

# Variability in Dietary Antioxidant Related Natural Product Supplements: The Need for Methods of Standardization

Ronald L Prior, PhD,<sup>1,2</sup> Guohua Cao, PhD<sup>1</sup>

<sup>1</sup>U.S. Department of Agriculture, Agriculture Research Service, Jean Mayer Human Nutrition Research Center on Aging at Tufts University, Boston, Massachusetts

## ABSTRACT

Forty-six commercial preparations of antioxidant related dietary supplements were evaluated for total antioxidant capacity using the Oxygen Radical Absorbance Capacity (ORAC) assay and for total anthocyanin and phenolic content. Products based upon bilberry, cranberry, chokeberry and elderberry were observed to have ORAC values ranging from 16 to 3985  $\mu\text{mol}$  Trolox Equivalents (TE)/g, total anthocyanin concentrations ranging from 0.2 to 204 mg/g, and total phenolic concentrations ranging from 1.8 to 464 mg/g. The percentage of total phenolics as anthocyanins ranged from 1.4 to 72.7%. ORAC per phenolic ranged from 3.9 to 17.1  $\mu\text{mol}$  TE per mg. Elderberry had the highest ( $49.5 \pm 1.5$ ; Mean  $\pm$  SEM) and cranberry had the lowest ( $8.7 \pm 0.7$ ) percentage of phenolics as anthocyanins. Both elderberry and chokeberry had two anthocyanins that accounted for more than 90% of the total anthocyanins based upon HPLC separation and visible detector response at 520 nm. Bilberry had 14 separate anthocyanin components with no one single component comprising more than 12% of the total. Proanthocyanin sources such as pine bark extract and grape seed extract had antioxidant capacities ranging from 16 to 8392  $\mu\text{mol}$  TE/g and total phenolics ranged from 1.9 to 804 mg/g. These antioxidant sources did not contain any anthocyanins. The results from this study clearly indicate that quality control measures of antioxidant capacity are needed for the determination of appropriate

levels of antioxidant intake and as a basis for standardization of antioxidant related dietary supplements.

## KEYWORDS

Bilberry, cranberry, chokeberry, elderberry, anthocyanins, phenolics, ORAC, HPLC

## INTRODUCTION

Production of free radicals and other reactive species in cells and body tissues has been linked to aging and more than one hundred disease states.<sup>1,2,3</sup> The body has various defense mechanisms to protect against the damaging effects of these reactive species on DNA, lipids, and proteins.<sup>4</sup> Dietary antioxidants serve as one of the sources of protection. We have demonstrated that flavonoid compounds, including anthocyanins, exhibit high antioxidant activity<sup>5,6</sup> as measured by the oxygen radical absorbance capacity (ORAC) assay and that the total antioxidant capacity of different fruits and vegetables can differ by as much as 20-fold.<sup>7,8</sup> The finding of potential health promoting aspects of dietary antioxidants has led to the development of numerous commercial products which contain phytonutrients with antioxidant activity and are marketed as dietary supplements. However, to date there have not been good markers applied to indicate the antioxidant potency of these natural products. In studies from our laboratory on the antioxidant phytonutrients in blueberries, we found considerable variability depending upon variety and maturity at harvest.<sup>9</sup> This observation led us to evaluate the amount of variation in total antioxidant capacity in some of these commercially prepared antioxidant supplements (primarily those based upon fruit or berries).

Therefore, the objective of the present study was to compare total antioxidant capacity (measured as ORAC), total anthocyanins, and total phenolics in various antioxidant related supplements.

<sup>1</sup>Mention of a trade name, proprietary product or specific equipment does not constitute a guarantee by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable.

<sup>2</sup>Correspondance:

Ronald L. Prior, USDA, ARS, HNRCA,  
711 Washington St. Boston, MA 02111.  
Phone 617-556-3310 Fax 617-556-3299  
E-Mail, prior@hnrc.tufts.edu

## MATERIALS AND METHODS

**Chemicals:** R-Phycoerythrin (R-PE), ascorbic acid, gallic acid, and acetonitrile (HPLC grade) were purchased from Sigma (St. Louis, Missouri). 6-Hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox) was obtained from Aldrich (Milwaukee, Wisconsin). 2,2'-Azobis (2-amidino-propane) dihydrochloride (AAPH) was obtained from Wako Chemicals USA, Inc. (Richmond, Virginia). Methanol (HPLC grade) was from Fisher Scientific (Boston, Massachusetts).

**Determination of ORAC, total anthocyanins, and total phenolics:** Samples of antioxidant preparations were obtained from commercial retail sources and from manufacturers or distributors of plant extracts. Powdered samples (1 g) were dissolved in 20 mL H<sub>2</sub>O for the analysis of ORAC, total anthocyanins, and total phenolics. If a residue remained following centrifugation, the pellet was extracted with 2 mL acetone. The acetone extract was also analyzed for ORAC, total anthocyanins and total phenolics. The analytical values from the aqueous and acetone extracts were added together to give the total value.

*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) (Emission filter = 565 nm). The procedure was based on a previous report of Cao and coworkers,<sup>10</sup> as modified for the COBAS FARA II.<sup>11</sup> Briefly, in the final assay mixture (0.4 mL total volume), R-PE (16.7 nM) was used as a target of free radical attack, and AAPH (4 mM) as a peroxy radical generator. Trolox (1.0 μmol/L), a water soluble analog of vitamin E, was used as a control standard. The analyzer was programmed to record the fluorescence of R-PE every 2 minutes 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 μmol Trolox equivalents (TE) per g.

*Total Anthocyanins Assay.* Total anthocyanins were estimated by a pH differential method.<sup>12</sup> Absorbance (A) was measured in the COBAS FARA II centrifugal analyzer at 510 nm, and 700 nm in buffers at pH 1.0 and 4.5. Results were calculated using the equation:  $A = [(A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5}]$  and expressed as mg of cyanidin-3-glucoside equivalent per g of extract with a molar extinction coefficient of cyanidin-3-glucoside of 29,600.

*Total Phenolics Assay.* Total soluble phenolics in the extracts were determined with Folin-Ciocalteu reagent by the method of Slinkard and Singleton<sup>13</sup> which was adapted for automatic analysis on the COBAS FARA II analyzer using gallic acid as a standard.

**HPLC Procedures:** A Bioanalytical Systems (BAS) PM-80 HPLC gradient pump (West Lafayette, IN) was cou-

pled with a ESA Coullarray coulometric detection system (ESA Inc., Chelmsford, MA), which consisted of two cell packs in series, each pack containing four porous graphite working electrodes with associated palladium reference electrodes and platinum counter electrodes. The potentials on the 8-channel electrochemical coulometric array detector were set with the potential of channel 1 equal to 300 mV and each subsequent channel 90 mV higher to a maximum of 840 mV on channel 7. Channel 8 was set in reduction mode at a potential of -50 mV. At the completion of every run all 8 channels were set to oxidize at 1000 mV for 12 seconds for cleaning purposes. Chromatographic separation was performed on an ODS (Octadecylsiloxane) Hypersil column (C<sub>18</sub>; 150 mm x 4.6 mm I.D.; particle size, 5 μm; pore size, 120Å) from Keystone Scientific Inc. (Bellefonte, PA). The system was used at ambient room temperature. A Spectra-Physics SP8780 refrigerated autosampler (San Jose, CA) was used to inject 20 μL samples. The autosampler temperature was maintained at 4°C.

A separate BAS gradient pump was coupled with a BAS UV-Visible detector (Model UV116A) (West Lafayette, IN) such that the column effluent could be monitored at 280 nm or 520 nm. Only anthocyanins absorb at 520 nm while phenolic type compounds, including anthocyanins, absorb at 280 nm.

All solvents were HPLC grade. The solvent flow rate in the pump was 1.0 mL/min. A binary linear gradient method was used as follows: 1) linear increase in B from 0% to 24% from time 0 to 50 minutes, 2) linear increase in B from 24% to 38% from time 50 to 110 minutes, and 3) 100% B from time 110 to 120 minutes. At 120 minutes, the program returned to initial conditions and the system was re-equilibrated for 10 minutes. Mobile Phase A was 25 mM sodium acetate in water. Mobile Phase B was 25 mM sodium acetate in methanol. Both mobile phases were adjusted to pH 1.5 with trichloroacetic acid.

## RESULTS:

Data for total antioxidant capacity (ORAC, μmol TE/g), anthocyanins (mg/g) and total phenolics (mg/g) for bilberry, cranberry, chokeberry, and elderberry products are presented in Table 1. Data for ORAC and total phenolics for proanthocyanin sources (grape seed and pine bark extracts) are presented in Figure 1. Total antioxidant capacity varies from 16 to 3984.5 μmol TE/g (a 249-fold difference) for the berry products and from 16 to 8392 μmol TE/g (a 525-fold difference) for all the products tested. ORAC values of more than approximately 200 indicate that a significant concentrating process has occurred in the preparation and manufacturing process.

Anthocyanins were not present at detectable levels in proanthocyanin sources (grape seed or pine bark extracts). In the berries, anthocyanin content varied from 0.2 to 204.4

mg/g (more than 1000-fold difference). Total phenolics ranged from 1.8 to 463.7 (a 258-fold difference) for berry products and from 1.8 to 804 mg/g (a 447-fold difference) for all the products tested. The extremes in both ORAC and total phenolics occurred in the grape seed extract products.

The ratio of total anthocyanins (mg/g) to total phenolics (mg/g) was calculated and expressed as a percentage (Table 1). The elderberry products, in general, have the highest percentage of phenolics as anthocyanins (18 - 73%) ( $49.5 \pm 1.5$ ; mean  $\pm$  SEM) with bilberry having less (22 - 62%) ( $43.1 \pm 0.9$ ) and cranberry having much less (1-14%) ( $8.7 \pm 0.7$ ). The individual anthocyanin composition, as determined at 520 nm, differed considerably between bilberry, elderberry and chokeberry (Figure 2). In bilberry, there were 14 different individual anthocyanin components (Figure 2), while in elderberry there were 5 easily detected anthocyanins and in chokeberry only 4 distinct anthocyanins with over 90% of the total in both elderberry and chokeberry accounted for by two compounds. Upon searching the literature for information on anthocyanins in elderberry, it became apparent that Peak 6, although it coelutes with delphinidin-3-O-arabinoside found in bilberry, is probably cyanidin-3-sambubioside.<sup>14</sup> The HPLC comparisons between bilberry, chokeberry and elderberry were made with preparations having similar ORAC values (1800-2000  $\mu\text{mol TE/g}$ ). Previously, we observed a good linear relationship between the ORAC measurement and the total peak areas across the entire chromatogram using the ESA Coularray electrochemical detector.<sup>15</sup> Figures 3 and 4 present the data obtained by using the Coularray electrochemical detector on two proanthocyanin sources containing 617 (Figure 3) (Sample PYC-080309) and 8392 (Figure 4) (Sample GRS-020403)  $\mu\text{mol TE/g}$  of ORAC activity. Peak areas reflect the much higher concentrations in the second sample relative to the first. Not only are the concentrations different, but the "fingerprints" also differ. Most notable are the relatively large peaks in the area between 35 and 40 minutes in sample GRS-020403 (Figure 4) compared to sample PYC-080309 (Figure 3). The lack of peaks in the area between 65 minutes and 105 minutes is also quite evident in these samples. It is in this area that the anthocyanins are eluted.

Anthocyanin standards were available for cyanidin-3-galactoside (Peak 5), cyanidin-3-glucoside (Peak 7), malvidin-3-galactoside (Peak 13) and malvidin-3-glucoside (Peak 14). Tentative identification of the other anthocyanins was obtained from relative retention times available in the literature.<sup>16,17,18,19</sup>

The ratio of antioxidant capacity to total phenolics was calculated for these analyzed supplements and expressed as  $\mu\text{mol TE/mg phenolic}$  (Table 1). As can be seen, this ratio varies from 3.9 to 17.1. As a point of reference, the same ratio was computed for various pure phenolic, polyphenolic, and anthocyanin compounds (Table 2). The mean ratio

$\pm$  SEM for the 41 compounds was  $6.76 \pm 0.60$  (Mean  $\pm$  SEM)  $\mu\text{mol TE ORAC/mg phenolic}$ . As is apparent from Table 2, the ORAC/phenolics ratio can be above 12  $\mu\text{mol TE/mg}$  for some compounds, such as caffeic acid, protocatechuic acid, fustin, luteolin and myricetin. Ratios of ORAC/phenolics ( $\mu\text{mol TE/mg}$ ) above 11 were observed in some samples of bilberry and elderberry (Table 1). Ratios greater than 12 have been observed in one commercial antioxidant supplement. Generally this is due to the addition of antioxidants to the supplement which do not have a phenolic structure. However, there was considerable variation in this ratio between samples. Preparations with a low ratio likely indicate 1) the use of a processing or manufacturing procedure that destroyed some of the antioxidant capacity, 2) an extraction procedure that extracted compounds not having much antioxidant capacity, or 3) dilution of the antioxidants with inactive components in the preparation of the final product.

There was a significant linear relationship between ORAC ( $\mu\text{mol TE/g}$ ) (Y) and total phenolics (mg/g) (X) (Figure 5) ( $Y = -31.5 + 9.21X$ ;  $r_{xy} = 0.98$ ). The relationship between ORAC ( $\mu\text{mol TE/g}$ ) (Y) and total anthocyanins (mg/g) (X) was described by the linear equation: ( $Y = 46.4 + 17.90X$ ;  $r_{xy} = 0.942$ ) (Figure 6).

## DISCUSSION

The potential effectiveness of herbal supplements in promoting various aspects of health depends upon their botanical and chemical composition and on the concentrations of active ingredients that they contain. The observation of a wide range in antioxidant potency of many commercial herbal supplements underscores the need for performing quality control of herbal supplements. This need is especially important in the U.S. because herbal supplements are not regulated as drugs but are instead sold as "food supplements." For antioxidant supplements, there have not been suitable analytical methods for measuring total antioxidant capacity until recently, when we adapted and automated the ORAC assay and utilized it for determination of antioxidant capacity in fruits and vegetables.<sup>5,7,8</sup> In addition to variability in the botanical source, another factor influencing antioxidant potency of a nutritional supplement is the type and quality of processing involved in production of the particular product.

In the current study we evaluated some common preparations of antioxidant supplements which were based upon berries and other sources. Bilberry and cranberry both belong to the *Vaccinium species* (*Vaccinium myrtillus*, *L.* and *Vaccinium macrocarpon* Ait., respectively), while chokeberry and elderberry belong to the *Aronia melanocarpa* and *Sambucus nigra* species, respectively.

**ORAC:** Natural products contain numerous antioxidants. The ORAC assay was used in this study to assess

**Table 1:** Total antioxidant capacity (measured as ORAC), total anthocyanins, and total phenolics of different commercial berry products.

Sample	ORAC	Anthocyanins	Phenolics	Anthocy/Phen <sup>d</sup>	ORAC/Phen <sup>e</sup>
	$\mu\text{mol/g}^a$	$\text{mg/g}^b$	$\text{mg/g}^c$	%	$\mu\text{mol/mg}$
<b>Bilberry</b>					
<b>Fresh Bilberry<sup>f</sup></b>	<b>282.3</b>	<b>19.0</b>	<b>33.2</b>	<b>57.1</b>	<b>8.50</b>
BIL-030302	40.0	2.0	4.6	43.5	8.79
BIL-010502	54.2	3.2	7.9	40.4	6.89
BIL-010602	53.6	2.3	9.6	23.5	5.57
BIL-020119	133.2	4.7	18.3	25.7	7.29
BIL-010211	146.5	8.2	15.8	51.7	9.27
BIL-010103	157.2	4.2	9.21	45.5	17.07
BIL-110101	191.0	11.0	48.9	22.4	3.90 <sup>g</sup>
BIL-010126 <sup>h</sup>	267.2	14.0	35.3	39.7	7.56
BIL-080113	918.0	47.8	82.1	58.2	11.18
BIL-010226 <sup>h</sup>	1555.8	71.0	184.6	38.5	8.43
BIL-010122	1577.0	111.5	179.2	62.3	8.80
BIL-010202	2056.3	96.4	173.2	55.7	11.87
BIL-010123	2592.6	146.9	236.3	62.2	11.00
BIL-010102	2988.8	116.0	349.6	33.2	8.55
BIL-010125	3984.5	204.4	463.7	44.1	8.53
			mean $\pm$ SEM	43.1 $\pm$ 0.9	9.0 $\pm$ 0.5
<b>Cranberry</b>					
<b>Fresh Cranberry<sup>i</sup></b>	<b>98.3</b>	<b>6.4</b>	<b>22.9</b>	<b>28.1</b>	<b>5.73</b>
CR-010605	16.0	0.2	1.8	8.5	9.12
CR-010405	36.6	0.3	4.9	6.8	7.56
CR-010705	64.9	0.9	7.6	11.6	8.53
CR-010902	91.1	0.3	11.7	2.7	7.77
CR-010102	94.4	2.6	19.0	13.7	4.97
CR-070505	101.5	0.7	11.6	6.2	8.72
CR-080213	107.0	1.6	17.7	9.1	6.05
CR-011602	121.4	2.6	19.0	13.7	6.39
CR-011502	127.5	0.3	21.7	1.4	5.87
CR-011702	151.4	3.0	22.0	13.5	6.88
			mean $\pm$ SEM	8.7 $\pm$ 0.7	7.2 $\pm$ 0.4

Table 1: Continued

<b>Chokeberry</b>					
<b>CH-SDA7</b>	<b>97.3</b>	<b>1.4</b>	<b>15.8</b>	<b>8.7</b>	<b>6.15</b>
<b>CH-011402</b>	<b>120.6</b>	<b>3.8</b>	<b>12.2</b>	<b>31.2</b>	<b>9.86</b>
<b>CH-010102</b>	<b>141.6</b>	<b>2.0</b>	<b>22.7</b>	<b>8.9</b>	<b>6.24</b>
<b>CH-011102</b>	<b>147.8</b>	<b>1.6</b>	<b>20.6</b>	<b>7.8</b>	<b>7.18</b>
<b>CH-011202</b>	<b>161.2</b>	<b>2.1</b>	<b>24.7</b>	<b>8.5</b>	<b>6.52</b>
<b>CH-010802</b>	<b>2087.1</b>	<b>93.7</b>	<b>225.9</b>	<b>41.5</b>	<b>9.24</b>
			mean±SEM	17.8±1.6	7.5±0.5
<b>Elderberry</b>					
<b>EL-102002</b>	<b>82.7</b>	<b>9.1</b>	<b>18.5</b>	<b>49.3</b>	<b>4.48</b>
<b>EL-011902</b>	<b>116.2</b>	<b>3.8</b>	<b>12.6</b>	<b>30.5</b>	<b>9.25</b>
<b>EL-010102</b>	<b>156.0</b>	<b>7.8</b>	<b>20.6</b>	<b>37.8</b>	<b>7.57</b>
<b>EL-020805</b>	<b>157.5</b>	<b>3.7</b>	<b>20.8</b>	<b>17.8</b>	<b>7.56</b>
<b>EL-012302</b>	<b>196.2</b>	<b>8.9</b>	<b>26.0</b>	<b>34.0</b>	<b>7.54</b>
<b>EL-082202</b>	<b>582.5</b>	<b>44.4</b>	<b>65.4</b>	<b>67.9</b>	<b>8.13</b>
<b>EL-010202</b>	<b>627.7</b>	<b>61.4</b>	<b>93.2</b>	<b>65.9</b>	<b>6.73</b>
<b>RU-012702</b>	<b>981.9</b>	<b>64.3</b>	<b>88.5</b>	<b>72.7</b>	<b>11.10</b>
<b>EL-011802</b>	<b>1823.0</b>	<b>140.8</b>	<b>202.9</b>	<b>69.2</b>	<b>8.98</b>
			mean±SEM	49.5±1.5	7.9±0.5

a ORAC expressed as  $\mu\text{mol TE/g}$  dried extract.

b Total anthocyanins expressed as mg cyanidin-3-glucoside equivalent per g dried extract.

c Total phenolics expressed as mg gallic acid equivalents per g dried extract.

d Ratio of total anthocyanins to total phenolics expressed as a percentage.

e Ratio of ORAC to total phenolics expressed as  $\mu\text{mol TE/mg}$  total phenolics.

f Data from fresh whole bilberry expressed on dry matter basis from Prior et al. (1998).

g Sample contained lutein which may account for the lowered ORAC/Phenolic ratio.

h Samples from same supplier. Manufacturer's claim was that sample BIL-010226 contained extra strength (4 times sample BIL-010126).

i Cranberries were analyzed to have 17.8% dry matter. On a fresh weight basis, ORAC was  $17.5 \pm 0.7 \mu\text{mol TE/g}$ ; Anthocyanin content was  $1.14 \pm 0.10 \text{ mg/g}$  and total phenolics were  $4.07 \pm 0.36 \text{ mg/g}$ .

total antioxidant capacity of a product because of the difficulty in measuring each antioxidant component separately and the interactions among different antioxidant components in the product. The ORAC value comes from all traditional antioxidants including  $\alpha$ -tocopherol,  $\beta$ -carotene, reduced glutathione (GSH), uric acid, bilirubin, albumin, melatonin, phenolic acids and flavonoids.<sup>5,6,10,11,15,20,21</sup> The ORAC assay depends on the detection of chemical damage to B- or R-PE through the decrease in its fluorescence emission. The fluorescence is highly sensitive to the confirmation and chemical integrity of the protein. Under appropriate conditions, the loss of PE fluorescence in the presence of free radicals is an index of oxidative damage to the protein. The inhibition of the free radical action by an antioxi-

dant, which is reflected in the protection against the loss of PE fluorescence in the ORAC assay, is a measure of its antioxidant capacity against the free radicals. In addition to the use of B- or R-PE as a sensitive target of free radical attack with Trolox as a calibrator, the ORAC assay uses AAPH as a free radical generating system and an area-under-curve (AUC) technique for the quantitation of antioxidant capacity. AAPH undergoes spontaneous decomposition and produces peroxy radicals with a rate primarily determined by temperature. Because of the very high molar ratio (more than 2000) of AAPH to antioxidant used in this procedure, the ORAC assay has high specificity and thus measures the capacity of an antioxidant to *directly quench free radicals*. The AUC technique com-

Table 2: Molecular weight and antioxidant capacity (ORAC) expressed as  $\mu\text{mol TE}/\mu\text{mol}$  compound or  $\mu\text{mol TE}/\text{mg}$  compound.

<b>Compound</b>	<b>Molecular Weight (g/mol)</b>	<b>ORAC <math>\mu\text{mol}/\mu\text{mol}</math></b>	<b>ORAC <math>\mu\text{mol}/\text{mg}</math></b>
<b>Phenolic Acids</b>			
caffeic acid	180.2	2.23	12.38
chlorogenic acid	354.3	3.00	8.47
ferulic acid	194.2	1.33	6.85
gallic acid	170.1	1.74	10.23
<i>p</i> -coumaric acid	164.2	1.09	6.64
protocatechuic acid (3,4-dihydroxybenzoic acid)	154.1	2.06	13.37
syringic acid	198.2	1.27	6.41
vanillic acid	168.1	1.11	6.60
gentisic acid (2,5-dihydroxybenzoic acid)	154.1	1.20	7.79
<b>Catechins</b>			
(-)-epicatechin	290.3	2.36	8.13
(+)-catechin	290.3	2.49	8.58
gallo catechin gallate	458.4	2.43	5.30
<b>Flavones/Flavanones</b>			
eriodictyol (3',4',5,7-tetrahydroxyflavanone)	288.3	3.41	11.84
fustin (3,3',4',7-tetrahydroxyflavanone)	288.3	3.91	13.55
hesperidin (3',5-dihydroxy-4'-methoxyflavanone-7-rutinoside)	610.6	0.04	0.07
kaempferol (3,4',5,7-tetrahydroxyflavone)	286.2	2.67	9.33
kaempferol-3,4'-dimethylether	314.3	1.22	3.88
kaempferol-7-neohesperidoside	594.5	1.65	2.77
luteolin (3',4',5,7-tetrahydroxyflavone)	286.2	3.57	12.49
myricetin (3,3',4',5,5',7-hexahydroxyflavone)	318.2	4.32	13.57
naringenin (4',5,7-trihydroxyflavanone)	272.3	2.67	9.80
naringin (naringenin-7-neohesperidoside)	580.5	0.37	0.63
quercetin (3,3',4',5,7-pentahydroxyflavone)	302.2	3.29	10.87
rutin (quercetin-3-rutinoside)	610.5	0.56	0.92
taxifolin (3,3',4',5,7-pentahydroxyflavanone)	304.3	3.59	11.79
<b>Anthocyanidin/Anthocyanins</b>			
cyanidin-Cl	322.7	2.24	6.94
cyanidin-3,5-diglucoside	647.0	1.69	2.61
cyanidin-3-galactoside	484.8	2.03	4.18
cyanidin-3-glucoside	484.8	3.49	7.20
cyanidin-3-rhamnoglucoside	631.0	2.99	4.74
delphinidin-Cl	338.7	1.81	5.34
malvidin-Cl	366.8	2.01	5.48
malvidin-3,5-diglucoside	690.0	1.55	2.25
malvidin-3-glucoside	528.9	1.40	2.65
pelargonidin-Cl	306.7	1.54	5.02
pelargonidin-3,5-diglucoside	631.0	1.07	1.69
callistephin (pelargonidin-3-glucoside)	468.8	1.33	2.83
peonidin-Cl	336.7	1.69	5.03
peonidin-3-glucoside	462.8	1.81	3.90
<b>Isoflavones</b>			
diadzein (4',7-dihydroxyisoflavone)	254.2	1.65	6.48
genistein (4',5,7-trihydroxyisoflavone)	270.2	2.38	8.79

<sup>a</sup> Data on ORAC ( $\mu\text{mol TE}/\mu\text{mol}$  compound) taken in part from Cao et al. 1997, Wang et al. 1997 and unpublished data.

bines both inhibition percentage and the length of inhibition time of free radical action by an antioxidant into a single quantity, which makes it superior to other similar methods that use either an inhibition percentage at a fixed time or a length of inhibition time at a fixed inhibition percentage.

The antioxidant capacity of a nutritional supplement is critical when it is being marketed as an antioxidant. For all of the extracts evaluated (Table 1) we have observed a range in ORAC from 16 to 8392  $\mu\text{mol}$  Trolox equivalents per gram. Although not definitive at this point, data are being obtained by our laboratory to define the optimal dietary antioxidant intake as measured by the ORAC procedure. Extrapolating from data from the USDA continuing survey of consumption of fruits and vegetables in the United States, the average ORAC intake from fruits and vegetables may be as low as 1200  $\mu\text{mol}$  TE/day (Prior, unpublished data). In other data from 36 elderly subjects who completed a food frequency questionnaire of intakes during the previous year, average ORAC intake was estimated to be  $1640 \pm 200$   $\mu\text{mol}$  TE/day.<sup>22</sup> In this study the number of servings of fruits and vegetables consumed was 5/day, which is higher than the national average. Thus, normal intakes of ORAC are likely in the range of 1200-1600  $\mu\text{mol}$  TE/day. However, we have observed in elderly individuals that ORAC intakes from self selected diets high in fruits and vegetables can be as high as 6000  $\mu\text{mol}$  TE/day.<sup>22</sup> In one experiment, we observed that by increasing ORAC intake to 3200  $\mu\text{mol}$  TE/day, serum antioxidant capacity increased by 10-15%.<sup>22</sup> In another clinical trial, we observed a significant increase in total plasma antioxidant capacity following a single meal providing 3700  $\mu\text{mol}$  TE from either spinach, strawberry, or phenolics from red wine.<sup>23</sup> Thus, from the data available at this point in time, we predict that increasing the ORAC intake by 1000-2500 units per day may be needed to increase serum and tissue antioxidant capacities sufficiently to improve health outcomes. Most manufacturers of antioxidant supplements recommend intakes of 2-3 capsules per day containing a total of 250-500 mg of supplement. Based upon the above information, it is clear that intakes of 1000-2500 units of ORAC could not be achieved with a supplement unless the supplement contained at least 3000  $\mu\text{mol}$  TE/g. Only eight of the 46 antioxidant supplements tested contained this level of total antioxidant capacity.

**Total Phenolics and Anthocyanins:** Total phenolics was measured in this study because phenolics are the dominant antioxidants found in the natural products of plant sources and anthocyanins are a main group of phenolics contained in the berry products. Among the most common and important phenolic compounds are simple phenols and flavonoids. In addition to anthocyanins, flavonoids also include flavones, isoflavones, flavanones, flavanols, and proanthocyanidins. Precise quantitation of anthocyanins continues to be a problem because of the lack of availabili-

ty of individual standards. The pH differential method used provides an estimation of total anthocyanins, but errors will be introduced as the composition of the individual anthocyanins differs between sources. Even with HPLC separation, quantitation is not precise as long as a single anthocyanin response curve is used to compute quantities of all anthocyanins, which historically has been done because of a lack of standards. We have observed a differential response of different anthocyanins to the electrochemical detector as well as in absorbance at 520 nm. However, until additional standards are available, quantitation will have to continue to be based upon a single or at most a few anthocyanins.

Mazza and coworkers<sup>17</sup> reviewed the literature on anthocyanin content of the bilberry and cranberry. Bilberry was reported to have anthocyanin concentrations of 3 to 7 mg per g of fresh fruit, which is similar to our observation on fresh bilberry.<sup>9</sup> Different investigators have isolated from 11 to 15 different anthocyanins in bilberry. We observed 14 anthocyanins in the bilberry extract that we analyzed (Figure 2). Cyanidin and delphinidin glycosides accounted for approximately 64% of the total anthocyanins with no single one accounting for more than 12% of the total anthocyanins. Mazza and Miniata<sup>17</sup> reported that quantitatively, the delphinidin glycosides were present in the largest quantities, and the peonidin glycosides were the least abundant. Cranberries were reported to have about 0.8 mg anthocyanins per g fresh fruit, with the cranberry being rich in the 3-galactosides and the 3-arabinosides of peonidin and cyanidin. We observed an anthocyanin content of 1.1 mg/g fresh cranberry. Little information is available on the absorption and metabolism of anthocyanins *in vivo*. We and others have recently demonstrated that the cyanidin anthocyanins from red fruit are absorbed and found unmetabolized in blood and urine.<sup>24,25</sup>

Morazzoni and Bombardelli<sup>26</sup> have reviewed the literature concerning some of the pharmacologic and physiologic properties of a highly purified extract of bilberry (Myrtocyan®) that contained 36% anthocyanins. In patients with altered capillary fragility, anthocyanins from Myrtocyan® in a dose of 160-800 mg/day had vasoprotective activity, reducing the clinical symptoms of decreased capillary resistance such as petechiae, bruising and fecal occult blood. A marked improvement in retinal sensitivity was observed in subjects consuming 150 mg/day for 15 days. The clinical efficacy of Myrtocyan® (320 mg/day for 90 days) in improving retinal function of normal and myopic patients was also demonstrated. Beneficial effects were observed in other studies where the Myrtocyan® intake was in the range of 150 to 320 mg/day. At 36% anthocyanosides, this translates to a dose of 50 - 100 mg of anthocyanosides per day. Of the bilberry extracts that we evaluated (Table 1) only five of them would provide more than 50 mg of anthocyanins if 500 mg of the extract were consumed per day.

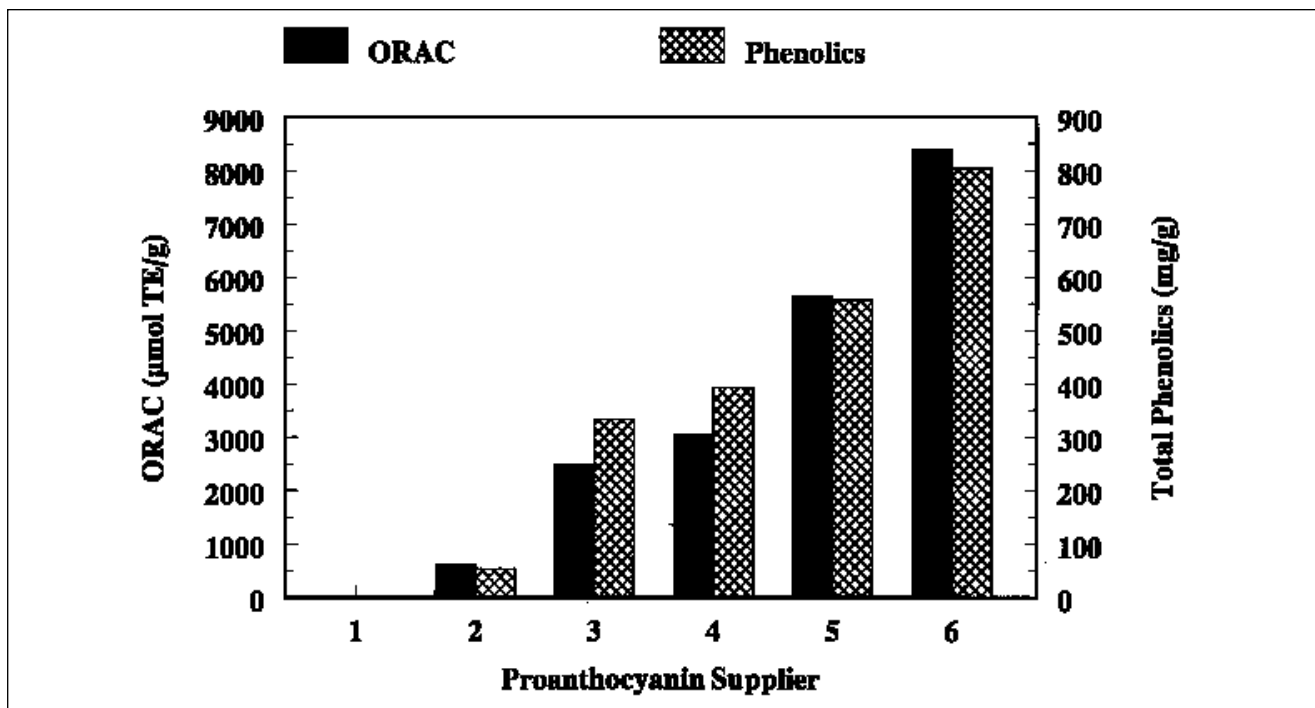
Although at least one of the bilberry products was labeled as being standardized to 25% anthocyanins, calculated as anthocyanidins, by weight, none of the samples we analyzed using the differential absorption method were found to have this level of anthocyanins. Furthermore, one sample which we obtained (data not presented), which was labeled as being bilberry, had no measureable levels of anthocyanins even though the total antioxidant capacity was above 1800  $\mu\text{mol TE/g}$ . Based upon the relationship observed in Figure 6, the sample should have contained nearly 100 mg anthocyanins/g. Thus, not only do preparations need to be monitored for antioxidant potency, but botanical source is also important, which can best be characterized by 'fingerprinting' using HPLC and measuring the total phenolics and anthocyanins in the preparation as we did for the samples of grape seed extract and of pine bark extract (Figures 3 and 4).

If 1000  $\mu\text{mol TE}$  of ORAC/day is a reasonable minimum effective quantity needed to provide added health benefits, then this would extrapolate to approximately 100 mg of total phenolics based upon the calculated regression equation observed (Figure 5). With a 50% anthocyanin to phenolic ratio, which could be obtained with some of the bilberry and elderberry products, an intake of approximately 50 mg of anthocyanins per day could be achieved.

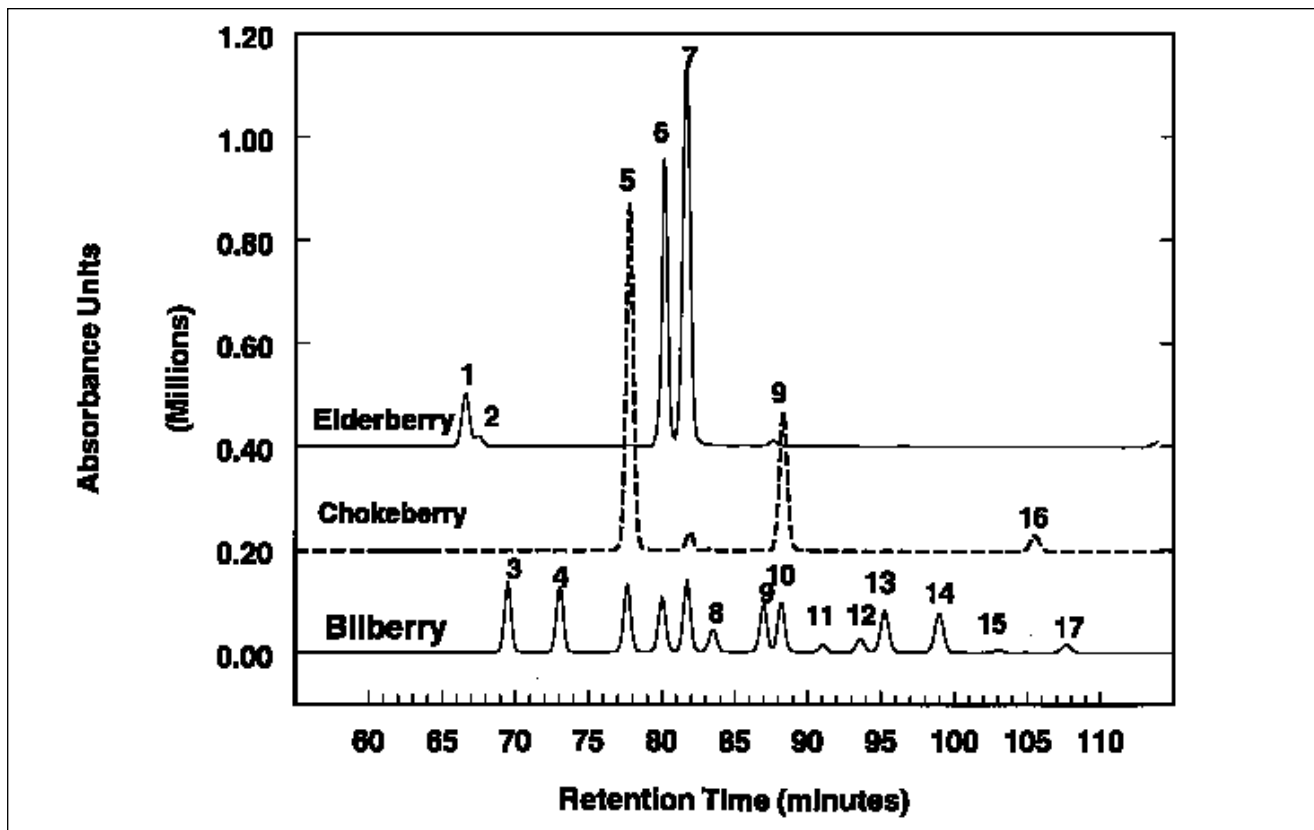
The important point is not that the more concentrated extracts are necessarily better, but that a standard measure of potency is needed in order to arrive at a reasonable intake

of the supplement to provide an efficacious dose. If sufficient intake cannot be provided in capsule form with the less potent preparations, then other means of delivery will be needed in order to obtain intakes equivalent to several grams. If a concentrated product is used, but diluted with inert materials during the final formulation process, the advantages of starting with a concentrated product are minimized or disappear completely.

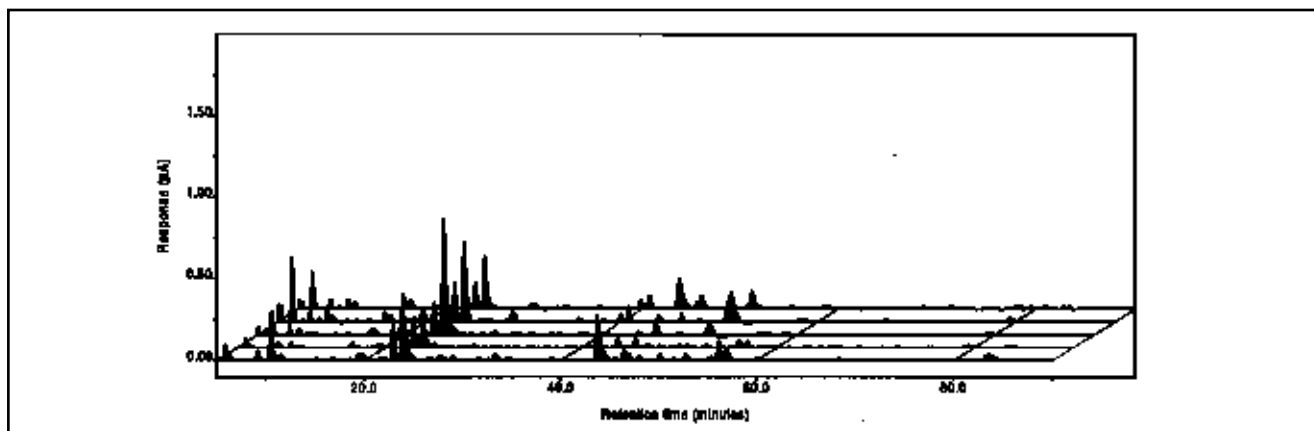
It seems clear from the data presented that a basis for standardizing the ingredients in nutritional supplements is critical and must be utilized by the nutritional supplement industry. For the antioxidant supplements, the measurement of ORAC and total phenolics provides a good measure of total potency. However, HPLC procedures will be needed if botanical source is to be verified. Although anthocyanins have strong antioxidant capacity, their mechanism(s) of action in biological systems may not be totally as antioxidants. For this reason, a separate estimation of anthocyanin content of the nutritional supplements utilizing berries may be critical. Andriambeloson and coworkers,<sup>27</sup> recently reported that among the anthocyanidins, delphinidin, but not malvidin or cyanidin, showed endothelium-dependent vasorelaxation. As we understand more about the biological activities of specific anthocyanins, standardization and quantitation of individual anthocyanins will become increasingly important.



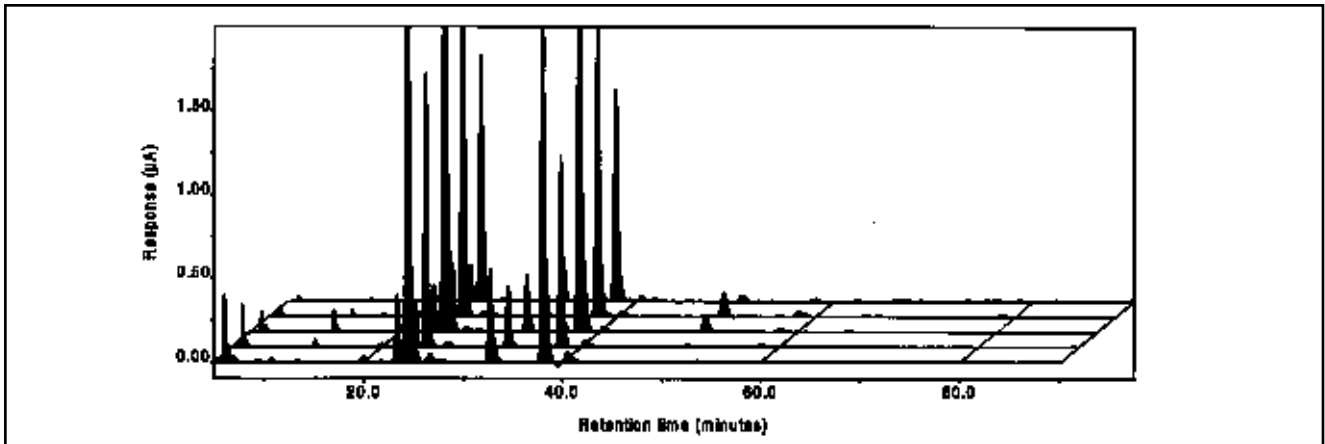
**Figure 1:** Total antioxidant capacity (ORAC in TE/g) and total phenolics in different sources of proanthocyanins such as grape seed and pine bark extracts, and pycnogenol®.



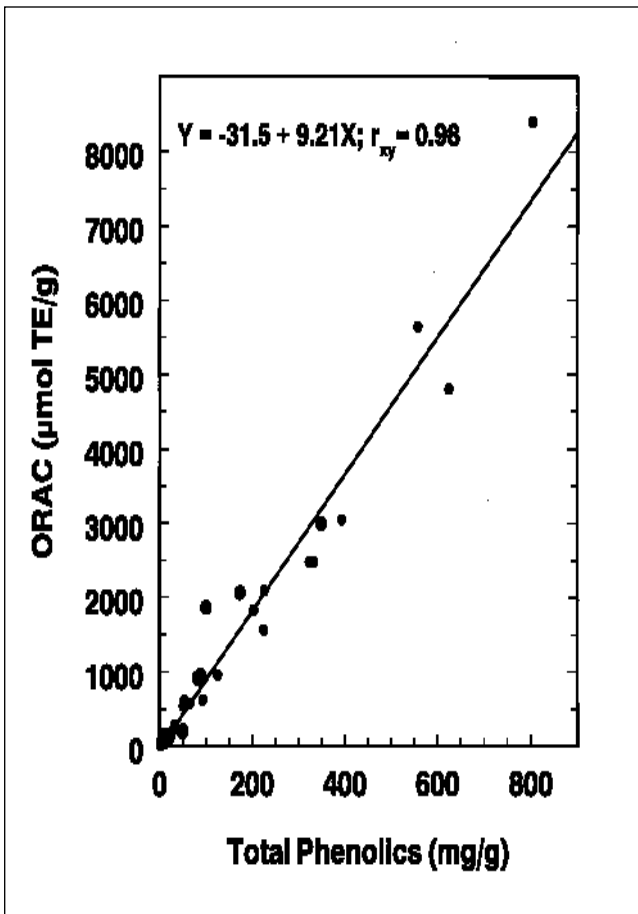
**Figure 2:** Comparison of anthocyanins from bilberry (BIL-010202), elderberry (EL-011802) and chokeberry (CH-010802) extracts using HPLC with the column effluent monitored at 520 nm wavelength from 45 minutes through the end of the chromatogram. Twenty  $\mu\text{L}$  of extract was injected onto the column. Identification of peaks as well as percentage of total anthocyanins based upon total area under the HPLC peaks using visible detector (520 nm) for bilberry, chokeberry and elderberry, respectively, were as follows: 1 - Cyanidin-3-sambubioside-5-glucoside (0,0,6.2%); 2 - Cyanidin-3,5-diglucoside (0,0,1.2%); 3 - Delphinidin-3-O-galactoside (11.1, 0, 0%); 4 - Delphinidin-3-O-glucoside (11.2, 0, 0%); 5 - Cyanidin-3-galactoside (11.6, 64.6, 0.1%); 6 - Delphinidin-3-O-arabinoside/Cyanidin-3-sambubioside (9.7, 0, 46.0%); 7 - Cyanidin-3-O-glucoside (12.2, 3.4, 45.9%); 8 - Petunidin-3-galactoside (4.2, 0, 0%); 9 - Cyanidin-3-arabinoside (8.2, 28.4, 0.6%); 10 - Petunidin-3-glucoside (8.7, 0, 0%); 11 - Petunidin-3-arabinoside (1.4, 0, 0); 12 - unknown (2.7, 0, 0%); 13 - Malvidin-3-galactoside (8.2, 0, 0%); 14 - Malvidin-3-glucoside (8.2, 0, 0%); 17 - Malvidin-3 arabinoside (2.0, 0, 0%). Identifications based upon relative retention time and information available in the literature. Although peak 6 coelutes with delphinidin-3-O-arabinoside, data from the literature indicates that this peak in elderberry is cyanidin-3-sambubioside (Inami et al., 1996).



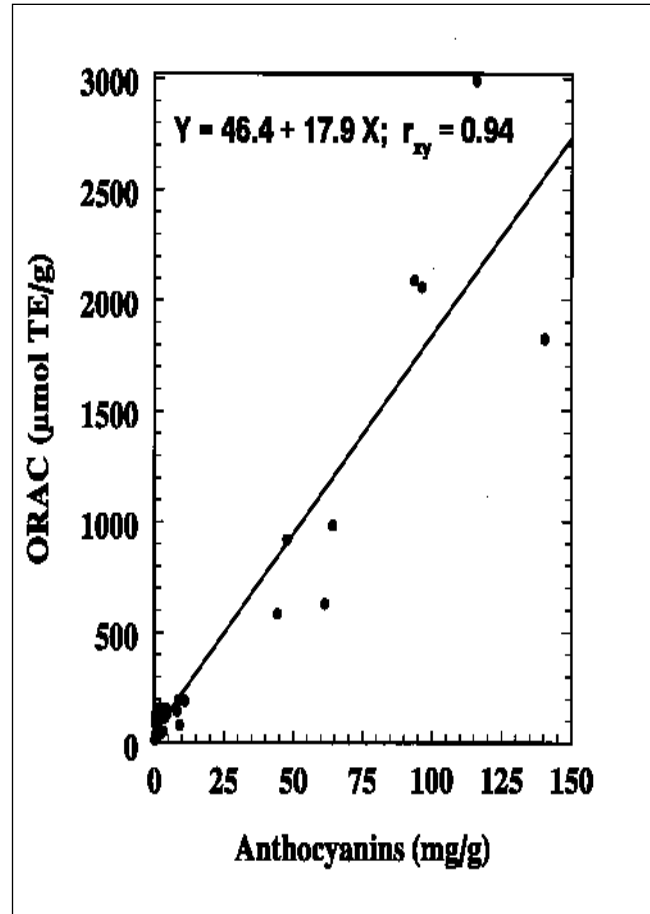
**Figure 3:** Chromatogram of pine bark extract (PYC-080309) (ORAC = 617  $\mu\text{mol TE/g}$ ; Total phenolics = 53.6 mg/g) showing channels 1-5 which were set at potentials of 300, 390, 480, 570 and 660 mV with a full scale output of 2.0  $\mu\text{C}$ . Twenty  $\mu\text{L}$  of extract was injected onto the column and an ESA coullarray detector was used for the detection.



**Figure 4:** Chromatogram of grapeseed extract (GRS-020403) (ORAC = 8392  $\mu\text{mol TE/g}$ ; Total phenolics = 804 mg/g) showing channels 1-5 which were set at potentials of 300, 390, 480, 570 and 660 mV with a full scale output of 2.0  $\mu\text{C}$ . Twenty  $\mu\text{L}$  of extract was injected onto the column and an ESA coularray detector was used for the detection.



**Figure 5:** Relationship between antioxidant capacity (ORAC -  $\mu\text{mol TE/g}$ ) (Y) and total phenolics (mg/g) (X) in antioxidant supplements. ( $Y = -31.5 + 9.21X$ ;  $r_{xy} = 0.98$ ;  $p < 0.01$ )



**Figure 6:** Relationship between antioxidant capacity (ORAC -  $\mu\text{mol TE/g}$ ) (Y) and total anthocyanins (mg/g) (X) in antioxidant supplements. ( $Y = 46.4 + 17.90X$ ;  $r_{xy} = 0.94$ ;  $p < 0.01$ )

## ABBREVIATIONS USED

ORAC, Oxygen Radical Absorbance Capacity; AAPH, 2,2'-Azobis (2-amidino-propane) dihydrochloride; Trolox, 6-Hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid; ODS, Octadecylsiloxane; TE, Trolox equivalents.

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