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# HPLC Method for the Quantification of Procyanidins in Cocoa and Chocolate Samples and Correlation to Total Antioxidant Capacity

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Monomeric and oligomeric procyanidins present in cocoa liquors and chocolates were separated and quantified in four different laboratories using a normal-phase high-performance liquid chromatography (HPLC) method with fluorescence detection. Procyanidin standards through decamers were obtained by extraction from cocoa beans, enrichment by Sephadex LH-20 gel permeation chromatography, and final purification by preparative normal-phase HPLC. The purity of each oligomeric fraction was assessed using HPLC coupled to mass spectrometry. A composite standard was then prepared, and calibration curves were generated for each oligomeric class using a quadratic fit of area sum versus concentration. Results obtained by each of the laboratories were in close agreement, which suggests this method is reliable and reproducible for quantification of procyanidins. Furthermore, the procyanidin content of the samples was correlated to the antioxidant capacity measured using the ORAC assay as an indicator for potential biological activity.

**Keywords:** HPLC; procyanidins; cocoa; chocolate; quantification; antioxidant

## INTRODUCTION

Procyanidins are a class of polyphenolic compounds found in several plant species and may be present as individual monomers or, in some cases, as oligomeric units (Porter, 1988). Recently, these compounds have attracted increased attention in the fields of nutrition, health, and medicine largely due to their potent antioxidant capacity and possible beneficial implications on human health (Rice-Evans and Packer, 1997; Waterhouse and Walzem, 1998). Certain members of this class of compounds can be found in several foods commonly consumed in the diet; however, detailed quantitative information on the procyanidin profiles present in many food products is lacking, especially with regard to the more complex oligomeric profiles (Peterson and Dwyer, 1998). Increasingly, this quantitative information will become important for understanding the impact of procyanidins on the total antioxidant capacity of foods and any health benefits associated with their consumption.

The analytical method normally employed to estimate the amount of catechins and procyanidins is the colorimetric measurement of their total content after reaction with aromatic aldehydes such as dimethylaminocinnamaldehyde (DMACA) or vanillin (Porter, 1989).

However, use of a spectrophotometric method typically gives estimations on the total flavanol content instead of the quantitative contribution of each compound within this class. Recently, this trend has changed to incorporate the use of high-performance liquid chromatography (HPLC) for the quantification of individual procyanidins in various food products. However, the challenge in quantification is the limited number of commercially available standards other than the catechins. Consequently, some researchers have used (+)-catechin or (–)-epicatechin as an external standard and estimated oligomers as catechin equivalents. For example, Spanos and Wrolstad (1990) used a linear regression of (+)-catechin concentration versus peak area at 280 nm to express procyanidins in grape juice as (+)-catechin equivalents. Similarly, Clapperton et al. (1992) used (–)-epicatechin as an external standard to estimate the quantity of dimers and trimers present in cocoa as detected by fluorescence.

However, use of catechins as external standards only allows for estimation of oligomeric forms since monomers have different detection response factors than do their oligomeric counterparts. Therefore, synthesis and rigorous purification techniques have been developed for obtaining oligomers for use as quantitative standards. For example, both Salagoity-Auguste and Bertrand (1984) and Oszmianski and Lee (1990) synthesized B-type procyanidins to quantify dimers in wine and red grapes, respectively. In contrast, McMurrough and Baert (1994) isolated prodelfinidin B3 and procyanidin B3 from barley to use as external standards for the quantification of dimers in beer.

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Regarding separation methods, reversed-phase HPLC with UV detection at 280 nm has been the primary method of analysis for the quantification of procyanidins in food samples. One limitation to this methodology is that reversed-phase methodologies are ineffective in separating the higher oligomers (>trimer), with these compounds eluting as a large unresolved hump. Thus, quantification of oligomeric and polymeric procyanidins is underestimated because only the resolved peaks corresponding to dimers and trimers are considered. Additionally, quantitative analysis based on UV detection at 280 nm is also not selective for procyanidins relative to other polyphenols such as phenolic acids. In these circumstances, a prepurification step is typically required to remove the interfering phenolic acids before the analysis of procyanidins (Spanos and Wrolstad, 1990; Ricardo da Silva et al., 1990). Alternatively, DMACA has been used as a postcolumn reagent that forms products unique to procyanidins that can be detected by UV at 640 nm, thus eliminating the need for prepurification (de Pascual Teresa et al., 1998a,b; Treutter, 1989).

In the current study, fluorescence detection (FLD) was used in place of UV detection at 280 nm since the FLD previously demonstrated increased sensitivity and selectivity for procyanidins (Lazarus et al., 1999). Furthermore, to ensure that contributions from all oligomeric classes were included, the normal-phase HPLC method reported by Hammerstone et al. (1999) was used for the separation and quantification of oligomers up to decamers in cocoa liquors and chocolates based on degree of polymerization. Finally, the quantitative information obtained was correlated to the antioxidant capacity of the samples as measured using the oxygen radical absorbance capacity (ORAC) assay as an indicator of potential health benefits of procyanidins present in foods.

## MATERIAL AND METHODS

**Samples.** Cocoa beans were provided by the Almirante Center for Cocoa Studies in Itajuipe, Brazil. Liquors (Sanchez and Ivory Coast) and chocolates (milk, dark, and noir-type) were made using Cocapro cocoa and provided by M&M/Mars (Elizabethtown, PA).

**Reference Compounds.** (–)-Epicatechin was obtained from Sigma Chemical Co. (St. Louis, MO).

**Preparation of Purified Oligomers from Brazilian Cocoa Beans.** *Extraction of Cocoa Procyanidins.* The fresh seeds were ground in a high-speed laboratory mill with liquid nitrogen until the particle size was reduced to approximately 90  $\mu\text{m}$ . Lipids were removed from 220 g of the ground seeds by extracting three times with 1000 mL of hexane. The lipid-free solids were air-dried to yield approximately 100 g of fat-free material. A fraction containing procyanidins was obtained by extraction with 1000 mL of 70 vol % acetone in water. The suspension was centrifuged for 10 min at 1500g. The acetone layer was decanted through a funnel with glass wool. The aqueous acetone was then re-extracted with hexane (~75 mL) to remove residual lipids. The hexane layer was discarded, and the aqueous acetone was rotary evaporated under partial vacuum at 40 °C to a final volume of 200 mL. The aqueous extract was freeze-dried to yield approximately 19 g of acetone extract material.

*Gel Permeation Chromatography of Cocoa Acetone Extract.* Approximately 2 g of acetone extract was suspended in 10 mL of 70% aqueous methanol and centrifuged at 1500g. The supernatant was semipurified on a Sephadex LH-20 column (70  $\times$  3 cm) that had previously been equilibrated with methanol at a flow rate of 3.5 mL/min. Two and a half hours after sample loading, fractions were collected every 20 min and

**Table 1. Gradient Profile for Preparative Normal-Phase HPLC**

time (min)	methylene chloride/ acetic acid/water (96:2:2 v/v) (%)	methanol/acetic acid/ water (96:2:2 v/v) (%)	flow rate (mL/min)
0	92.5	7.5	10
10	92.5	7.5	40
30	91.5	8.5	40
145	78.0	22.0	40
150	14.0	86.0	40
155	14.0	86.0	50
180	0	100	50

**Table 2. Oligomeric Profile of Composite Standard**

oligomer	% contribution by weight	oligomer	% contribution by weight
monomer	9.82	hexamer	12.68
dimer	13.25	heptamer	7.98
trimer	9.85	octamer	8.44
tetramer	10.49	nonamer	11.56
pentamer	10.51	decamer	5.42

analyzed by HPLC for theobromine and caffeine (Clapperton et al., 1992). Once the theobromine and caffeine were eluted off the column (~3.5 h), the remaining eluate was collected for an additional 4.5 h and rotary evaporated under partial vacuum at 40 °C to remove the organic solvent. Then the extract was suspended in water and freeze-dried.

*Purification of Procyanidin Oligomers by Preparative Normal-Phase HPLC.* Approximately 0.7 g of semipurified acetone extract was dissolved in 7 mL of acetone/water/acetic acid in a ratio by volume of 70:29.5:0.5, respectively. Separations were effected at ambient temperature using a 5  $\mu\text{m}$  Supelcosil LC-Si 100 Å (50  $\times$  2 cm). Procyanidins were eluted by a linear gradient under the conditions shown in Table 1. Separations of oligomers were monitored by UV at 280 nm, and fractions were collected at the valleys between the peaks corresponding to oligomers. Fractions with equal retention times from several preparative separations were combined, rotary evaporated under partial vacuum, and freeze-dried.

**Mass Spectrometry Analysis of Partially Purified Oligomers.** Purified fractions were analyzed by HPLC/mass spectrometry (MS) using the parameters described by Lazarus et al. (1999). Purities of each fraction were determined by peak area using UV detection at 280 nm in combination with comparing the ratio of ion abundances between each oligomeric class.

**Standard Stock Solutions.** A composite standard was made using commercially available (–)-epicatechin for the monomer and the purified oligomers for dimers through decamers, and its oligomeric profile is shown in Table 2. Stock solutions were made at the following concentrations: 20, 10, 5, 2, 1, and 0.4 mg/mL.

**Preparation of Cocoa Liquors and Chocolate Samples.** Approximately 8 g of sample was extracted three times with 45 mL of hexane to remove lipids. After air-drying in a hood to remove residual hexane, approximately 1 g of defatted material was extracted with 5 mL of acetone, water, and acetic acid in a ratio by volume of 70:29.5:0.5, respectively. The solids were pelletized by centrifuging for 10 min at 1500g, then the supernatant was filtered through a 0.45  $\mu\text{m}$  nylon filter into an HPLC vial for injection. All defatted samples were weighed, extracted, and injected in duplicate.

**Determination of Percent Fat Composition of Cocoa Liquors and Chocolates.** The AOAC Official Method 920.177 was used for determination of percent fat for the cocoa liquors and chocolates. A slight modification to the sample size was needed which incorporated the use of 1 g for the chocolate samples and 0.5 g for the liquor samples.

**High-Performance Liquid Chromatography Analysis of Procyanidins.** Chromatographic analyses were performed using four instruments in different laboratories. Laboratory 1 used a HP 1100 Series HPLC (Hewlett-Packard, Palo Alto, CA) equipped with an autoinjector, quaternary HPLC pump,



column heater, fluorescence detector, and HP ChemStation for data collection and manipulation. Laboratory 2 used a similar HP 1100 Series HPLC with the minor difference being the use of a binary pump instead of a quaternary pump. Laboratory 3 used Bioanalytical systems pump and degasser (West Lafayette, IN), an Eppendorf column heater (Westbury, NY), a Dionex fluorescence detector (Sunnyvale, CA), and ESA Coullarray for Windows Software (Chelmsford, MA) for data collection and manipulation. Laboratory 4 used a HP 1100 Series HPLC equipped with an autoinjector, quaternary HPLC pump, column heater, fluorescence detector, and Perkin-Elmer TurboChrom (Nieuwerkerk aan de IJssel, The Netherlands) for data collection and manipulation. Fluorescence detection was recorded at excitation wavelength 276 nm and emission wavelength 316 nm. Normal-phase separations of the procyanidin oligomers were performed using a Phenomenex (Torrance, CA) 5  $\mu$ m Lichrosphere silica column (250  $\times$  4.6 mm) at 37 °C with a 5  $\mu$ L injection volume. The ternary mobile phase consisted of (A) dichloromethane, (B) methanol and (C) acetic acid and water (1:1 v/v). Separations were effected by a series of linear gradients of B into A with a constant 4% C at a flow rate of 1 mL/min as follows: elution starting with 14% B in A; 14–28.4% B in A, 0–30 min; 28.4–39.2% B in A, 30–45 min; 39.2–86% B in A, 45–50 min. However, for those laboratories equipped with binary pumps, the mobile phase, and gradients were adjusted accordingly. The binary mobile phase consisted of (A) dichloromethane, methanol, water, and acetic acid (82:14:2:2 v/v) and (B) methanol, water, and acetic acid (96:2:2 v/v). Separations were effected by a series of linear gradients of B into A at a flow rate of 1 mL/min as follows: elution starting with 0%B in A; 0–17.6% B in A, 0–30 min; 17.6–30.7% B in A, 30–45 min; 30.7–87.8% B in A, 45–50 min. In all cases, the columns were reequilibrated between injections with the equivalent of 25 mL (10 column volumes) of the initial mobile phase.

**Quantification of Procyanidins in Cocoa Liquors and Chocolates.** Calibration curves were made from the stock solutions using a quadratic fit for the relationship of area sum versus concentration for the peaks corresponding to each oligomeric class.

**Automated ORAC<sub>ROO</sub> Assay.** The automated ORAC assay was carried out on a COBAS FARA II spectrofluorometric centrifugal analyzer (Roche Diagnostic System, Inc., Branchburg, NJ) with an emission filter of 565 nm. The procedure was based on a previous report by Cao et al. (1993), as modified for the COBAS FARA II (Cao et al., 1995). Briefly, in the final assay mixture (0.4 mL total volume), R-phycoerythrin (R-PE) (16.7 nM) (Sigma Chemical Co., St. Louis, MO) was used as a target of free-radical attack, with AAPH (4mM) as a peroxy radical generator. Trolox (1.0  $\mu$ mol/L), a water-soluble analogue of vitamin E, was used as a control standard. The analyzer was programmed to record the fluorescence of R-PE every 2 min after addition of AAPH. All fluorescent measurements are expressed relative to the initial reading. Final results were calculated using the differences of the normalized areas under the R-PE decay curves between the blank and a sample, and expressed as  $\mu$ mol of Trolox equivalents (TE) per gram. The measurements were made on the materials extracted into the acetone, water, and acetic acid (70:29.5:0.5 v/v) extraction mixture and expressed on a defatted weight basis.

## RESULTS AND DISCUSSION

One of the challenges in quantifying procyanidins in plant materials and food samples is the lack of commercial availability of the oligomeric forms. Additionally, there is difficulty in developing methodology for the separation and subsequent purification of individual oligomeric forms, especially with regard to the higher oligomers. In part, this difficulty is due to the traditional use of reversed-phase chromatography, which is unable to separate oligomers higher than the trimers. In the current study, purified oligomers through the decamers

**Table 3. Percent Fat Composition for Cocoa Liquor and Chocolate Samples**

sample	% fat by weight	sample	% fat by weight
milk chocolate	32.1	Ivory Coast liquor	53.9
dark chocolate	32.5	Sanchez liquor	52.0
high liquor chocolate	44.5		

were obtained by extracting procyanidins from cocoa beans and using a combination of gel permeation chromatography and preparative normal-phase HPLC for purification from sugars, alkaloids and other non-polyphenolic compounds. The purity of each fraction collected was assessed using the HPLC/MS method described by Lazarus et al. (1999) and a composite standard generated. This composite and its oligomeric profile (Table 2) were provided to each laboratory for preparation of stock solutions for use as external standards.

To assess the robustness of the quantitative method, sample preparation and analysis were performed by four laboratories. During sample preparation, it was necessary to ensure complete removal of lipids in order to achieve maximum extraction efficiency of the procyanidins. All of the defatted samples were weighed, extracted, and analyzed in duplicate using the slightly modified normal-phase HPLC method previously reported by Hammerstone et al. (1999). The minor modifications made to the HPLC method were to ensure that the column was well flushed and completely reequilibrated prior to the next sample injection.

For the quantification of the samples, calibration curves were made from the standard stock solutions, which were injected before and after sample analysis. The areas for the peaks corresponding to each oligomeric class as previously determined by Hammerstone et al. (1999) were summed, and a quadratic relationship was determined when plotted against concentration. Area summation or peak grouping was used in order to include the contributions from all of the isomers within an oligomeric class. It is interesting to note that the curves for monomers and smaller oligomers were nearly linear while the higher oligomers exhibited a more pronounced quadratic shape. This observation is consistent with previous reports that have predominantly used linear regression to generate calibration curves when using monomers and dimers as external standards (Clapperton et al., 1992; Bronner and Beecher, 1998; Salagoity-Auguste and Bertrand, 1984). Additionally, it is important to note that each laboratory generated calibration curves with correlation coefficients greater than 0.97.

Using the calibration curves, the procyanidin content of the defatted samples was calculated in each laboratory and then converted to a total weight basis using the determined percent fat composition (Table 3). The average results for procyanidin content per gram of sample are shown in Table 4. The results indicated that the Sanchez liquor contains substantially more procyanidins than the Ivory Coast liquor. This is not surprising since Ivory Coast traditionally produces well-fermented beans versus Sanchez, which are typically underfermented, and it is widely known that procyanidin levels decrease during fermentation (Kim and Keeney, 1984). Additionally, it can also be seen in Table 4 that the high cocoa liquor chocolate contains a substantial level of procyanidins probably because it contains approximately 70% liquor. In contrast, the relatively low levels

**Table 4. Quantification of Procyanidins (Mean  $\pm$  Standard Deviation)<sup>a</sup> in Cocoa Liquors and Chocolate Samples**

	milk chocolate	dark chocolate	high liquor chocolate	Ivory Coast liquor	Sanchez liquor
monomers	0.2 $\pm$ 0.05	0.8 $\pm$ 0.08	4.0 $\pm$ 0.4	2.0 $\pm$ 0.1	4.9 $\pm$ 0.3
dimers	0.2 $\pm$ 0.05	0.5 $\pm$ 0.04	3.2 $\pm$ 0.2	1.8 $\pm$ 0.2	4.2 $\pm$ 0.3
trimers	0.1 $\pm$ 0.00	0.2 $\pm$ 0.04	2.2 $\pm$ 0.1	1.3 $\pm$ 0.1	2.8 $\pm$ 0.3
tetramers	0.1 $\pm$ 0.00	0.2 $\pm$ 0.09	1.9 $\pm$ 0.1	1.0 $\pm$ 0.1	2.2 $\pm$ 0.1
pentamers	0.1 $\pm$ 0.05		1.4 $\pm$ 0.2	0.8 $\pm$ 0.1	1.7 $\pm$ 0.1
hexamers	0.1 $\pm$ 0.05		1.2 $\pm$ 0.3	0.6 $\pm$ 0.1	1.4 $\pm$ 0.1
heptamers			0.6 $\pm$ 0.1	0.3 $\pm$ 0.1	0.7 $\pm$ 0.1
octamers			0.6 $\pm$ 0.1	0.4 $\pm$ 0.1	0.6 $\pm$ 0.1
nonamers			0.6 $\pm$ 0.1	0.4 $\pm$ 0.1	0.7 $\pm$ 0.1
decamers			0.3 $\pm$ 0.1	0.2 $\pm$ 0.1	0.3 $\pm$ 0.1
total	0.7 $\pm$ 0.17	1.7 $\pm$ 0.08	15.8 $\pm$ 0.4	8.6 $\pm$ 0.4	19.4 $\pm$ 1.2
coeff of variation (%)	24	4.7	2.5	4.7	6.2

<sup>a</sup> Mean  $\pm$  standard deviation from four laboratories expressed as mg/g.

**Table 5. Limits of Detection and Quantification<sup>a</sup> for Each Oligomer**

	limit of detection	limit of quantification
monomer	0.01	0.03
dimer	0.01	0.03
trimer	0.01	0.03
tetramer	0.01	0.03
pentamer	0.02	0.05
hexamer	0.01	0.04
heptamer	0.01	0.03
octamer	0.01	0.04
nonamer	0.03	0.08
decamer	0.02	0.06

<sup>a</sup> Expressed as mg/mL.

found in the dark chocolate could be explained by the fact that the liquor used to prepare the chocolate was partially alkalinized, which is known to cause chemical alterations to polyphenols (Meursing, 1994). Furthermore, it would be expected that the milk chocolate would contain the lowest levels of procyanidins since it consists of the least amount of liquor component.

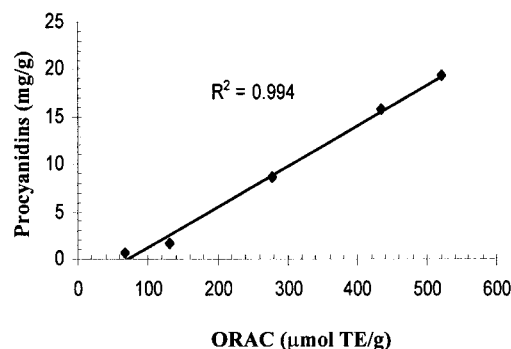
It is interesting to note that the coefficients of variation for total procyanidin content for all of the samples except the milk chocolate are relatively small, ranging from approximately 4 to 6% (Table 4). However, the milk chocolate has a significantly higher coefficient of variation of approximately 24%. This phenomenon could possibly be due to the fact that the small quantities of higher oligomers in the milk chocolate were approaching the lower limit of quantification under the conditions used in the study. This is most pronounced by the fact that not all of the laboratories were able to quantify the higher oligomers in the milk chocolate sample. Therefore, the limits of detection and quantification were calculated and the results shown in Table 5 (Krull and Swartz, 1998). Indeed, the levels for the higher oligomers in the milk chocolate are at the limit of quantification, thus accounting for the larger coefficient of variation. Adjusting the fluorescence detector's photomultiplier tube gain and the lamp output could improve signal-to-noise and subsequently lower the limits of detection and quantification. Since the other four samples analyzed contained much higher levels of procyanidin oligomers, their results were not significantly affected by detector limitations.

Next, the procyanidin content of the cocoa samples was correlated to antioxidant potential as measured

**Table 6. ORAC Value<sup>a</sup> for Chocolate and Liquor Samples**

	milk chocolate	dark chocolate	high liquor chocolate	Ivory Coast liquor	Sanchez liquor
ORAC	67.4	131.2	434.0	278.5	520.1

<sup>a</sup>  $\mu$ mol Trolox equivalents per gram.

**Figure 1.** Linear relationship between oxygen radical absorbance capacity (ORAC) and procyanidins as measured by HPLC.

using the ORAC assay previously developed (Cao et al., 1995). The ORAC values of the various samples can be seen in Table 6, and in calculating the ratio of antioxidant capacity to procyanidins, it appears that the more fermented sources have a lower antioxidant capacity relative to procyanidin content. Indeed, plotting procyanidin content versus ORAC value (Figure 1) yields a linear regression that indicates a close correlation ( $r^2 = 0.994$ ) between procyanidin levels and cocoa antioxidant capacity. Since many of the potential health benefits generally associated with polyphenols may be attributed to their potent antioxidant capacity, procyanidin content could be an indicator of potential biological activity.

In conclusion, the normal-phase HPLC method previously reported by Hammerstone et al. (1999) has been slightly modified for the quantification of procyanidins in cocoa liquors and chocolates. By multiple laboratories performing analysis on an identical sample set and producing results within a small coefficient of variation, this quantification method has shown to be a reliable and reproducible tool. Finally, it was determined that the procyanidin content in cocoa samples closely correlates to their ORAC values, which indicates that procyanidins are the primary contributors to cocoa antioxidant capacity. This finding may in part explain some of the suggested health benefits of cocoa and chocolate consumption.

#### ABBREVIATIONS USED

DMACA, dimethylaminocinnamaldehyde; HPLC, high-performance liquid chromatography; MS, mass spectrometry; HP, Hewlett-Packard; R-PE, R-phycoerythrin; TE, Trolox equivalents; ORAC, oxygen radical absorbance capacity

#### LITERATURE CITED

- Bronner, W. E.; Beecher, G. R. Method for determining the content of catechins in tea infusions by high-performance liquid chromatography. *J. Chromatogr. A* **1998**, 805, 137–142.
- Cao, G.; Alessio, H. M.; Cutler, R. G. Oxygen-radical absorbance capacity assay for antioxidants. *Free Radical Biol. Med.* **1993**, 14, 303–311.

- Cao, G.; Verdon, C. P.; Wu, A. H. B.; Wang, H.; Prior, R. L. Automated oxygen radical absorbance capacity assay using the COBAS FARA II. *Clin. Chem.* **1995**, *41*, 1738–1744.
- Clapperton, J.; Hammerstone, J. F.; Romanczyk, L. J.; Yow, S.; Lim, D.; Lockwood, R. Polyphenols and Cocoa Flavour. *Proceedings, 16th International Conference of Groupe Polyphenols*; Lisbon, Portugal, Groupe Polyphenols: Norbonne, France, 1992; Tome II, pp 112–115.
- de Pascual Teresa, S.; Treutter, D.; Rivas Gonzalo, J. C.; Santos Buelga, C. Qualitative and Quantitative Analysis of Dietary Flavanols. *Proceedings, 19th International Conference on Polyphenols*, Lille, France, Polyphenols Communications 98, Villeneuve d'Ascq: France, 1998a; Vol. 2, pp 257–258.
- de Pascual Teresa, S.; Mendez Arroyo, A.; Santos Buelga, C.; Rivas Gonzalo, J. C. Determination of Flavan-3-ols in some Spanish Wines. *Proceedings, 19th International Conference on Polyphenols*, Lille, France, Polyphenols Communications 98: Villeneuve d'Ascq: France, 1998b; Vol. 2, pp 333–334.
- Hammerstone, J. F.; Lazarus, S. A.; Mitchell, A. E.; Rucker, R.; Schmitz, H. H. Identification of Procyanidins in Cocoa and Chocolate Using High Performance Liquid Chromatography/Mass Spectrometry. *J. Agric. Food Chem.* **1999**, *47*, 490–496.
- Kim, H.; Keeney, P. G. (–)-Epicatechin Content in Fermented and Unfermented Cocoa Beans. *J. Food Sci.* **1984**, *49*, 1090–1092.
- Krull, I.; Swartz, M. Frequently Asked Questions about Analytical Method Validation. *LC·GC*. **1998**, *16*, 464–467.
- Lazarus, S. A.; Adamson, G. E.; Hammerstone, J. F.; Schmitz, H. H. High Performance Liquid Chromatography/Mass Spectrometry Analysis of Proanthocyanidins in Food Stuffs. *J. Agric. Food Chem.* **1999**, *47*, 3693–3701.
- McMurrough, I.; Baert, T. Identification of Proanthocyanidins in Beer and their Direct Measurement with a Dual Electrode Electrochemical Detector. *J. Inst. Brew.* **1994**, *100*, 409–416.
- Meursing, E. H. Cocoa mass, cocoa butter, cocoa powder. In *Industrial Chocolate Manufacturing and Use*; Beckett, S. T., Ed.; Blackie Academic & Professional: Glasgow, U.K., 1994.
- Official Methods of Analysis*, 16<sup>th</sup> ed.; AOAC International: Gaithersburg, MD, 1996; method 920.117.
- Oszmianski, J.; Lee, C. Y. Isolation and HPLC Determination of Phenolic Compounds in Red Grapes. *Am. J. Enol. Vitic.* **1990**, *41*, 204–206.
- Peterson, J.; Dwyer, J. Taxonomic Classification Helps Identify Flavonoid-containing Foods on a Semiquantitative Food Frequency Questionnaire. *J. Am. Diet. Assoc.* **1998**, *98*, 677–682.
- Porter, L. J. Flavans and Proanthocyanidins. In *The Flavonoids Advances in Research since 1980*; Harborne, J. B., Ed.; Chapman and Hall Ltd.: New York, 1988.
- Porter, L. J. Tannins. In *Methods in Plant Biochemistry, Volume I Plant Phenolics*; Dey, P. M., Harborne, J. B., Eds.; Academic Press Inc.: San Diego, CA, 1989.
- Rice-Evans, C.; Packer, L., Eds. *Flavonoids in Health and Disease*; Marcel Dekker: New York, 1997.
- Ricardo da Silva, J. M.; Rosec, J.-P.; Bourzeix, M.; Heredia, N. Separation and Quantitative Determination of Grape and Wine Procyanidins by High Performance Reversed Phase Liquid Chromatography. *J. Agric. Food Chem.* **1990**, *53*, 85–92.
- Salagoity-Auguste, M.-H.; Bertrand, A. Wine Phenolics – Analysis of Low Molecular Weight Components by High Performance Liquid Chromatography. *J. Sci. Food. Agric.* **1984**, *35*, 1241–1247.
- Spanos, G. A.; Wrolstad, R. E. Influence of Processing and Storage on the Phenolic Composition of Thompson Seedless Grape Juice. *J. Agric. Food Chem.* **1990**, *38*, 1565–1571.
- Treutter, D. Chemical Reaction Detection of Catechins and Proanthocyanidins with 4-Dimethylaminocinnamaldehyde. *J. Chromatogr.* **1989**, *467*, 185–193.
- Waterhouse, A. L.; Walzem, R. L. Nutrition of Grape Phenolics. In *Flavonoids in Health and Disease*; Rice-Evans, C. A., Packer, L., Eds.; Marcel Dekker: New York, 1998.

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