Anthocyanin-Based Natural Colorants: A New Source of Antiradical Activity for Foodstuff

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The antiradical capacity (radical scavenger capacity, RSC) of anthocyanin-based fruit extracts prepared in the laboratory (black chokeberry, black-thorn, and strawberry) was studied by using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•). To determine their RSC, the second-order rate constant (k_2) for the oxidation of these extracts by DPPH• was calculated. The value of k_2 was compared to that used in the food industry as natural (α-tocopherol) or synthetic (butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA)) antioxidants, as well as for a commercial elderberry concentrate and a synthetic colorant (Ponceau 4R). The k_2 values ($(mg/mL)^{-1}$ s^{-1}), in methanol at 25 °C, were 1.87, 0.7, 0.42, 0.2, 0.05, 0.03, and 0.008 for α-tocopherol, black chokeberry, BHA, black-thorn, BHT, strawberry, and elderberry, respectively. Ponceau 4R lacked RSC. Therefore, these natural colorants proved to be a combined source of color and RSC for food material.

Keywords: *Anthocyanins; colorants; radical scavenger; DPPH; kinetics*

INTRODUCTION

Natural antioxidants and colorants present in foods have attracted interest because of their safety and potential nutritional and therapeutic effect.

It is recognized that besides a role in endogenous defense of plants, human consumption of dietary antioxidants affords protection against some pathological events. In the past years, much attention has been devoted to ascorbic acid, tocopherol, tocotrienols, and *β*-carotene (Rice-Evans and Miller, 1995). It must also be admitted that free radical scavenging is generally the accepted mechanism for antioxidants inhibiting lipid oxidation (Brand-Williams et al., 1995).

Anthocyanins are well-known alternatives to synthetic dyes (Bridle and Timberlake, 1997). Moreover, as for flavonoids and related phenolics, both the antiradical and antioxidant activities of these compounds have been recently reported (Tsuda et al., 1994, Sauté-Gracia et al., 1997, Lapidot et al., 1999). These activities contribute to explaining the protective effect of vegetable-rich diets on coronary diseases (Hertog et al., 1993).

The number of articles published on the RSC of these natural colorants are scarce (Kaack and Austed, 1998; Cao et al., 1998) which prompted us to carry out the present work. For comparison, natural anthocyanin-based dyes (black chokeberry, elderberry, strawberry, and black-thorn), some commonly used by the food industry and others which colorant capability is still under study (García-Viguera, unpublished data), the synthetic colorant Ponceau 4R (E-124, $C_{20}H_{11}N_2O_{10}S_3Na$), the natural antioxidant α-tocopherol (vitamin E), and the synthetic antioxidants butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), were studied.

The aim of the present work was to characterize kinetically the free radical scavenger capacity of these natural colorants by using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) to consider them as an alternative source of both color and antioxidant capacity for food products.

MATERIALS AND METHODS

Samples. Commercial Ponceau 4R colorant was provided by Franmosan S. L. (Murcia, Spain). Commercial elderberry (*Sambucus nigra*, L.) concentrate was provided by Hero S.A. (Murcia, Spain). Freeze-dried black chokeberry fruits (*Aronia melanocarpa*, Elliot) cv. Viking, harvested in June 97, were kindly supplied by Dr. N. Jeppsson (SLU Balsgard, Sweden), and freeze-dried black-thorn (*Prunus spinosa*, L.), harvested in October 96, by Dr. O. Urrutia (University of Navarra, Spain). Strawberries (*Fragaria* × *ananassa*, Duch) cv. Oso Grande were harvested ripe, in July 95 from Lepe (Huelva, Spain).

Quercetin 3-rutinoside was supplied by Merck (Darmstad, Germany), cyanidin 3-rutinoside, chlorogenic acid, ellagic acid, DPPH•, ABTS, α-tocopherol, BHT, and BHA were supplied by Sigma (St. Louis, MO). All other reagents (citric acid, diethyl ether, formic acid, and methanol) were of analytical grade and were also supplied by Sigma. DPPH• had a molar absorptivity of 12 500 M^{-1} cm^{-1} at 515 nm (Brand-Williams et al., 1995).

Extracts Preparation. All the extracts were obtained on the year of harvested, and stored at 4 °C until analyzed. Frozen chokeberry, blackthorn and strawberry fruits were thawed, crushed, weighed, and extracted with 3% formic acid in methanol (1:4 w/v) for 24 h at 4 °C. The extraction procedure was repeated three times. The extracts were filtered through glass wool, the methanol was removed under reduced pressure, with a rotary evaporator (35 °C), and the residues redissolved in acidified water (3% formic acid). These aqueous solutions were adsorbed onto activated C_{18} Sep-Pak cartridge (Waters Associates, Milford, MA) for solid-phase extraction (SPE). The cartridges were washed with 3% formic acid, and the pigments

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eluted with 3% formic acid in methanol. The methanolic extracts were concentrated to dryness leaving a red residue. Diethyl ether was added to the red residue. Addition of diethyl ether transformed this red concentrate into a powder because of its insolubility in this solvent. Finally, the diethyl ether layer was removed and the precipitated powders, containing the anthocyanins, were dried under nitrogen, collected and kept at 4 °C.

The dried anthocyanin chokeberry, blackthorn, and strawberry extracts, Ponceau 4R, and commercial elderberry concentrate (2 mg each) were redissolved in a mixture of aqueous 5% formic acid containing 20% methanol (1 mL) for HPLC analysis.

HPLC Analysis of Anthocyanins. For identification and quantification of anthocyanins and other phenolics, the same method as in García-Viguera et al. (1997, 1998) was used. Each sample was analyzed on a liquid chromatograph Merck-Hitachi L-6200 intelligent pump equipped with a diode array detector Shimadzu SPD-M6A. Chromatograms were recorded and processed on a LC Workstation Class M10A Shimadzu PC based chromatographic data system.

A 20 μL sample was analyzed on a Lichrochart 100 RP-18 reversed-phase column (12.5 × 0.4 cm, particle size 5 μm) using a mobile phase 5% formic acid (v/v) (solvent A) and methanol (solvent B). Elution was performed at a flow rate of 1 mL/min using a gradient starting with 15% (B), increasing to 30% (B) at 15 min, isocratic elution at 30% (B) for 5 min, increasing to 50% (B) at 30 min, and 80% (B) at 33 min. Detection was achieved at 520, 360, and 280 nm, to detect anthocyanins, flavonols, and phenolic acids. All analyses were done in duplicate and results expressed as mean value. Repeatability of the HPLC analyses was ~5%.

Identification and Quantitation of Anthocyanins and Noncolored Phenolics. The different phenolics were characterized by chromatographic comparison with standards and quantified by absorbance of their corresponding peaks in the chromatograms. The anthocyanins were quantified as cyanidin 3-rutinoside (detected at 520 nm); phenolic acids, as chlorogenic acid (detected at 280 nm); and flavonols, as quercetin 3-rutinoside (detected at 360 nm), due to insufficient amount of the other standards.

The total levels of anthocyanins and phenolics were calculated by addition of the amounts detected in each chromatogram, as previously reported (Zafrilla et al., 1998).

Ascorbic Acid Determination. Ascorbic acid (AA) and dehydroascorbic acid (DHAA) contents were determined as described by Zapata and Dufour (1992). The HPLC analysis was achieved after derivatization of DHAA into the fluorophore 3-(1,2-dihydroxyethyl)furo[3,4-*b*]quinoxaline-1-one (DFQ), with 1,2-phenylenediamine dihydrochloride (OPDA). The samples (20 μL) were analyzed with a Merck-Hitachi (Tokyo, Japan) liquid chromatograph equipped with a L-4000 UV detector and a L-6000 pump. Separations of DFQ and AA were achieved on a Kromasil 100 C-18 column (250 × 4 mm; 5 μm particle size, Technokroma, Barcelona, Spain). The mobile phase was methanol−water (5:95 v/v) containing 5 mM cetrimide and 50 mM potassium dihydrogen phosphate at pH 4.5. The flow rate was 0.9 mL/min. The detector wavelength was initially set at 348 nm, and after DFQ elution, it was shifted to 261 nm for AA detection

Antiradical Activity Assay. The ABTS radical (ABTS$^\bullet$) was generated in the medium by using MnO_2 as described elsewhere (Miller and Rice-Evans, 1997). DPPH$^\bullet$ was dissolved in methanol (Brand-Williams et al., 1995). Experiments were always performed on freshly prepared solutions of both radicals, which in the absence of radical scavengers were stable for more than 1 day.

The different extracts were dissolved in methanol immediately before use. The presence of methanol was required to enhance the solubility of the extract to reach pseudo-first-order assay conditions: initial radical concentration ≪ initial natural colorant (or standard antioxidant) concentration, ([DPPH$^\bullet$ or ABTS$^\bullet$]$_0$ ≪ [(AH)$_n$]$_0$).

Specific antiradical activity assay conditions were as follows: DPPH$^\bullet$ 26 μM and strawberry extract (0.065−0.32) mg/mL; DPPH$^\bullet$ 42 μM and α-tocopherol (0.026−0.096) mg/mL; DPPH$^\bullet$ 38 μM and BHT (0.044−0.22) mg/mL; BHA (0.018−0.072) mg/mL; elderberry (0.38−1.15) mg/mL; black-thorn (0.01−0.04) mg/mL; and black chokeberry (0.004−0.012) mg/mL.

Kinetic Analysis. Graphs and fitting of the experimental data were carried out by using a Gauss−Newton algorithm (Marquardt, 1963) implemented in the Sigma Plot 2.01 program for Windows (Jandel Scientific, 1994). A total of 1000 data points were taken per spectrophotometric recording of the disappearance of DPPH$^\bullet$ in the presence of the natural colorants.

Second-order rate constants (k_2) were calculated to determine the RSC of the different natural colorants and standards assayed. DPPH$^\bullet$ was depleted from the medium under pseudo-first-order conditions, ([DPPH$^\bullet$]$_0$ ≪ [(AH)$_n$]$_0$), (Figure 1) following the equation:

$$[\mathrm{DPPH}^\bullet] = [\mathrm{DPPH}^\bullet]_0 \, e^{-k_{obsd}t} \quad (1)$$

where [DPPH$^\bullet$] is the radical concentration at any time, [DPPH$^\bullet$]$_0$ is the radical concentration at time zero and k_{obsd} the pseudo-first-order rate constant. This constant (k_{obsd}) was linearly dependent on the concentration of the natural colorant and antioxidant standards, and from the slope of this plot, the second-order rate (k_2) was determined (Mukai et al., 1993; Shi and Niki, 1998).

The mean of six separate determinations of k_2 (duplicate for three different weighs of each natural colorant extract or antioxidant standard) is shown. The initial estimations of [DPPH$^\bullet$]$_0$ and k_{obsd} which are necessary to fit the experimental data by nonlinear regression to eq 1, were obtained from the slope and ordinate on the origin of the straight line resulting from the fit by linear regression of the experimental data of [DPPH$^\bullet$] and t to

$$\ln [\mathrm{DPPH}^\bullet] = \ln [\mathrm{DPPH}^\bullet]_0 - k_{obsd}t \quad (2)$$

Spectrophotometric Assays. Kinetic assays were carried out by measuring the disappearance of DPPH$^\bullet$ at 515 nm. The spectrophotometric assays were recorded in an UV−vis Perkin-Elmer Lambda-2 spectrophotometer (Überlingen, Germany), on-line interfaced to a Pentium-100 microcomputer (Ede, The Netherlands). Temperature was controlled at 25 °C with a circulating bath with heater/cooler and checked using a precision of ±0.1 °C. The reference cuvette contained all the components except the radical with a final volume of 1 mL.

RESULTS AND DISCUSSION

Phenolic Composition of the Natural Colorants. All the extracts were characterized and quantified with respect to the anthocyanin composition. Also, some minor noncolored phenols were quantified.

Aronia extract (black-chokeberry) presented the highest anthocyanin concentration (623 μg/mg), characterized by the presence of cyanidin 3-galactoside (57%), cyanidin 3-arabinoside (29%), cyanidin 3-xyloside (7%), cyanidin 3-glycoside (4%), and cyanidin 3-glucoside (3%). Also caffeic acid derivatives (3.22 μg/mg) and quercetin derivatives (0.82 μg/mg) were found.

Black-thorn extract presented also a high total anthocyanin concentration (151 μg/mg), represented by cyanidin 3-glucoside (39%), cyanidin 3-rutinoside (21%), peonidin 3-glucoside (19%), and peonidin 3-rutinoside (21%). Also a caffeic acid derivative was detected in high proportion (12.27 μg/mg), as well as a small amount of flavonols (0.98 μg/mg).

In the strawberry extract, a smaller amount of anthocyanin was found (54.8 μg/mg), represented by pelargonidin 3-glucoside (82%), pelargonidin 3-rutinoside (9%), and cyanidin 3-glucoside (9%). When analyzing the

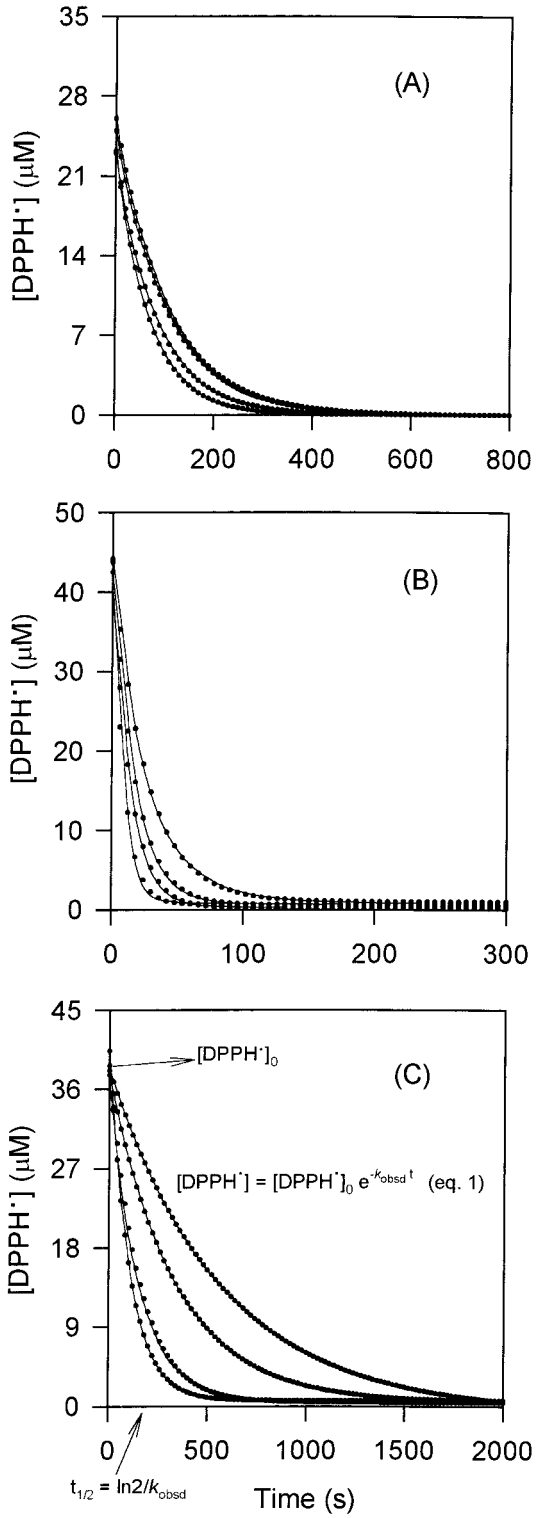


Figure 1. Spectrophotometric recordings of the disappearance of DPPH• in the presence of increasing concentrations of (A) strawberry extract, (B) α-tocopherol, and (C) BHT. Lines: (—) experimental data and (···) nonlinear regression fitting of experimental data to eq 1. Conditions were as detailed in the Antiradical Activity Assay section for (A) strawberry extract; (B) α-tocopherol, and (C) BHT.

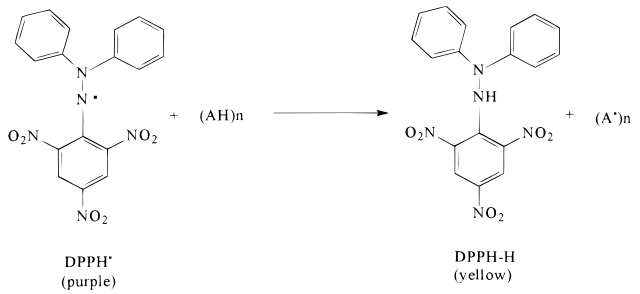
noncolored phenols, caffeic acid derived compounds (1.25 μg/mg), ellagic acid (0.26 μg/mg) and quercetin- and kaempferol-derived flavonols (0.55 μg/mg) were also detected.

The commercial elderberry concentrate had the smallest anthocyanin concentration (5 μg/mg). The predominant anthocyanins were cyanidin 3-monoglycoside (97%) and cyanidin 3,5-diglycoside (3%), in accordance with other authors previous findings for fresh fruit (Inami et al., 1996). This concentrate was also characterized by the presence of a caffeic acid derivative (0.11 μg/mg) and the presence of rutin (0.55 μg/mg).

All the extracts contained other noncolored phenols in trace amounts (which represented all together, less than 0.1% of the total weight of the extract).

No ascorbic acid was detected in any of the extracts by following the method detailed in the Materials and Methods section.

Scheme 1. Scavenging of Free DPPH• by Antiradical (Free Radical Scavenger) Species (Yamaguchi et al., 1998)[a]



[a] Abbreviations: DPPH-H, reduced 2,2-diphenyl-1-picrylhydrazyl radical; $(AH)_n$, antiradical species (the subscript "*n*" indicates the presence of one, $n = 1$ or various, $n > 1$ radical species).

Scavenging Effect on DPPH•. The model of scavenging stable DPPH• is a widely used method to evaluate the RSC in a relatively short time compared to other methods (Brand-Williams et al., 1995; Sánchez-Moreno et al., 1998). Free radical scavenging is the generally accepted mechanism for antioxidants inhibiting lipid oxidation.

Under pseudo-first-order assay conditions, in the presence of both the natural colorants and standard antioxidants used here, ABTS• disappeared from the medium so fast that stopped-flow equipment should be used. However, the scavenging of DPPH• by the different radical scavengers in these assay conditions was slower, allowing characterization by conventional spectrophotometry. Therefore, DPPH• was used throughout this study.

The scavenging of DPPH• by radical scanvengers can be represented as depicted in Scheme 1 (Yamaguchi et al., 1998) which can be summarized as

$$\mathrm{DPPH}^{\bullet} + (\mathrm{AH})_n \rightarrow \mathrm{DPPH{-}H} + (\mathrm{A}^{\bullet})_n \qquad (3)$$

The newly formed radical (A•) can mainly follow radical−radical interaction to render stable molecules via radical disproportionation (collision of radicals with abstraction of an atom by one radical to another, DPPH• + A• → DPPH-A; A• + A• → A−A) although these secondary reactions are greatly hindered (Chimi et al., 1991; Aruoma, 1998).

Taking into account that we are studying partially purified extracts of natural colorants, the subscript "*n*" represents the existence of several possible radical scavenger species in these extracts.

Absorbance decreases as a result of a color change from purple to yellow as the radical is scavenged by antiradicals through donation of hydrogen to form the reduced form DPPH−H (eq 3).

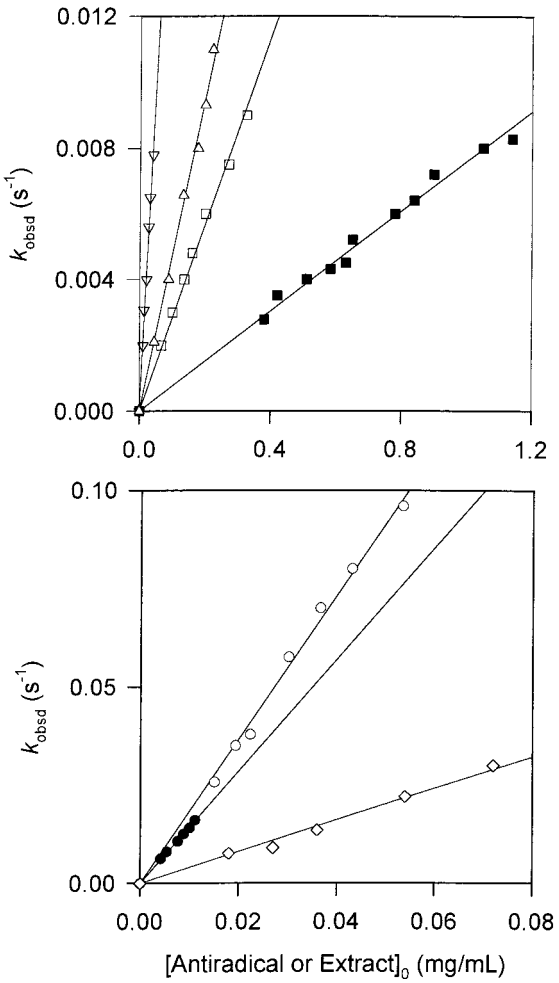


Figure 2. Dependence of the pseudo-first-order rate constant (k_{obsd}) on the concentration of antiradical standards or colorant extracts: (○) α-tocopherol, (●) black chokeberry, (◊) BHA, (▽) black-thorn, (△) BHT; (□) strawberry, and (■) Elderberry. The solid line (—) shows the linear regression fitting of the experimental data.

Radical Scavenger Capacity (RSC) of Natural Colorants. In the presence of the natural colorants, a decrease in the absorbance at 515 nm was measured until the radical was depleted under pseudo-first-order assay conditions ($[DPPH^{\bullet}]_0 \ll [(AH)_n]_0$). Therefore, taking into account eq 3:

$$-\frac{d[DPPH^{\bullet}]}{dt} = k_{obsd}\,[DPPH^{\bullet}] = k_2\,[(AH)_n][DPPH^{\bullet}] \quad (4)$$

The pseudo-first-order rate constant, k_{obsd} was linearly dependent on initial radical scavenger (natural colorant) concentration ($[AH]_0$) (Figure 2). From these plots, the second-order rate constants k_2 were calculated in the scavenging reaction of the different natural colorants studied here. This rate constant is related to the RSC present in the colorant extracts.

The RSC of natural colorants was compared to that of other synthetic (BHA and BHT) and natural (α-tocopherol) antioxidants used in the food industry. α-Tocopherol (vitamin E) is a natural antioxidant abundant in oils and other foodstuffs with high fat content such as butter, margarine, etc. (Shahidi and Wanasundara, 1992). Vitamin E was the best antioxidant assayed with a k_2 value of 1.87 $(mg/mL)^{-1}$ s^{-1} (Table 1). The order of RSC, according to k_2 values (the higher k_2 value, the better RSC) was α-tocopherol > black chokeberry > BHA > black-thorn > BHT > strawberry > elderberry, (Table 1).

Table 1. Second-Order Rate Constants (k_2) for the Reactions between DPPH$^{\bullet}$ and Antiradical Standards or Colorant Extracts[a]

antiradical standard or colorant extract	k_2, $(mg/mL)^{-1}$ s^{-1}
vitamin E (α-tocopherol)	1.87 ± 0.10
chokeberry (*Aronia melanocarpa*, Elliot)	0.70 ± 0.05
BHA (butylated hydroxyanisole)	0.42 ± 0.02
black-thorn (*Prunus spinosa* L.)	0.20 ± 0.01
BHT (butylated hydroxytoluene)	0.05 ± 0.003
strawberry (*Fragaria* × *ananassa*, Duch)	0.03 ± 0.002
elderberry (*Sambucus nigra* L.)	0.008 ± 0.0004
Ponceau 4R	

[a] Conditions were as detailed in the Antiradical Activity Assay section.

The natural colorants used here presented k_2 values (in our assay conditions) which ranged from 0.008 to 0.7 $(mg/mL)^{-1}$ s^{-1} (Table 1). The black-chokeberry extract showed the highest RSC among the natural colorants with a k_2 value of 0.7 $(mg/mL)^{-1}$ s^{-1}, higher than the RSC of both BHT and BHA (synthetic antioxidants without colorant properties; Shahidi and Wanasundara, 1992). The extracts from black-thorn showed RSC higher than BHT (Table 1).

Phenolic Content vs RSC. In the present study ascorbic acid was removed in the SPE, together with the sugars. Thus, it seems that the RSC is due to the total anthocyanin concentration (TAC) of the extracts, as there is a good correlation between these two factors, according to the following relationship:

$$RSC = 0.0011\ TAC - 0.0019 \qquad R^2 = 0.994$$

Nevertheless, some possible synergistic effects with the other compounds present in the extracts, such as caffeic acid derivatives, ellagic acid, or flavonols, cannot be excluded.

From our results we can conclude that the synthetic colorant "Ponceau 4R" showed no RSC. Therefore, the natural extracts assayed here combined the usefulness as potential colorants and as antiradicals. This could be an interesting opportunity for the food industry to solve two problems at the same time: color and oxidative stability of foodstuffs.

ABBREVIATIONS USED

AH, (antiradical = radical scavenger) or antioxidant; $[AH]_0$, initial antiradical or antioxidant concentration; BHA, butylated hydroxyanisole or 2[3]-*tert*-butyl-4-hydroxyanisole; BHT, butylated hydroxytoluene or 2,[6]-di-*tert*-butyl-*p*-cresol; DPPH$^{\bullet}$, 2,2-diphenyl-1-picrylhydrazyl radical; $[DPPH^{\bullet}]_0$, initial DPPH$^{\bullet}$ concentration; k_{obsd}, pseudo-first-order rate constant; k_2, second-order rate constant; RSC, radical scavenger capacity; TAC, total anthocyanin concentration.

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