Characterization of Grape Procyanidins Using High-Performance Liquid Chromatography/Mass Spectrometry and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

Ying Yang* and Mingjien Chien‡

Science and Technology, Givaudan Flavors Corporation, 1199 Edison Drive, Cincinnati, Ohio 45216

High-performance liquid chromatography/mass spectrometry (HPLC/MS) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) were used to characterize the procyanidin composition of the grape seed extract. The detection of the oligomers composed of (+)-catechin, (−)-epicatechin, and their galloylated derivatives in the grape seeds is demonstrated. With MALDI-TOF MS, oligomers up to nonamers were observed. The potential of the MALDI-TOF MS technique as a quantification tool is also discussed. The information presented in this study could lead to the determination of procyanidin content and their molecular weight distribution in grape seeds.

Keywords: *Grape seed; catechin; procyanidins; HPLC/MS; MALDI-TOF MS*

INTRODUCTION

In the past several years, a large number of scientific reports have presented data showing the beneficial health effects of beverages made from grape (*Vitis vinifera*) related products (Criqui, 1996; Facino et al., 1996; Goldberg et al., 1995). Correlation between the consumption of red wine and the low rate of cardiovascular disease has long been recognized (Renaud and de Lorgeril, 1992; Criqui and Ringel, 1994). The beneficial effects of grape are attributed to the phenolic components located in the skin, stems, and seeds, which exhibit strong antioxidant activity (Hagerman et al., 1998; Saint-Cricq de Gaulejac et al., 1999a,b; Yamaguchi et al., 1999) and play a critical role in reducing the oxidation of low-density lipoproteins (LDL) (Meyer et al., 1997; Tebib et al., 1994, 1997; Teissedre et al., 1996; Vinson and Hontz, 1995; Vinson et al., 1999). The oxidation of LDL is believed to be a crucial step toward the advance of atherosclerosis (Steinberg et al., 1989; Steinberg, 1992).

Among the most abundant phenolic compounds found in grape are procyanidins, a class of flavonoids that yield cyanidin upon oxidation under strongly acidic conditions (Porter et al., 1986; Prieur et al., 1994; Fuleki and Ricardo da Silva, 1997). They consist mainly of (+)-catechin, (−)-epicatechin, and their 3-*O*-gallates, linked by either 4−8 or 4−6 bonds (B type) (Figure 1) (Thompson et al., 1972; Ricardo da Silva et al., 1991; de Freitas et al., 1998). Also identified in many plants are the oligomers with a second interflavonoid bond formed between 2 and 7-*O* (A type) (Jacques and Haslam, 1974; Vivas et al., 1996). In addition to the (+)-catechin and (−)-epicatechin polymers, prodelphinidins, i.e., the polymerized gallocatechins or epigallocatechins, were identified in the grape skin (Souquet et al., 1996). Some of the mononers and dimers in grape are shown in Figure 1.

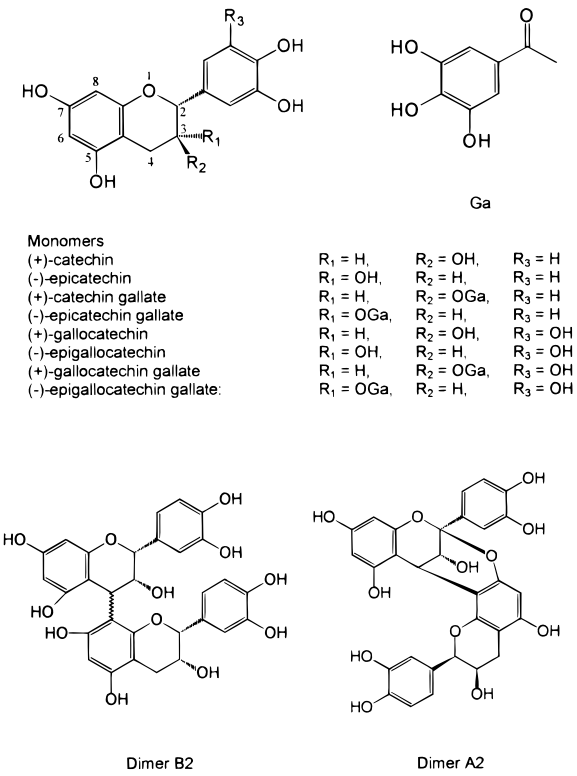

Figure 1. Structures of catechin monomers and dimers.

Because of their complex nature, a complete characterization of grape procyanidins has so far eluded analytical chemists despite the amount of effort devoted to it. Presently, reliable methods for total procyanidin quantification and for information regarding the molecular weight distribution are still lacking. Classically, the total procyanidin content is determined by one of several colorimetric methods. A butanol/HCl assay,

* Correspondence regarding the analytical procedures should be addressed to Y.Y. [telephone (513) 948-4984; fax (513) 948-4889; e-mail ying.yang@givaudan.com].

‡ Correspondence regarding grape seed chemistry and processing should be addressed to M.C.: [telephone (513) 948-3508; fax (513) 948-3582; e-mail mingjien.chien@givaudan.com].

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which has become popular in the health food industry, was developed on the basis of the acid-catalyzed degradation of procyanidins to the red-colored cyanidin ion (Bate-Smith, 1981). However, the assay can be difficult to reproduce due to the competitions from side reactions and the yields vary greatly depending on temperature, acid concentration, water content, the type and condition of solvents, and the presence of air, catalysts, or other oxidizing reagents (Porter et al., 1986). All of these conditions have to be carefully controlled in order to obtain reproducible results. The reaction yield of the acid-catalyzed degradation of procyanidins may be improved in the presence of nucleophiles, which form adducts with the extension units of the polymers. By analyzing these adducts by HPLC, additional information, including the average degree of polymerization and the nature of both the extension and the termination units, may be obtained (Matthews et al., 1997; Guyot et al., 1998). A few other colorimetric assays are also commonly used in the health food industry, especially the Folin−Ciocalteu test for total phenol (Singleton and Rossi, 1965), and the acidic vanillin test for flavanols (Broadhurst and Jones, 1978). The Folin−Ciocalteu test is not specific to procyanidins, as it responds to many types of phenols with varying degrees of sensitivity. The acidic vanillin assay is specific to flavonols and is useful in determining catechins and proanthocyanidins. However, lack of reproducibility between samples, days, and laboratories has been reported (Broadhurst and Jones, 1978; Sun et al., 1998b).

The most common method used to study the molecular weight distribution of procyanidins is HPLC. Attempts by others to separate and quantify procyanidins by both reverse- and normal-phase HPLC have enjoyed only limited success. In reverse-phase HPLC, compounds smaller than trimers are well resolved, but those larger than trimers still appear as a broad peak (Guyot et al., 1997; Sun et al., 1998a). There does not appear to be any correlation between the elution order and the degree of polymerization. Normal-phase HPLC was applied to simple procyanidins derived from cocoa (Rigaud et al., 1993; Hammerstone et al., 1999) with more success. Monomers and oligomers up to decamers were well resolved into equally spaced peaks in an order consistent with the degree of polymerization. However, for the more complex procyanidins extracted from grape, the resolution for the large oligomers was still less than satisfactory. Monomers through trimers appear as multiple peaks, but the higher oligomers and the polymers are still not resolved [see data in Rigaud et al. (1993)].

More recently, normal-phase HPLC has been used in combination with mass spectrometry, allowing the identification of oligomeric procyanidins. For example, Hammerstone and co-workers (Hammerstone et al., 1999; Lazarus et al., 1999) reported successful separation and identification of monomeric and oligomeric procyanidins in cocoa and other plant materials by HPLC/MS using atmospheric pressure ionization electrospray (API-ES). Fulcrand et al. (1999) analyzed wine tannins by HPLC coupled to an electrospray ionization mass spectrometry (ESI-MS) and concluded that each peak separated by HPLC may contain either or both catechin and gallocatechin units.

In comparison to apple and cocoa, the composition of grape procyanidins appears to be much more complex. Thiolysis studies revealed that both apple and cocoa procyanidins are composed of mainly of (−)-epicatechin units (Guyot et al., 1998) while grape procyanidins were shown to contain (+)-catechin, (−)-epicatechin, their gallates (seeds), and all the gallo counterparts (skin). There was also evidence suggesting the existence of copolymers of catechins and gallocatechins (Fulcrand et al., 1999). Thus, with increasing degree of polymerization, the number of isomers increases exponentially, making the HPLC signals of the large oligomers overly broad and thus undetectable by the current HPLC methodologies.

Direct mass spectrometric techniques have also been used to characterize the procyanidin components without prior separation by HPLC. For example, Guyot et al. (1997) used ESI-MS to show a complete series of the apple cider procyanidins with a degree of polymerization up to 17. Ohnishi-Kameyama et al. (1997) applied fast-atom bombardment mass spectrometry (FAB-MS) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) techniques to identify catechin oligomers up to the pentadecamer in apple.

In the present study, we tried several chromatographic and mass spectrometric techniques for characterizing the procyanidins extracted from grape seeds. While continuing to refine the HPLC conditions for better separation of the oligomers, we focused on a direct MS technique, MALDI-TOF MS, which has been applied very successfully to the studies of the biological molecules and polymers. Because of its soft ionization energy and high ion transmission yield, MALDI-TOF MS should be an ideal tool to study procyanidins. Furthermore, it is highly tolerant toward contaminants and thus particularly suitable for directly studying complex mixtures. We have also attempted to determine whether MALDI-TOF MS is useful for the quantification of the individual oligomers in the grape extract.

MATERIALS AND METHODS

Standards and Raw Materials. (+)-Catechin, (−)-epicatechin, (−)-epicatechin gallate, 2,5-dihydroxybenzoic acid (DHB), ACTH (fragment 18−39), insulin B chain oxidized, angiotensin II, and angiotensinogen (fragment 1−13) were purchased from Sigma (St. Louis, MO). *trans*-3-Indoleacrylic acid (IAA) was purchased from Aldrich (Milwaukee, WI). Dried grape seeds were obtained from Canandaigua Wine Co. (Madera, CA). Cocoa nibs were purchased from a local supplier.

Extraction Procedures. *Grape Seeds.* Dried whole grape seeds (500 g) were extracted with 1 kg of acetone/water mixture (50/50 w/w) at 50 °C. This extraction was repeated once and the extracts were combined. After evaporation of the acetone at 50 °C under reduced pressure, the aqueous solution was further concentrated until the total weight was reduced to 300 g. After removal of the insoluble residue by filtration, the clear aqueous solution was further extracted with three portions of ethyl acetate (180 g each). The ethyl acetate extracts were combined and dried under vacuum to give 5.0−7.5 g of light tan-colored powder.

Cocoa. Cocoa nibs were extracted using the same procedure described for grape seeds except that the aqueous concentrate was washed with an equal amount of hexane prior to the ethyl acetate extraction. Typical yields were between 0.5 and 0.8%.

HPLC/MS Analysis. Normal-phase HPLC analysis of the grape seed and cocoa extracts were carried out under conditions similar to those used by Hammerstone et al. (1999). An HP 1100 series HPLC (Hewlett-Packard, Palo Alto, CA) equipped with quaternary pumps and interfaced to an HP mass-selective detector was used. A Phenomenex Luna 5 μm Silica column (250 × 4.6 mm) was used for all these analyses. Detection was made by UV at $\lambda = 280$ nm and by MS in the

negative atmospheric pressure chemical ionization (APCI) mode. The ternary mobile phase consisted of (A) dichloromethane, (B) methanol, and (C) acetic acid and water (1:1 v/v). The series of linear gradients used in the analysis were as follows: 0−30 min, 82−68% A, 14−28% B; 30−60 min, 68−46% A, 28−50% B; 60−65 min, 46−10% A, 50−86%B; 65−70 min, isocratic. The concentration of C was kept constant at 4%. The flow rate was kept at 1 mL/min and column temperature at 37 °C.

Conditions for the mass spectroscopic detection in the negative APCI mode were as follows: capillary voltage at 2000 V, nitrogen as the drying gas at 6.0 L/min and 350 °C, vaporizer temperature at 450 °C, nebulizer pressure at 20 psig, corona current at 7 μA, and fragmentor voltage at 75 V. Grape seed and cocoa extracts were dissolved in methanol (5 mg/mL), filtered through a 0.45-μm nylon filter, and then injected at 10 μL volume.

MALDI-TOF MS Analysis. Reflectron mode MALDI-TOF MS experiments were performed on a Perkin-Elmer Biosystems Voyager-Elite instrument at the Department of Chemistry of the University of New Orleans (New Orleans, LA). Positive ion spectra (128 summed acquisitions) were acquired in the delayed-extraction (50 ns) and reflectron modes. The accelerating voltage was set at 20 000 V, and the grid voltage was set at 75% of the accelerating voltage. A nitrogen laser (337 nm) was used with an attenuation at 2400. The low mass gate was set at *m*/*z* 200. The singly charged molecular ions of two peptides, ACTH (fragment 18−39) (FW 2465.7) and angiotensin II (FW 1046.2), were used as external standards for the mass calibration procedure that was performed prior to the analysis of each sample.

Linear mode MALDI-TOF MS experiments were performed on a Reflex III TOF-MS (Bruker Daltonics, Billerica, MA) equipped with a two-stage gridless reflectron at the Department of Chemistry and Biochemistry of Miami University (Oxford, OH). The effective ion flight path was 290 cm. The 355 nm line of a Nd−yttrium−aluminum−garnet (YAG) laser (MiniLase-10, New Wave Research, Sunnyvale, CA) was employed. Positive mass spectra were acquired by summing the spectra generated from 100 laser shots. The singly charged molecular ions of insulin B chain oxidized (FW 3495.9) and angiotensin II (FW 1046.2) were used as external standards for the mass calibration procedure performed prior to the analysis of each sample.

Grape seed and cocoa extract samples were dissolved in acetone or methanol at 2 mg/mL. The matrix systems (DHB or IAA) were prepared in tetrahydrofuran at 20 mg/mL. The sample and matrix solutions were mixed at 1/1 (v/v) ratio immediately before the analysis.

RESULTS AND DISCUSSION

HPLC/MS Studies. The normal-phase HPLC traces for the grape seed extract are shown in Figure 2. The UV trace shows a clear separation for gallic acid, catechin, epicatechin, the dimers, and their corresponding galloylated derivatives. The presence of gallocatechins and gallocatechin gallates that are hidden under other larger peaks became evident by plotting the extracted ion chromatograms for *m*/*z* 305 and 457 (Figure 3). The UV chromatogram also shows weak peaks at 27 and 31 min that may very well be the tetramers and pentamers, respectively. The corresponding MS total ion chromatogram (TIC), however, was not strong enough to produce the anticipated negative ions at *m*/*z* 1153 and 1441. Oligomers larger than pentamers were not observed in either the UV or TIC chromatograms.

The normal-phase HPLC profile of the grape seed extract is consistent with that reported for the cocoa extract (Rigaud et al., 1993; Hammerstone et al., 1999). The monomers and the oligomers eluted in the order of

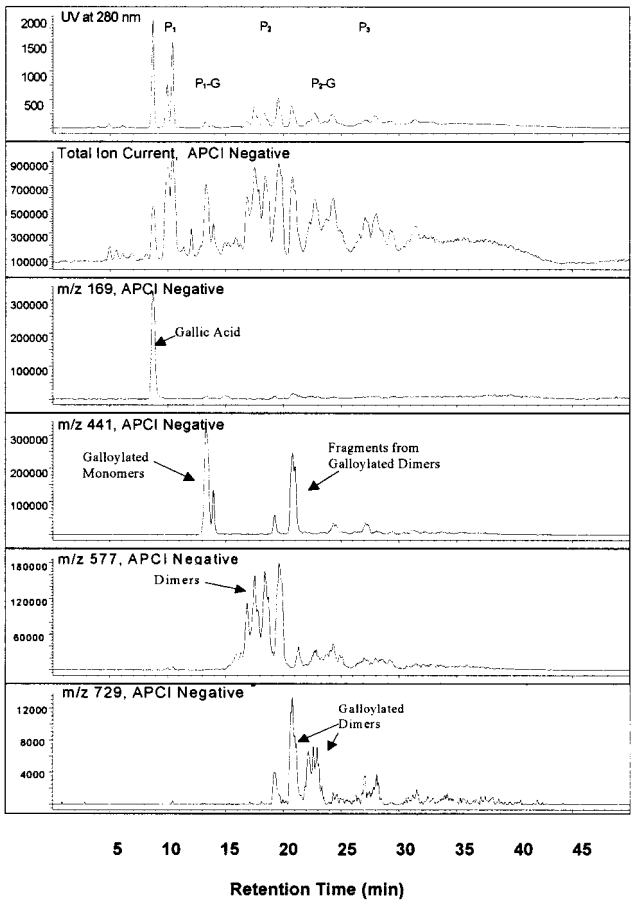

Figure 2. HPLC/UV/MS chromatograms of grape seed extract. Selected ions showing monomers, dimers and their galloylated derivatives are indicated (P_1, monomers, P_2, dimers; P_3, trimers; P_1-G, galloylated monomers; P_2-G, galloylated dimers).

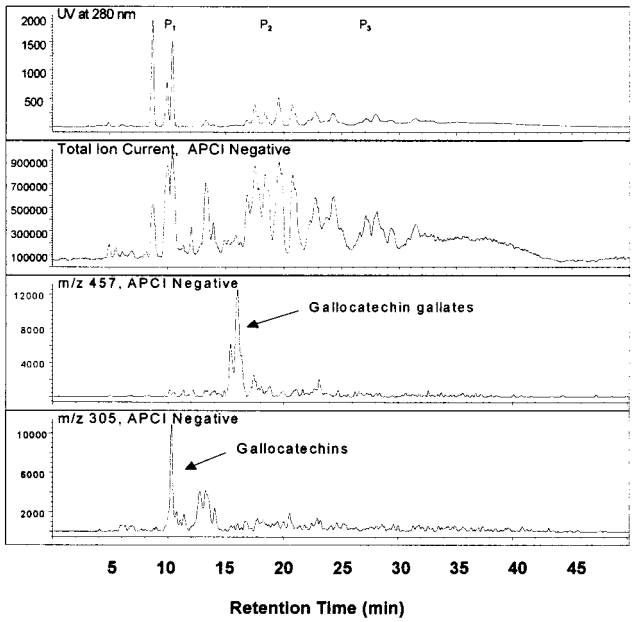

Figure 3. HPLC/UV/MS traces of grape seed extract, selected ions showing gallocatechins and gallocatechin gallates are indicated (P_1, monomers; P_2, dimers; P_3, trimers).

their molecular weights. However, the HPLC profiles of the cocoa extract reported by those authors, and replicated in our laboratory, were very simple, consisting of several well-resolved peaks that were equally

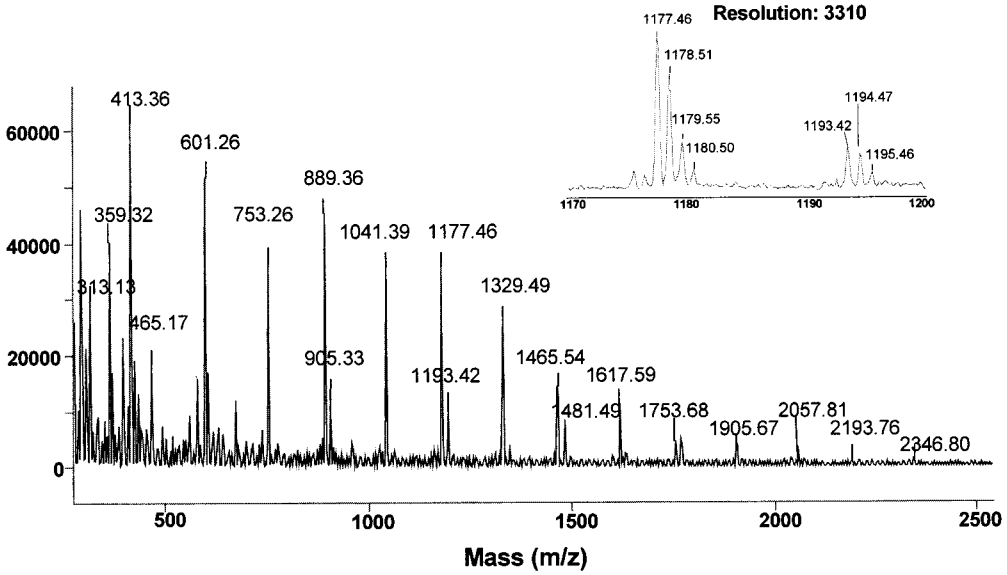

Figure 4. Positive-ion MALDI-MS spectrum of grape seed extract with DHB matrix in the reflectron mode.

spaced in order of increasing molecular weight. There were no more than two or three isomers shown in the cocoa extract for each degree of polymerization. This simplicity in procyanidin composition makes it possible to detect the higher oligomers in cocoa by HPLC/MS. In contrast to cocoa, the composition of the grape seed procyanidins is much more complex. Numerous diastereomers consisting of catechin, epicatechin, and their corresponding galloylated derivatives exist at each degree of polymerization. As the molecular weight increases, the number of isomers becomes so large that the separation and detection of individual isomers become almost impossible. Instead of forming well-resolved peaks, the signals of the larger oligomers were so broad that they became undetectable by HPLC/MS. Although Lazarus et al. (1999) listed the oligomers up to the octamers as present in the grape seeds, presumably detected in their LC/MS study, they did not present the actual data.

MALDI-TOF MS Studies. The positive-ion MALDI-TOF MS spectrum of the same grape seed extract sample with DHB matrix acquired in the reflectron mode is shown in Figure 4. The observed mass ions are summarized in Table 1 under the column head FW (observed). The dominating features of the spectra are two major series of ions separated by 152 Da. The first series (series A in Table 1) can be attributed to the sodium adducts of monomers and the oligomers composed purely of (+)-catechin and (−)-epicatechin, with the molecular weight of 290 + 288 (n − 1), n being the degree of polymerization. The second series (series B in Table 1) consists of ions with the molecular weight of 442 + 288 (n − 1), which are the sodium adducts of oligomers containing one galloylated unit, i.e., either a (+)-catechin gallate or an (−)-epicatechin gallate. In addition to the observed ions, the calculated molecular weight and the proposed compositions are also listed in Table 1. Up to hexamer and heptamer were observed for these two series, respectively. In agreement with Ohnishi-Kameyama et al. (1997), we observed no protonated molecular ions. Instead, only the sodium adducts were detected. For example, the ion observed at 1041.30 is the sodium adduct of the trimer with molecular weight of 1018.89, which consists of two catechin/epicatechin and one galloylated catechin/epicatechin.

Table 1. Positive Ions of Grape Seed Procyanidins Detected by MALDI-TOF MS in the Reflectron Mode

	composition		MW	Na adduct	FW	
n[a]	C/EC[b]	CG/ECG[c]	(calcd)	(calcd)	(obsd)[d]	series[e]
1	1		290.27	313.26	313.13	A
1		1	442.38	465.37	465.17	B
2	2		578.53	601.52	601.26	A
2	1	1	730.64	753.63	753.26	B
2		2	882.74	905.73	905.33	C1
3	3		866.79	889.78	889.36	A
3	2	1	1018.89	1041.88	1041.30	B
3	1	2	1171.00	1193.99	1193.42	C1
3		3	1323.11	1346.10	1345.50	D1
4	4		1155.04	1178.03	1177.46	A
4	3	1	1307.15	1330.14	1329.49	B
4	2	2	1459.26	1482.25	1481.49	C1
4	1	3	1611.36	1634.35	1633.60	D1
5	5		1443.30	1466.29	1465.54	A
5	4	1	1595.41	1618.40	1617.59	B
5	3	2	1747.51	1770.50	1769.50	C1
6	6		1731.56	1754.55	1753.68	A
6	5	1	1883.66	1906.65	1905.67	B
6	4	2	2035.79	2058.76	2057.81	C1
7	6	1	2171.92	2194.91	2193.76	B
7	5	2	2324.03	2347.02	2346.80	C1

[a] n = degree of polymerization. [b] C = (+)-catechin; EC = (−)-epicatechin. [c] CG = (+)-catechin gallate; ECG = (−)-epicatechin gallate. [d] Observed as sodium adducts. [e] Tentative compositions: series A, 290 + 288 (n − 1); series B, 442 + 288 (n − 1); series C1, 882 + 288 (n − 2); series D1, 1322 + 288 (n − 3).

Since no sodium salt was used in the preparation of grape seed extract, the sodium apparently arises from the seeds themselves. Desalting the samples by cation exchange and by repeated precipitating in anhydrous methanol had no effect on the overall quality of the spectrum. On the other hand, adding sodium chloride to the sample did not change the signal sensitivity either. Apparently, only a minute amount of sodium is needed. This is in agreement with the general observation in MALDI-TOF MS that sodium greatly enhances the signal sensitivity (Chen and Guo, 1997; Ohnishi-Kameyama et al., 1997).

In addition to the two major series of ions described above, there are other ions at lower intensity that are also detectable in the spectrum. Most notable is a series of ions that are16 Da higher than those in series A. Most probably, they are the sodium adducts of oligomers

containing two galloylated units, with the molecular weights of 882 + 288 (n − 2) (series C1 in Table 1). Theoretically, this series may be interpreted as the oligomers containing one gallo-(+)-catechin or gallo-(−)-epicatechin, with the molecular weights of 306 + 288 (n − 1) (series C2). Of the two series, C1 is more likely because grape seeds are known to be rich only in procyanidins composed of caetechins and the galloylated catechins. Gallocatechins, which make up the prodelphinidins, are found only in the skin. However, we did notice the presence of the corresponding monomers for series C2, gallocatechins and gallocatechin gallates, in our HPLC/MS study (Figure 3).

Similar to series C, ions that are 16 Da higher than those in series B can be found by carefully searching the MALDI-TOF spectrum. For example, the presence of the trimer and tetramer containing three galloylated units became evident only after searching for their respective sodium adducts at m/z 1345 and 1633 [series D1 in Table 1 with the molecular weight of 1322 + 288 (n − 3)]. The same ions could theoretically be interpreted as a tetramer and a pentamer with one gallocatechin and one catechin gallate unit with the molecular weight of 458 + 288 (n − 1) (series D2). However, such copolymers were never reported to be present in the seeds. Similarly, the presence of some of the higher oligomers, such as the hexamers and heptamers, containing one and two galloylated units (FW of sodium adducts 1905, 2057, 2193 and 2345, respectively) could be established by searching for their specific molecular ions in the MALDI-TOF spectrum. Among these four series of procyanidin oligomers, only series A has been reported in apple by Ohnishi-Kameyama et al. (1997). The other series proposed in this study have never been observed by MALDI-TOF MS before.

It is important to note that series C and D could be explained alternatively as the potassium adducts of the same ions that give rise to series A and B, respectively. The potassium adducts were previously observed in apple by Ohnishi-Kameyama et al. (1997). However, if these ions are indeed potassium adducts, one would expect series C and D to follow the same pattern. This is not what we have observed. While ions in series C becomes more predominant as the mass increases, series D appears to be random in intensity. This indicates that series A, B, C, and D are more likely four distinct components, rather than two components bound to two different alkali ions.

The mass resolution of the positive-ion MALDI-TOF MS in the reflectron mode achieved by the Perkin-Elmer Biosystems Voyager Elite instrument was greater than 3000, allowing the separations of individual ions of different isotope composition. For example, the ion at m/z 1177.46 is further resolved into a group of four peaks in the expanded spectrum, representing its isotopic pattern (see inset of Figure 4). Likewise, the other ion clusters in this MALDI-TOF MS spectrum are well resolved into the similar isotopic patterns.

To detect ions higher than those shown above, it is necessary to operate the MALDI-TOF MS in the linear mode. Ohnishi-Kameyama et al. (1997) have reported that the largest oligomer detected in the linear mode was usually three units higher than that detected in the reflectron mode. The lower sensitivity of the reflection mode for the larger ions is due to the breakdown of these ions as the results of longer flight path and the postacceleration process. Conversely, the sensitivity for the large ions is higher in the linear mode operation because the flight path is shorter and the postacceleration process is not involved. In a separate linear mode experiment using IAA matrix, we were able to extend the mass range beyond 3000 Da at the expense of some resolution. Sodium adduct ions of oligomers as large as the nonamers containing multiple galloylated units were detected, although the quality of spectrum became less satisfactory (Table 2).

Table 2. Positive Ions of Large Grape Seed Procyanidins Detected by MALDI-TOF MS in the Linear Mode

	composition			
n[a]	C/EC[b]	CG/ECG[c]	MW (calcd)	FW (obsd)[d]
6	1	5	2490	2513
6		6	2642	2665
7	4	3	2474	2497
7	3	4	2626	2649
7	2	5	2778	2801
8	6	2	2610	2633
8	5	3	2762	2785
8	4	4	2914	2937
8	3	5	3066	3089
8	2	6	3218	3241
9	7	2	2898	2921
9	6	3	3050	3073

[a] n = degree of polymerization. [b] C = (+)-catechin; EC = (−)-epicatechin. [c] CG = (+)-catechin gallate; ECG = (−)-epicatechin gallate. [d] Observed as sodium adducts.

Optimization of MALDI-TOF MS Conditions. Since MALDI-TOF MS is capable of detecting individual oligomers with great resolution, it would be very desirable to examine the sensitivity and reproducibility of this technique, which are essential for quantification method development. The sensitivity and reproducibility of MALDI-TOF MS depend on a number of factors that need to be examined individually. Two of the most important factors are the selection of matrix systems and the sample/matrix preparation method. The best combination of these two should offer shot-to-shot repeatability and sample-to-sample spectral reproducibility.

Matrix Selection. Based on the results of separate studies using other matrix systems, as well as the information reported by Ohnishi-Kameyama et al. (1997), we concluded that DHB is the best matrix for studying grape procyanidins by the reflectron mode MALDI-TOF MS. Compared with other matrix systems commonly used, such as *trans*-3-indoleacrylic acid (IAA), α-cyano-4-hydroxycinnamic acid (CCA), sinapinic acid (SA), 9-nitroanthracene (9NA), 5-chlorosalicylic acid (5CSA), 2-(4-hydroxyphenylazo)benzoic acid (HABA), and dithranol, DHB provides the broadest mass range with the least background noise. Although IAA matrix provides a similar mass range as DHB, it tends to generate a very high background of noise in the mass range below 500 blocking out the signals from the monomers and the dimers, even though a lower laser power is applied. Table 3 summarizes the detectability of procyanidin components by MALDI-TOF MS in various matrix systems.

Sample Preparation. During the course of our MALDI-TOF MS study, it became clear that the solvents used for the sample and matrix preparation have strong effects on the overall quality of the spectrum. Normally, rapid evaporation of the solvents leads to the formation of fine crystals of the solutes, allowing a homogeneous incorporation of the sample into the matrix. Greater sample homogeneity results in better shot-to-shot re-

Table 3. Matrix Systems Investigated for MALDI-TOF MS

analyte	DHB	IAA	CCA[a]	SA[a]	9NA[a]	5CSA[a]	HABA[a]	Dithranol[a]
oligomers	X[b]	X	nd[b]	nd	nd	nd	nd	nd
dimers	X	X	X	X	nd	nd	nd	nd
monomers	X	nd	X	nd	nd	nd	nd	nd

[a] Ohnishi-Kameyama et al. (1997). [b] X = detected; nd = not detected.

peatability and sample-to-sample reproducibility. In the case of grape procyanidins, good results were obtained with anhydrous tetrahydrofuran, acetone, or methanol. Presence of water, on the other hand, makes it difficult to evaporate the solvents uniformly, resulting in poor sample−matrix distribution and weak mass ion signals.

Quantification Consideration. With the MALDI-TOF MS conditions optimized for its best sensitivity and reproducibility, it is possible to determine whether this technique may be used for the quantification. A series of grape seed extract samples were prepared at various concentrations. The peptide angiotensinogen (fragment 1−13) was selected as an internal standard (IS), because it is not susceptible to fragmentation under our operating conditions. The relative intensity (sample intensity/IS intensity) of each oligomer shows a general increasing trend with increasing concentration. This indicates that MALDI-TOF MS may be useful for quantification, although major obstacles remain for a full quantification. First, the exact amounts of each oligomer in the sample may be determined only if pure standards are available. Unfortunately, this is not the case except for the small oligomers. Without the standards, one could only assume equal molar ionization efficiencies for all oligomers and calculate their amounts in a catechin-equivalent unit. Further difficulty in the quantification of the higher oligomers arises from the fact that only singly charged ions are formed by MALDI and as the molecular weight increases the charge density decreases proportionally. Under the current operating conditions, only the peaks from hexamers and lower oligomers are quantitatively significant; the peaks from heptamers and up are too small to be useful. A more complete characterization of procyanidins by the MALDI-TOF MS may be achieved when these obstacles are eventually overcome.

In conclusion, HPLC/MS is able to separate the procyanidin isomers of the lower degree of polymerization but fails to resolve oligomers larger than pentamers. MALDI-TOF MS, on the other hand, allows a rapid analysis of such complex mixtures. The individual oligomers of procyanidins in grape seeds are well resolved in MALDI-TOF MS spectra with their molecular weight determined with great accuracy.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; MS, mass spectrometry; API-ES, atmospheric pressure ionization electrospray; APCI, atmospheric pressure chemical ionization; TLC, thin-layer chromatography; FAB-MS, fast atom bombardment mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; TIC, total ion chromatogram; DHB: 2,5-dihydroxybenzoic acid; IAA, *trans*-3-indoleacrylic acid; C, (+)-catechin; EC, (−)-epicatechin; CG, (+)-catechin gallate; EGC, (−)-epigallocatechin; GC, (+)-gallocatechin; EGC, (−)-epigallocatechin.

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