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Procyanidin Composition of Selected Fruits and Fruit Byproducts Is Affected by Extraction Method and Variety

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Fruits and fruit byproducts are rich sources of polyphenols, including procyanidins, which are known to have numerous potential health benefits. This study investigated if varietal differences existed in the procyanidin composition of grape seed and if soaking in extraction solvent overnight prior to extraction improved the recovery of procyanidins from grape seed, grape pomace, and blueberry and cranberry powders. Riesling contained the highest amount of procyanidins, including the lower molecular weight monomers and dimers, followed by Chardonnay (60%), whereas Merlot contained much lower levels (14%) of individual and total procyanidins. A modified method of extraction whereby selected fruits and fruit byproducts were soaked in the extraction solvent overnight before the extraction process was begun increased procyanidins extracted by 24−100% from grape seeds and by 0−30% with berry procyanidin sources. The results indicate a wide variation in the procyanidin contents among different varieties of grape seeds that could have implications in the selection of procyanidin-rich germplasm. Soaking samples in the extraction solvent for 16 h resulted in increased procyanidins extracted and thus higher calculated concentrations in the food samples tested.

KEYWORDS: Procyanidins; grape seed; grape pomace; cranberry; blueberry; extraction method; variety

INTRODUCTION

Proanthocyanidins, better known as condensed tannins, are oligomeric and polymeric flavan-3-ols, a class of flavonoids. Proanthocyanidins containing exclusively (epi)catechin as subunits are procyanidins (PCNs), and those containing (epi)afzelechin or (epi)gallocatechin as subunits are named propelargonidins or prodelphinidins, both of which are less common in nature than PCNs and coexist with PCNs. Although their primary role in plant physiology may have been protection against pathogens or grazing herbivores (*1*, *2*), there has been a growing interest in PCNs due to their antioxidant, anti-inflammatory, antibacterial, and antiarthritic activities and their potential role in the prevention of heart disease, skin aging, and various cancers (*3*−*7*).

Procyanidins are commonly found in many fruits and vegetables as well as barks and leaves. They are abundantly present in grapes and berries and probably more concentrated in seeds. Because of the diversity in structure and occurrence, PCN contents in many fruits and their byproducts, such as the seeds, may vary greatly. Although numerous studies have investigated PCN occurrence in foods of plant origin, their astringentic properties, their effects on food quality, antinutrient properties, and metabolic pathways upon ingestion by animals, and, more recently, their effect on health and modulation of chronic diseases, etc., details about the varietal differences in complete PCN profiles of grape seeds are not available. Studies that have investigated the variation in grape seed PCNs were confined to lower molecular weight compounds, up to tetramers at most (*8*, *9*).

Diversity in occurrence and the structure and complexity of PCNs have also created difficulties in developing analytical tools and appropriate and commercially available standards for accurate estimation of their concentrations. Moreover, a large portion of PCNs may be unextractable (*10*), suggesting that most of the reported PCN contents in the literature may be underestimated. More commonly available methods (*11*−*13*) of PCN extraction and quantification involve extraction with an acetone−water solution, purification in a Sephadex column, and high-performance liquid chromatography (HPLC) separation based on degree of polymerization using normal or diol phases. Neither method involves soaking samples for an extended period of time prior to initiation of the extraction process. The hypothesis here is that soaking in the extraction solvent for an extended period of time would facilitate the solubilization of PCNs in the food matrix and thus enhance the overall amount extracted.

The objective of this study was to compare the genotype differences in PCN composition of grape seed and to assess whether soaking enhances the extraction of PCN from grape seeds and PCN-rich fruits and fruit byproducts, such as cranberry and blueberry and grape pomace. Varietal differences in PCN contents of grapes can be employed by geneticists to enhance the contents of chemical components beneficial to human health, and

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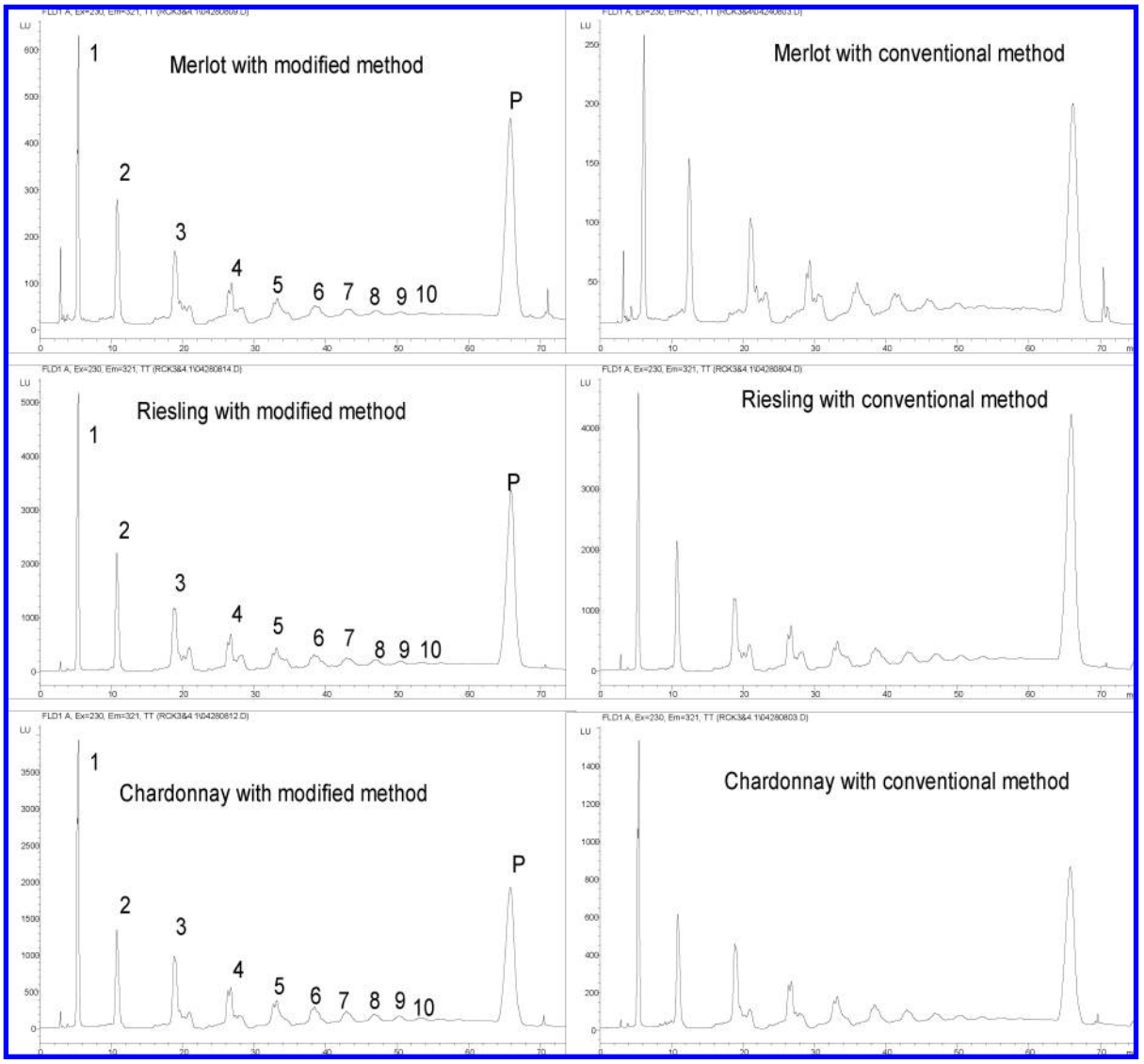

Figure 1. Representative HPLC chromatogram (diol phase) showing the differences in procyanidin composition of three grape varieties, Merlot, Riesling, and Chardonnay, extracted with (modified method) or without (conventional method) overnight soaking in extraction solvent. Numbers above peaks indicate degree of polymerization (DP).

an improved method of extraction will be useful for providing better estimates of PCNs in food products and intake by humans.

MATERIALS AND METHODS

Plant Materials. Grape seed samples (varieties Merlot, Chardonnay, and Riesling) were obtained from FruitSmart (Prosser, WA), and red grape pomace (variety Sunbelt) was obtained from the University of Arkansas Enology program (Fayetteville, AR). Grape seed was screened through a kitchen sieve to exclude any extraneous material and ground in a coffee grinder. All three varieties of grape seeds were obtained from grapes grown during the same season under similar growing conditions. Blueberry powder (low bush type, *Vaccinium angustifolium*) was obtained from Wild Blueberry Association of North America (Old Town, ME), whereas cranberry powder was obtained from Decas Cranberry Products (Carver, MA). Grape pomace consisting of stems, skins, and seeds was dried in a Virtis Genesis freeze dryer (Gardiner, NY) before grinding in a coffee grinder.

Experimental Protocol, Sample Preparation, Extraction, and Purification. To study the effect of variety and extraction method in grape seeds, three varieties of grape seed (Merlot, Chardonnay, Riesling) were used to extract PCNs using two methods of sample extraction in a 3 × 2 factorial arrangement with three replicates in each treatment. First, a method described earlier by Kelm et al. (*12*), in which samples were extracted three times with extraction solvent by sonication for 10 min at 50 °C, was compared with the modified method in which samples were soaked overnight (approximately 16 h) in extraction solvent prior to PCN extraction. In short, 2 g of sample was soaked in 15 mL of extraction solvent of acetone/water/acetic acid (70:29.5:0.5, v/v/v) in 50 mL conical tubes. Tubes were then left at room temperature for overnight soaking with the caps on to avoid evaporation of acetone. PCNs were extracted three times from the soaked sample in the extraction solvent while homogenizing for 1 min using a Turrax T18 Tissuemizer (Tekmar-Dohrmann Corp., Mason, OH). Extracts were filtered through Miracloth (CalBiochem, LaJolla, CA) and adjusted to 100 mL with extraction solvent. Samples were loaded onto a Sephadex LH-20 (Sigma, St. Louis, MO) column, and soluble components were eluted with 40 mL of 30% methanol. Procyanidins were then eluted from the column with 70 mL of 70% acetone. Further details about this extraction method have been provided previously (*14*). The only modification made to this process in the modified method being overnight soaking in extraction solvent before proceeding to the extraction process. In the second experiment, we included cranberry powder, blueberry powder, and grape pomace (all freeze-dried) to compare the method of extraction of Gu et al. (*11*)—which is similar in certain respects to that of Kelm et al. (*12*) (except samples were sonicated at 37 °C for 10 min and then allowed to remain at room temperature for 50 min)—with the modified method in a 3 × 2 factorial arrangement. Again, the modified method involved soaking samples overnight (approximately 16 h) as described above in extraction solvent prior to PCN extraction.

Table 1. Effect of Variety and Extraction Method on Procyanidin Composition (Milligrams per Kilogram of Dry Weight) of Grape Seed[a]

	variety					extraction method			
procyanidin	Merlot	Chardonnay	Riesling	SEM	*P*	conventional	modified	SEM	*P*
monomer	594 b	3285 a	3652 a	228	<0.01	1825 b	3195 a	187	<0.01
dimer	220 c	890 b	1636 a	78	<0.01	610 b	1221 a	64	<0.01
trimer	244 c	1029 b	1776 a	91	<0.01	734 b	1298 a	74	<0.01
tetramer	243 c	998 b	1485 a	81	<0.01	680 b	1137 a	66	<0.01
pentamer	180 c	815 b	1157 a	59	<0.01	543 b	892 a	48	<0.01
hexamer	127 c	623 b	973 a	64	<0.01	413 b	735 a	53	<0.01
heptamer	124 c	652 b	1053 a	38	<0.01	494 b	725 a	31	<0.01
octamer	103 c	612 b	951 a	27	<0.01	480 b	631 a	22	<0.01
nonamer	85 c	519 b	866 a	31	<0.01	437 b	544 a	25	0.01
decamer	90 b	688 a	874 a	66	<0.01	448 b	654 a	54	0.02
polymer	1122 c	3639 b	8622 a	279	<0.01	3461 b	5501 a	228	<0.01
total	3133 c	13809 b	23045 a	517	<0.01	10126 b	16532 a	423	<0.01

[a] Values represent means ($n = 3$). Means within rows with different letters are significantly different ($p < 0.05$).

Table 2. Effect of Extraction Method on Procyanidin Composition (Milligrams per Kilogram of Dry Weight) of Cranberry Powder, Blueberry Powder, and Grape Pomace[a]

	food					extraction method			
procyanidin	grape pomace[b]	cranberry	blueberry	SEM	*P*	modified	conventional	SEM	*P*
monomer	578 a	62 b	77 b	6.4	<0.001	206 a	161 b	4.5	<0.001
dimer	390 b	545 a	125 c	15.4	<0.001	300 a	243 b	10.9	0.002
trimer	370 a	400 a	159 b	13.2	<0.001	269 a	219 b	9.3	0.002
tetramer	309 a	316 a	220 b	13.2	<0.001	256 a	222 b	9.3	0.021
pentamer	211 a	184 b	176 b	7.7	<0.001	199 a	163 b	5.4	<0.001
hexamer	125 b	194 a	154 b	9.0	<0.001	188 a	145 b	6.4	<0.001
heptamer	100 b	188 a	195 b	9.4	<0.001	201 a	181 b	6.7	0.050
octamer	97 b	ND	207 a	14.3	<0.001	216	202	11.7	0.394
nonamer	77 b	ND	256 a	21.1	<0.001	249	244	17.2	0.852
decamer	100 b	ND	295 a	20.8	<0.001	322	293	17.0	0.251
polymer	1064 b	2271 a	2508 a	76.3	<0.001	1914	1909	54.0	0.952
total	3730 b	4476 a	4593 a	152.0	0.003	4380 a	4019 b	107.0	0.030

[a] Values represent means ($n = 3$). Means within rows with different letters are significantly different ($p < 0.05$). [b] Variety Sunbelt.

HPLC Analysis of PCNs. PCNs were analyzed using an Agilent 1100 HPLC system consisting of a quaternary pump, a solvent degasser, an autosampler, a thermostat column compartment, a diode array detector, a fluorescence detector, and ChemStation for data collection and manipulation (Agilent Technologies, Palo Alto, CA). Extracts were passed through a 0.45 μm filter before loading onto the HPLC column. Separations based on degree of polymerization (DP) were conducted using a Develosil Diol column (250 × 4.6, 5 μm, Phenomenex, Torrence, CA). Details about the HPLC conditions, mobile phase, gradient, standards, and peak identification were given previously (*14*, *15*).

Determination of Moisture Content. The moisture content of all samples was analyzed by drying for 8 h in a forced air oven at 103 °C. All data related to PCN contents are presented on a dry weight (DW) basis.

Statistical Analysis. Statistical analysis was carried out in SigmaPlot (Systat Software Inc., San Jose, CA). Variety, extraction method, and their interactions were included in a 3 × 2 factorial model for grape seed PCNs, whereas a similar factorial model was used in two extraction methods involving cranberry powder, blueberry powder, and grape pomace. Means were separated using Student's *t* test. Significance level was set at $p \leq 0.05$.

RESULTS AND DISCUSSION

Varietal and Extraction Method Differences in Grape Seed PCNs. The representative HPLC chromatograms (**Figure 1**) obtained from Merlot, Chardonnay, and Riesling grape seeds indicated distinct differences in their PCN profiles. Although peak shapes were essentially the same in all varieties, they were noticeably greater for the modified method. Although peak size alone may not be of any significance on its own, it is relevant in the current study given that the injection volume, initial sample weight, dilution factor, integration technique, and all other quantitative aspects of the two methods were virtually identical. Merlot, a red grape variety, had the lowest amount of PCNs irrespective of the level of polymerization (**Table 1**). Total PCN content was also lowest in Merlot, which was 3133 mg/kg of DW as compared to 13809 and 23045 mg/kg of DW, respectively for Chardonnay and Riesling, both white grape varieties. All three varieties were significantly different ($P \leq 0.01$) from each other for all of the PCNs determined in the current study, except the monomer and decamer between Chardonnay and Riesling. Irrespective of the differences in PCN profiles, the results suggested two very important findings: (1) they are an excellent source of PCNs, and (2) they have appreciable amounts of the lower oligomeric forms, such as monomers, dimers, and trimers, that are associated with potential health benefits and have varying degrees of bioavailability when ingested (*16*−*18*). Such variation indicates the potential for genetic selection of grape varieties with higher PCN contents. Another important aspect is the possibility of using grape seeds in value-added food products, such as nutraceuticals, thereby bringing this important source of polyphenols back to the human food chain.

Previously, Yilmaz and Toledo (*9*) reported wide variations in catechin and epicatechin contents among Merlot and Chardonnay grapes, with Merlot containing much less than Chardonnay in both skin and seeds. They showed that skins contained much less monomeric PCNs than seeds. Similarly, Guendez et al. (*19*)

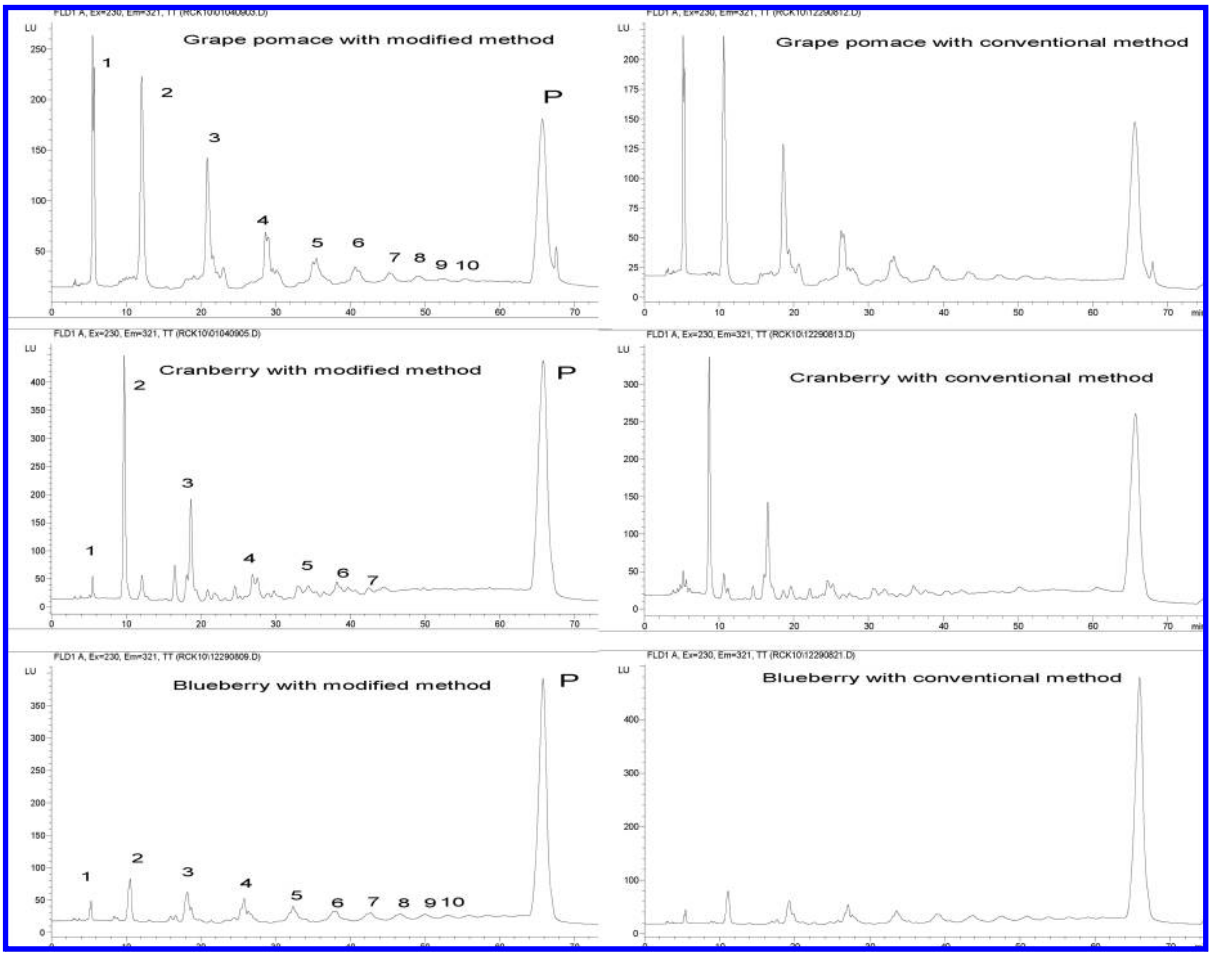

Figure 2. Representative HPLC chromatograms (diol phase) obtained from grape pomace, cranberry powder, and blueberry powder extracted with (modified method) or without (conventional method) overnight soaking in extraction solvent.

reported wide variations in total polyphenol contents, including catechin, epicatechin, and procyanidin dimers B_1 and B_2, among white and red grape seed varieties cultivated in Greece. de Freitas et al. (*8*) studied the differences in PCNs in two red grape varieties (Merlot and Cabernet Sauvignon) and found that Merlot contained fewer monomers, dimers, and trimers than Cabernet Sauvignon. It should be noted that these studies measured only the lower oligomers, such as monomer, dimers, or trimers at the most, and not other oligomers and polymers, which are present in various amounts as can be seen in the current study as well as others we have reported previously (*15*). We are not aware of other studies comparing the different varieties of grapes or their seeds for PCN contents or their detailed profiles. Polymer content is usually high and may commonly be the major PCN constituent present in many fruits and fruit byproducts including grape seeds (*20*). This was true in the current study as well.

When the conventional extraction method as per Kelm et al. (*12*) was compared with the modified method, the two methods were significantly different ($P < 0.05$) in their ability to extract PCNs from grape seeds (**Table 1**). It was true for all three varieties included in the study. The differences were broad across all of the constituent PCNs and ranged from slightly more than 30% in octamer to as high as 100% in dimers. Whereas the polymer content increased by about 59% with the modified method, monomer content increased by 75%. The total PCNs also increased by about 63%.

Effect of Extraction Method on PCN Contents in Selected Fruits and Fruit Byproducts. When another method (*11*) was modified by soaking samples overnight in the extraction solvent for estimating the PCN contents in blueberry, cranberry, and grape pomace, it resulted in significantly higher ($P < 0.05$) overall estimates of total and most of the individual PCNs, except octa-, nona-, deca-, and polymers (**Table 2**). Although not always significant, total and individual PCN contents were usually higher when extracted according to the modified method compared with the previously described method (*11*) for all of the samples tested. However, the extent of increase in PCNs was smaller compared to what was observed for grape seed. One of the reasons is probably the lack of difference in PCNs DP8−DP10 and polymers, which may have been loosely bound in grape pomace and blueberry and cranberry powders compared to grape seed; thus, no improvement in their extraction when soaked overnight was seen. Indeed, binding of PCNs with the cell walls has been shown to increase with increasing DP and percent of galloylation (*21*, *22*), which have been demonstrated to be higher in grape seeds (*23*). Another potential reason may be the small difference between the two methods of Gu et al. (*14*) and Kelm et al. (*11*). Whereas the former allowed samples to remain at room temperature for 50 min, essentially soaking, after sonication, the latter did not include this step. There was also an interaction (data not presented) between extraction method and food samples used, suggesting that PCNs from grape pomace, cranberry powder, and blueberry powder (as well as high-tannin sorghum bran, for which no data are presented) behaved differently when exposed to the two methods of extraction employed in the current study. Except for the polymer content in blueberry powder, which was numerically

higher with the conventional method, all other constituents in all of the food samples tested were higher, although not always significantly so, when extracted after overnight soaking in the extraction solvent. Representative HPLC chromatograms for the two methods are presented in **Figure 2**. Careful inspection of the chromatograms indicates slightly greater peaks for the modified method, which is relevant given the injection volume, initial sample weight, dilution factor, and all other quantitative aspects of the two methods were essentially the same. We, however, did not compare the two methods described by Gu et al. (*11*) and Kelm et al. (*12*) in their ability to extract PCNs.

Our results demonstrate that previously reported values for PCN contents may have been underestimated. These results are noteworthy considering the different types of food samples used and the fact that the modification is very simple and does not cost anything extra. All it needs is a relatively longer time for soaking in the extraction solvent, which could be accomplished the night prior to sample extraction. Moreover, a recent paper has suggested that a large part of the PCNs may be unextractable with the conventional methods (*10*) that do not involve soaking of the samples for an extended period of time. At least part of these unextractable PCNs may have become extractable by overnight soaking, thus increasing the estimates of PCN contents.

In conclusion, Merlot grape seeds had considerably lower levels of total and individual procyanidins irrespective of the degree of polymerization among the three varieties investigated, whereas Riesling, a white grape variety, had the highest procyanidin content, with Chardonnay being closer to Riesling than to Merlot. Wide variation in the procyanidin contents among different varieties of grape seeds suggested potential implications in the selection of procyanidin-rich germplasm. Soaking samples overnight before the extraction process is begun, as is usually not the case with conventional methods, resulted in increased quantities of procyanidins, suggesting that conventional methods may have underestimated procyanidin contents in certain foods and food products.

ABBREVIATIONS USED

PCN, procyanidins; DW, dry weight; DP, degree of polymerization; HPLC, high-performance liquid chromatography.

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