power compared to that of (+)-catechin and B_3 procyanidin dimer, suggesting that the increasing degree of polymeriation enhances the effectiveness of proanthocyanidins against radicals.

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