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Aronia-Enriched Lemon Juice: A New Highly Antioxidant Beverage

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Lemon juice (LJ) was enriched with aronia concentrate (AC) in two different proportions (2.5 and 5%, v/v) to design new beverages rich in bioactive ingredients. The phytochemical composition (anthocyanins, flavanones, flavones, flavonols, and hydroxycinnamic acids) and stability of the beverages were analyzed by high-performance liquid chromatography with a diode array detector (HPLC−DAD), as well as color alterations and *in vitro* antioxidant activity (DPPH$^{\bullet}$ assay). Results showed that, although anthocyanin degradation was higher than 90% after 60 days of storage, the new beverages retained an attractive red color. Also, the *in vitro* antioxidant activity of the new mixtures was 2-fold higher when 5% AC was added compared to pure LJ. Thus, an addition of only 5% AC could effectively increase the antioxidant properties of LJ, as well as improving certain organoleptic characteristics, rendering an interesting beverage in the growing market of food for health.

KEYWORDS: ***Citrus limon*; *Aronia melanocarpa*; beverage; antioxidant; health; DPPH$^{\bullet}$; flavonoids; storage**

INTRODUCTION

Fruits and vegetables are very important items of a balanced diet, particularly for their role in prevention of diseases, including certain types of cancer, mainly because of their flavonoid content (*1*). Flavonoids are important phytonutrients because of their antioxidative, anti-inflammatory, antiallergic, antiviral, antiproliferative (*2*), antimutagenic, cardiovascular (*3*), and anticarcinogenic activities (*4*).

Lemons (*Citrus limon* (L.) Burm. f.) are a rich source of nutrients, including ascorbic acid (vitamin C), citric acid, minerals, and flavonoids (*5*), which provide many health-related properties (*2, 6*). With its high flavor and nutritive value, it is little wonder that lemon juice is successful in non-alcoholic beverages.

Chokeberry (*Aronia melanocarpa* (Michx.) Elliott; family Rosaceae) is also a natural source of phenolic antioxidants, because of its high concentration (even over 1 g/100 g fw) in cyanidin 3- glycoside anthocyanins (Cy 3-glucoside, Cy 3-galactoside, Cy 3-arabinoside, and Cy 3-xyloside) (*7*−*11*), hydroxycinnamic acids (neochlorogenic and chlorogenic acids) (*12*), quercetin derivatives (quercetin 3-galactoside, 3-glucoside, and 3-rutinoside) (*8*), as well as vitamin C (*13*).

Nowadays, there is a trend and worldwide pursuit of designing new functional foods and healthy food products. In this sense, a design of new beverages combining lemon juice with aronia concentrate, a natural colorant, was intended on the basis of the acidic pH of the lemon juice that could naturally stabilize the anthocyanins of the chokeberry. Anthocyanins are responsible for the red color of many berries and numerous other fruits (*14*) with health-promoting properties (*4, 11, 15, 16*). Nevertheless, stability of the red color is a big problem in food processing (*17*) because the color stabilization depends upon the structure and concentration of the pigment, pH, temperature, presence of co-pigments, ascorbic acid, metallic ions, etc. (*18, 19*). However, there is evidence of the influence of anthocyanins on the color stability in different foods and food products (*20*−*22*).

Besides, the highly antioxidant and rich composition in bioactive compounds of the resulting beverage could provide an interesting healthy product. The aim of this work was to evaluate the stability of the bioactive compounds present in the new beverages/mixtures, as well as their antioxidant activity, as a suitable assay for a health-promoting activity of the food products (*23*−*25*).

MATERIALS AND METHODS

Material. *Lemon Juice (LJ).* Lemons were collected from the CEBAS−CSIC's Experimental Farm ("La Matanza", Santomera, Murcia, SE Spain) of "Fino" clones harvested in December 2005. Freshly prepared lemon juice was obtained using a domestic squeezer ("Citromatic", Braun Española S.A., Barcelona, Spain).

Aronia Concentrate (AC). Commercial frozen aronia concentrate (62.3 °Brix; pH 3.58) was provided by "Juver Alimentación S.A." (Churra, Murcia, Spain) and thawed at 5 °C, previous to beverage preparation.

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Standards and Reagents. Rutin (quercetin 3-β-D-rutinoside) (Fluka AG, Switzerland), cyanidin 3-glucoside (Polyphenols, Norway), chlorogenic acid (Sigma, Steinheim, Germany), hesperidin (Merck, Darmstadt, Germany), diosmin (Genay, France), 2,2-diphenyl-1-picryl-hydrazyl (DPPH$^{\bullet}$) (Sigma, Steinheim, Germany), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Fluka Chemika, Neu-Ulm, Switzerland), and gallic acid (Doesder. Chem. Co., Barcelona, Spain) were obtained. Other reagents were Folin−Ciocalteau's reagent (Sigma, Steinheim, Germany), sodium carbonate anhydrous (Panreac Quimica S.A., Barcelona, Spain), potassium dihydrogen phosphate (Panreac Quimica S.A., Barcelona, Spain), citric acid (Sigma, Steinheim, Germany), benzoic acid (Sigma, St. Louis, MO), dimethylsufoxide (Merck, Darmstadt, Germany), formic acid and methanol, which were of analytical grade (Merck, Darmstadt, Germany), ascorbic acid and dehydroascorbic acid, both from Sigma-Aldrich (Steinheim, Germany), and 1,2-phenyilenediamine dihydrochloride (OPDA) (Fluka Chemika, Neu-Ulm, Switzerland). Milli-Q water used was produced using a Elix 3 Millipore water purification system (Molsheim, France).

Beverage Preparation. Aronia concentrate (as used industrially) was added to a volume of fresh lemon juice to obtain a final concentration of 2.5 or 5% (v/v) of aronia concentrate in the beverage.

To study the behavior of the aronia phytochemicals, blank solutions were prepared in a 0.18 M citric acid buffer (pH 2.46), with the same aronia concentrate proportions as before. Finally, lemon juice was also studied as a control (LJC).

Benzoic acid was added (100 mg L^{-1}) (*26*) to all of the samples to prevent spoilage.

The samples were shacken and centrifuged at 2894*g* for 10 min at 4 °C. Juices and blanks (25 mL) were stored in transparent glass vials (60 × 30 mm Ø; 28 mL volume) with plastic screw caps, after removal of air under N_2 atmosphere, and stored at room temperature in the dark for 60 days. Triplicate solutions were prepared for each experiment, and all of the analytical measurements were performed in triplicate, with the mean values reported in the data. Analyses were performed every 7 days during the first 21 days and every 15 days during the rest of the experiment.

The samples were labeled as follow: LJC (lemon juice control), LJ-AC5% (lemon juice with 5% aronia concentrate addition), LJ-AC2.5% (lemon juice with 2.5% aronia concentrate addition), AC5% (blank solution containing 5% aronia in citric acid buffer), and AC2.5% (blank solution containing 2.5% aronia in citric acid buffer).

Quality Parameters. Titratable acidity (TA), pH, and total soluble solid (TSS) contents were evaluated as quality indexes. The TA was determined by titrating 2 mL of mixture (60 mL final volume with Milli-Q water) with 0.1 N NaOH (pH 8.1). Results were expressed as grams of citric acid per 100 mL of sample, in accordance to the Association of Official Analytical Chemists (AOAC) (*27*). The pH values were measured using a pH-meter (GLP 21, Crison Ltd., Barcelona, Spain). The TSS contents were recorded in a refractometer (Abbe WYA-S, Optic Ivymen System, Barcelona, Spain) at 20 °C, with values being expressed as °Brix. Triplicate solutions were prepared for each experiment, and all of the analytical measurements were performed in triplicate. All analyses were performed every 7 days during the first 21 days and every 15 days during the rest of the experiment.

High-Performance Liquid Chromatography (HPLC) Analysis of Vitamin C. Ascorbic acid (AA) and dehydroascorbic acid (DHAA) contents were determined as described in ref *5*. Briefly, the juice samples were centrifugated at 10500*g* (model Sigma 1-13, B. Braun Biotech International, Osterode, Germany) for 5 min at 4 °C. The supernatant was filtered through a 0.45 μm filter (Millex HV13, Milipore, Bedford, MA), and then phenolic compounds in this aqueous solution were absorbed onto a C_{18} Sep-Pak cartridge (Waters Associates, Milford, MA). Then, 250 μL of 1,2-phenilenediamine dihydrochloride (OPDA) solution (18.8 mM) was added to 750 μL of extract for DHAA derivatization into the fluorophore 3-(1,2-dihydroxietyl)furo[3,4-b]quinoxaline-1-one (DFQ). After 37 min in darkness, the samples were analyzed by HPLC. AA and DHAA was evaluated using a HPLC system (Merck-Hitachi, Tokyo, Japan) equipped with an isocratic L-6000 pump, injection valve, and sample loop 20 μL (Rheodyne, Rohnert Park, CA) and coupled to a L-4000 UV detector. Samples were analyzed on a Lichrospher 100 RP-18 reversed-phase column (250 × 4 mm, particle size of 5 μm) (Teknokroma, Barcelona, Spain) with a C_{18} precolumn (Teknokroma, Barcelona, Spain). The mobile phase was MeOH/H_2O (5:95, v/v), 5 mM cetrimide, and 50 mM KH_2PO_4 (pH 4.59). The flow rate was kept at 0.9 mL min^{-1}. The detector wavelength was initially set at 348 nm, and after DFQ eluted, it was manually shifted to 261 nm, for AA detection. L-AA and L-DHAA were identified and quantified by a comparison to pattern areas from L-AA and L-DHAA. The vitamin content was calculated by adding AA and DHAA contents, and results are expressed as milligrams per 100 mL. All analyses were performed in triplicate, and the mean values are reported in the data.

Qualitative and Quantitative Analysis of Phenolic Compounds by HPLC. All samples were centrifuged, 5 min at 10500*g* (model Sigma 1-13, B. Braun Biotech International, Osterode, Germany), at 4 °C. The supernatant (soluble fraction) was filtered through a 0.45 μm filter (Millex HV13, Millipore, Bedford, MA) prior to injection into the HPLC.

For identification and quantification of phenolic compounds, the method previously reported by Pérez-Vicente et al. (*28*) was followed. Each sample was analyzed on a Merck-Hitachi L6200 liquid chromatograph (Tokyo, Japan) equipped with a diode array detector (DAD) UV−vis Shimadzu SPD-M6A (Kyoto, Japan) and an autoinjector (Gilson International, model 234, Barcelona, Spain). Chromatograms were recorded and processed on a LC Workstation Class M10A Shimadzu PC-based chromatography data system.

A 20 μL sample was analyzed on a Lichrocart RP-18 reversed-phase column (250 × 4 mm, particle size of 5 μm) with a precolumn C_{18} (Lichrocart 4-4, Lichrospher 100 RP-18-5 μm) from Merck (Darmstadt, Germany), using mobile-phase 5% formic acid (v/v) (solvent A) and HPLC-grade methanol (solvent B) (Merck, Darmstadt, Germany). Elution was performed at a flow rate of 1 mL min^{-1} using a gradient starting with 15% B, increasing to 30% B at 15 min, isocratic elution at 30% B for 5 min, and increasing to 50% B at 30 min. Chromatograms were recorded at 280, 360, and 520 nm.

The different phenolics were characterized by chromatographic comparison to analytical standards and quantified by absorbance of their corresponding peaks in the chromatograms. The flavanones were quantified as hesperidin (detected at 280 nm); flavones were quantified as diosmin (at 360 nm); phenolic acids were quantified as chlorogenic acid (at 360 nm); quercetin derivatives were quantified as rutin (at 360 nm); and anthocyanins were quantified as cyanidin 3-glucoside (at 520 nm) (**Figure 1**).

Total Phenolic Compound Analysis. Total phenol (TP) content was determined with the Folin−Ciocalteu method, adapted to a microscale by Arnous et al. (*29*). In a 1.5 mL Eppendorf microtube, 790 μL of Milli-Q water, 10 μL of sample appropriately diluted with MeOH, and 50 μL of Folin−Ciocalteu reagent were added and vortexed. After exactly 1 min, 150 μL of aqueous 20% sodium carbonate (Merck, Darmstadt, Germany) were added, vortexed again, and allowed to stand at room temperature in the dark for 120 min. The absorbance was recorded at 750 nm and quantified using gallic acid as a standard. Results were expressed as milligrams per 100 mL of gallic acid equivalents (GAE).

Color Measurements. Solutions were measured in glass cells of 2 mm path length (CT-A22) at 520 nm using a Minolta CM-508i tristimulus color spectrophotometer (Osaka, Japan) coupled with a CM-A760 transmittance adaptor. The values of CIEL^*, CIEa^*, and CIEb^*, were calculated using illuminant D65 and a 10° observer according to the CIE$L^*a^*b^*$ 76 convention. Data were recorded and processed using the Minolta Software Chromacontrol S, PC-based colorimetric data system (*28*).

***In Vitro* Antioxidant Activity Evaluation.** All samples were centrifuged at 10500*g* (Sigma 1-13, B. Braun Biotech International, Osterode, Germany) for 5 min at 4 °C. The free-radical-scavenging activity was determined using the free-radical DPPH$^{\bullet}$ (Sigma, Steinheim, Germany) according to Llorach et al. (*30*). It was evaluated by measuring the variation in absorbance at 515 nm after 50 min of reaction in parafilm-sealed glass cuvettes (to avoid methanol evaporation) at 25 °C. All reactions started by adding 5 μL of the corresponding diluted sample and 45 μL of MeOH to the cuvette containing the diluted stock

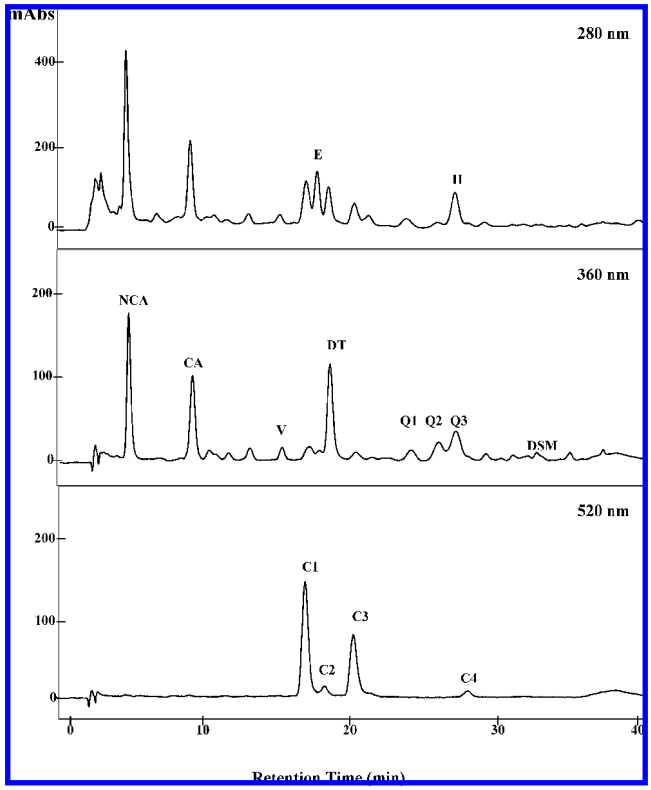

Figure 1. Chromatograms of the new mixtures based on lemon juice and aronia concentrate registered at 280, 360, and 520 nm. At 280 nm, E, eriocitrin; and H, hesperidin. At 360 nm, NCA, neochlorogenic acid; CA, chlorogenic acid; V, vicenin-2; Q1, quercetin 3-galactoside, Q2, quercetin 3-glucoside, Q3, quercetin 3-rutinoside; Dt, diosmetin 6,8-diglc; and Dsm, diosmin. At 520 nm, C1, cyanidin 3-galactoside; C2, cyanidin 3-glucoside; C3, cyanidin 3-arabinoside; and C4, cyanidin 3-xyloside.

solution up to absorbance 1 (950 μL, from 0.094 mM free-radical DPPH$^{\bullet}$). The final volume of the assay was 1 mL. The reaction was followed with a spectrophotometer (UV-1603 Shimadzu, Tokyo, Japan). Results were expressed as millimolar Trolox.

Statistical Analyses. All data were subjected to analyses of variance (ANOVA) using the MS-DOS version of STATGRAPHICS version 7.0 program. The data shown are mean values ($n = 3$), and the significance of the differences was compared using a multiple range test [least significance difference (LSD)] at $p < 0.05$ probability level (Duncan's test).

RESULTS AND DISCUSSION

Quality Parameters. It is remarkable that the pH, TA, and TSS content of the mixtures were unaffected over the storage period as previously reported in plant-based food products (i.e., jams) (*21*). In this section, the results are presented as average values for each beverage/mixture for the whole experiment (**Table 1**).

The pH values were slightly higher in the mixtures elaborated with AC than in those with LJ alone. TSS values ranged from 2.23 to 11.33 °Brix for AC2.5% and LJ-AC5%, respectively. The TSS content of the new beverages was in accordance with the range established by the Spanish National Regulations for commercial drinks/beverages (**Table 1**) (*31*).

Vitamin C Content. The vitamin C content (as the sum of AA and DHAA) of the mixtures was provided by LJ. No vitamin C was detected in AC, probably because of the thermal treatment used for concentration. Thus, the vitamin C analyses were only carried out in mixtures containing LJ. Vitamin C was the same ± 30 mg 100 mL^{-1} at time zero of storage in LJC, LJ-AC2.5%, and LJ-AC5%. When analyzing the degradation rate, results showed that, after the 7 days of storage, vitamin C loss was slightly higher in LJC (50%) than in the new beverages containing LJ and AC (40%) (**Figure 2**). Nevertheless, after 14 days, the vitamin C content was negligible in all mixtures, as also found by other authors (*26, 28*).

Table 1. Average Quality Indexes through the Storage Period in the Different Mixtures[a]

mixtures	pH	TA[b]	TSS[c]
LJ-AC5%	2.47 ± 0.07 c	5.81 ± 0.76 a	11.33 ± 1.34 a
LJ-AC2.5%	2.41 ± 0.04 d	5.95 ± 0.10 a	9.99 ± 0.06 b
AC5%	3.00 ± 0.03 a	0.63 ± 0.01 b	4.45 ± 0.12 d
AC2.5%	2.84 ± 0.05 b	0.50 ± 0.00 b	2.23 ± 0.10 e
LJC	2.37 ± 0.04 e	5.83 ± 0.03 a	8.27 ± 0.07 c

[a] Means in the same column followed by different letters are significantly different at $p < 0.05$ according to Duncan's test. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). [b] Titratable acidity (TA) is expressed as grams of citric acid per 100 mL of juice. Values are mean ± standard deviation ($n = 21$). [c] TSS is expressed as °Brix (20 °C).

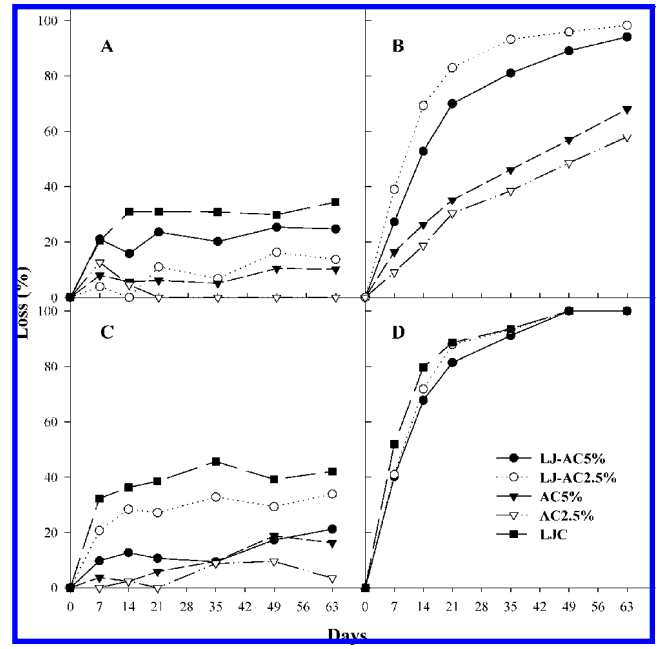

Figure 2. Percentage loss of (A) total phenolic compounds, (B) total anthocyanins, (C) antioxidant activity, and (D) vitamin C in beverages stored at 25 °C in the dark. LJ-AC5%, lemon juice containing 5% aronia; LJ-AC2.5%, lemon juice containing 2.5% aronia; AC5%, 5% of aronia concentrate; AC2.5% aronia concentrate; and LJC, lemon juice control.

Stability of Phenolic Compounds. LJ provided flavanones and flavones. On the other hand, the AC showed four monomeric anthocyanins with decreasing content as follows: cy 3-gal > cy 3-arab > cy 3-glc ~ cy 3-xyl, as well as additional noncolored phenolics (chlorogenic and neochlorogenic acids and quercetin derivatives). No changes were observed in the qualitative anthocyanin patterns through the manufacture of the mixtures (**Figure 1**). The initial composition of the mixtures is shown in **Table 2**, in accordance with the data reported for lemon juice (*5*) and aronia concentrate (*8*).

The degradation rate of all monomeric anthocyanins followed the same pattern. Thus, the degradation of total anthocyanin (the sum of individual anthocyanins with initial values ca. 26 mg/100 mL for those samples containing 5% AC and 13 mg/100 mL for those with 2.5% AC) was a reflection of all of the individual ones, ranging in values from 55 to 65% loss in aronia control samples (AC5% and AC2.5%, respectively) after 63 days of storage (**Figure 2**). Greater losses (over 70%) were reached

Table 2. Initial Values of Flavanones, Flavones, Anthocyanins, Hydroxycinnamic Acids, and Quercetin Derivatives of the Different Mixtures through the Storage Period at 25 °C in the Dark[a]

		LJC	AC2.5%	AC5%	LJ-AC2.5%	LJ-AC5%
flavanones						
	eriocitrin	9.73 ± 0.07			10.44 ± 0.10	11.45 ± 0.08
	hesperidin	11.80 ± 0.11			12.17 ± 1.20	13.54 ± 0.14
flavones						
	vicenin-2	0.63 ± 0.01			0.62 ± 0.03	0.59 ± 0.02
	Dt[b]	6.48 ± 0.22			7.13 ± 0.32	7.75 ± 0.19
	diosmin	<0.25			<0.25	<0.25
anthocyanins						
	Cy 3-gal[c]		7.86 ± 0.10	15.50 ± 0.13	7.43 ± 0.30	15.08 ± 0.10
	Cy 3-glc[d]		0.64 ± 0.01	1.27 ± 0.10	0.83 ± 0.33	1.31 ± 0.02
	Cy 3-arab[e]		4.63 ± 0.04	9.37 ± 0.47	4.38 ± 0.06	9.19 ± 0.20
	Cy 3-xyl[f]		0.62 ± 0.07	1.07 ± 0.03	0.51 ± 0.03	1.11 ± 0.03
	TAnth[g]		13.75 ± 0.22	27.21 ± 0.73	13.16 ± 0.72	26.70 ± 0.35
hydroxycinnamic acids						
	NCA[h]		9.67 ± 0.92	19.69 ± 0.92	9.84 ± 0.08	19.72 ± 0.17
	CA[i]		7.28 ± 0.08	14.87 ± 0.33	7.35 ± 0.17	14.69 ± 0.13
quercetin derivatives						
	∑quercetins[j]		1.47 ± 0.16	3.23 ± 0.16	2.61 ± 0.05	4.31 ± 0.04

[a] Values are mean ± standard deviation ($n = 3$), expressed as mg 100 mL^{-1} juice at time zero. [b] Dt = diosmetin 6,8 diglucoside. [c] Cy 3-gal = cyanidin 3-galactoside. [d] Cy 3-glc = cyanidin 3-glucoside. [e] Cy 3-arab = cyanidin 3-arabinoside. [f] Cy 3-xyl = cyanidin 3-xyloside. [g] TAnth = total anthocyanins. [h] NCA = neochlorogenic acid. [i] CA = chlorogenic acid. [j] Quercetins were quantificated as the sum of quercetin 3-galactoside, 3-glucoside, and 3-rutinoside.

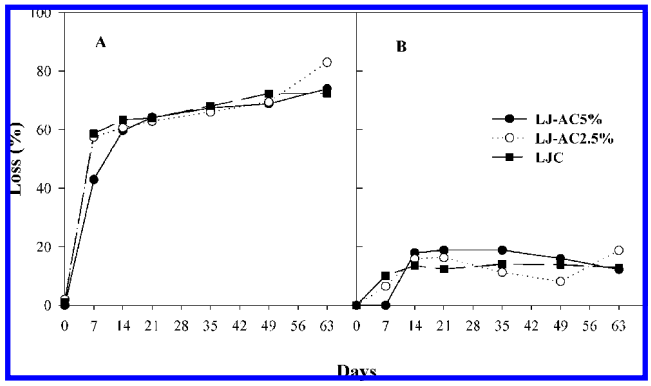

Figure 3. Percentage loss of (A) hesperidin and (B) eriocitrin content in beverages stored at 25 °C in the dark. LJ-AC5%, lemon juice containing 5% aronia; LJ-AC2.5%, lemon juice containing 2.5% aronia; and LJC, lemon juice control.

in LJ-AC beverages in only 21 days and even 90%, after 49 days. This could be explained by a decrease in the vitamin C content of the LJ until the total degradation registered in the first 14 days. Then, the vitamin C content was negligible, and the anthocyanin degradation was slowed down, as previously reported (*18, 26*). Moreover, it is also well-known that anthocyanins may react with ascorbic acid, resulting in the degradation of both components (*19*).

With regard to the losses over time for the hydroxycinnamic acids (initial values in **Table 2**), these were always lower than 15%. Moreover, the high stability of quercetin derivatives (±1%) in the solutions during storage (initial values in **Table 2**) is also remarkable (*21*).

Lemon flavones, vicenin-2 ($p < 0.001$) and diosmetin 6,8-diglc ($p < 0.001$), did not suffer significant reductions through the storage after the addition of AC, with losses smaller than 7 and 5%, respectively (initial values in **Table 2**). Diosmin flavone was only detected as traces. On the other hand, hesperidin content, after the first 7 days of storage, decreased by 50% in all of the mixtures, with slight losses by the end of the experiment (**Figure 3**), while the flavanone eriocitrin ($p < 0.001$) presented only a 18% loss (initial values in **Table 2**). Similar trends were also previously found in orange juices in the first 24 h of storage at 4 °C, revealing that hesperidin, rather insoluble in acidic water solutions, tends to form white crystals that precipitate, explaining the large variability reported for its content in citrus juices (10−80 mg 100 mL^{-1}) (*32*).

Changes in Color Quality during Beverage Storage. A general increase in color lightness (CIEL^* value) was detected, yielding bright colored solutions, probably resulting from the precipitation of different compounds (hesperidin, polymers, etc.) during the storage period.

Alteration in redness (CIEa^* value) was also observed, during the storage period, showing a general decrease for the new beverages, which was lower for those containing 2.5% aronia (ca. 80% loss) than for those containing 5% aronia (ca. 45% loss), while less than 15% loss was detected for the aronia control solutions. Finally, yellowness (CIEb^* value) showed a general decrease for all of the samples, including LJC, except for AC2.5%, which remained unaltered. These results produced a parallel decrease in Croma, showing a lower saturation for those mixtures containing LJ (**Table 3**).

When these results are compared to the total anthocyanin concentration, it can be observed that a decrease in pigments, during storage period, is highly correlated with a decrease in Croma ($R^2 \geq 0.900$, $p < 0.001$) and CIEa^* ($R^2 \geq 0.700$) for all mixtures (**Table 4**), confirming that the red color in these mixtures was related to their anthocyanin content. However, the rate of browning, determined by hue angle values (less than 20° differences between initial and end values, for all of the mixtures), was much slower than the rate of anthocyanin degradation, in agreement with previous findings in other fruit products (*21, 26*). This could be explained on the basis of the increased concentration of polymeric pigments over time, as demonstrated in fruit juices and red wines (*33*).

Nevertheless, changes in the color of the new beverages were impossible to evaluate by the human eye.

Total Phenolics. The total phenolics in the mixtures decreased as follow: LJ-AC5% > AC5% > LJ-AC2.5% > AC2.5% > LJ ($p < 0.001$), with initial values of 242, 176, 135, 96, and 78 mg of gallic acid 100 mL^{-1}, respectively (**Figure 2A**). Losses were lower than 40% in all mixtures, especially in the 7 first days, and higher for LJC and LJ-AC5% (50%), at least in part, because of hesperidin precipitation. The initial values of total phenolics in LJ-AC mixtures were

Table 3. Stability of CIE$L^*a^*b^*$ Values in the Prepared Mixtures through the Storage Period[a]

	days	LJ-AC5%	LJ-AC2.5%	AC5%	AC2.5%	LJC
CIEL^*	0	34.46 ± 0.23 d	43.25 ± 0.38 c	43.10 ± 0.25 c	59.12 ± 0.15 b	78.82 ± 0.07 a
	7	46.15 ± 0.04 d	51.78 ± 0.50 c	44.13 ± 1.02 e	60.42 ± 0.23 b	82.71 ± 0.13 a
	14	47.00 ± 0.18 c	61.13 ± 0.03 b	45.79 ± 1.07 d	61.13 ± 0.03 b	84.11 ± 1.40 a
	21	51.74 ± 0.75 d	70.53 ± 0.40 b	47.55 ± 0.09 e	61.11 ± 0.16 c	86.18 ± 0.72 a
	35	57.49 ± 0.83 d	75.95 ± 0.24 b	48.87 ± 0.28 e	62.25 ± 0.78 c	88.55 ± 0.70 a
	49	61.42 ± 0.33 d	78.83 ± 0.23 b	50.4 ± 0.30 e	65.17 ± 0.96 c	90.72 ± 0.33 a
	63	65.10 ± 0.35 c	80.56 ± 0.34 b	53.83 ± 1.20 d	66.18 ± 0.30	89.97 ± 1.52 a
CIEa^*	0	60.36 ± 0.13 b	59.21 ± 0.28 c	63.78 ± 0.24 a	56.83 ± 0.14 d	1.22 ± 0.04 e
	7	67.17 ± 0.01 a	58.27 ± 0.31 c	62.96 ± 0.32 b	53.72 ± 0.24 d	0.62 ± 0.04 e
	14	67.15 ± 0.09 a	52.96 ± 0.08 c	63.98 ± 0.12 b	52.96 ± 0.08 c	0.38 ± 0.23 d
	21	63.39 ± 0.97 a	30.29 ± 1.91 c	62.88 ± 0.01 a	52.61 ± 0.52 b	−0.18 ± 0.08 d
	35	51.58 ± 1.86 b	18.24 ± 0.58 d	62.93 ± 0.13 a	49.30 ± 1.39 c	−0.45 ± 0.12 e
	49	42.31 ± 0.94 c	14.15 ± 0.50 d	61.47 ± 0.22 a	45.00 ± 1.95 b	−0.75 ± 0.07 e
	63	34.03 ± 0.41 c	11.64 ± 0.29 d	57.92 ± 1.81 a	43.28 ± 0.43 b	−0.75 ± 0.19 e
CIEb^*	0	59.26 ± 0.39 a	42.59 ± 0.99 b	59.51 ± 0.63 a	15.41 ± 0.20 c	12.34 ± 0.08 d
	7	53.77 ± 0.63 a	19.75 ± 0.76 b	53.44 ± 2.80 a	16.15 ± 0.27 c	10.89 ± 0.17 d
	14	43.73 ± 0.64 b	15.26 ± 0.03 c	47.76 ± 2.59 a	15.26 ± 0.03 c	10.05 ± 0.91 d
	21	25.16 ± 1.66 b	12.63 ± 0.24 bc	39.55 ± 11.73 a	14.02 ± 0.14 bc	8.58 ± 0.61 c
	35	22.38 ± 0.19 b	13.49 ± 0.08 d	43.08 ± 0.87 a	18.36 ± 0.57 c	7.22 ± 0.43 e
	49	22.62 ± 0.06 b	13.86 ± 0.07 d	38.12 ± 0.78 a	17.63 ± 0.49 c	6.08 ± 0.28 e
	63	23.49 ± 0.90 b	14.57 ± 0.19 c	28.00 ± 0.83 a	15.45 ± 0.13 c	6.04 ± 0.10 d
Croma	0	84.59 ± 0.34 b	72.94 ± 0.46 c	87.24 ± 0.37 a	58.88 ± 0.10 d	12.34 ± 0.16 e
	7	86.06 ± 0.38 a	61.53 ± 0.04 c	82.69 ± 1.43 b	56.09 ± 0.30 d	10.89 ± 0.18 e
	14	80.14 ± 0.27 a	55.12 ± 0.07 b	79.85 ± 1.45 a	55.12 ± 0.07 b	10.05 ± 0.10 c
	21	66.24 ± 1.51 b	31.12 ± 1.11 d	77.53 ± 0.08 a	54.44 ± 0.47 c	8.58 ± 0.48 e
	35	59.23 ± 1.78 b	22.69 ± 0.43 d	76.27 ± 0.57 a	52.60 ± 1.47 c	7.22 ± 1.21 e
	49	47.98 ± 0.85 b	19.81 ± 0.40 c	72.33 ± 0.57 a	48.33 ± 1.99 b	6.13 ± 1.00 d
	63	41.35 ± 0.84 c	18.64 ± 0.26 d	64.32 ± 1.99 a	45.96 ± 0.45 b	6.09 ± 1.92 e
hue	0	44.47 ± 0.15 b	35.72 ± 0.72 d	43.02 ± 0.36 c	15.18 ± 0.21 e	84.31 ± 0.16 a
	7	38.67 ± 0.33 c	18.72 ± 0.77 d	40.72 ± 1.04 b	16.74 ± 0.20 e	86.76 ± 0.18 a
	14	33.07 ± 0.41 c	16.07 ± 0.01 d	36.72 ± 1.53 b	16.07 ± 0.01 d	87.87 ± 1.10 a
	21	18.18 ± 1.00 c	18.34 ± 0.26 c	39.29 ± 0.02 b	18.04 ± 3.14 c	90.21 ± 0.48 a
	35	22.48 ± 0.59 d	36.51 ± 1.01 b	34.39 ± 0.50 c	20.43 ± 0.34 d	93.64 ± 1.21 a
	49	28.13 ± 0.47 d	44.43 ± 0.93 b	31.80 ± 0.45 c	21.44 ± 0.10 e	97.04 ± 1.00 a
	63	34.61 ± 0.72 c	51.03 ± 0.53 b	25.80 ± 0.05 d	19.65 ± 0.02 e	97.08 ± 1.92 a

[a] Values are mean ($n = 3$) ± standard deviation. Means in the same column followed by different letters are significantly different at $p < 0.05$ according to Duncan's test. (∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$).

Table 4. Analysis of Correlations between Total Anthocyanin Content and Color Parameters (Croma and a^* Values) in the Prepared Mixtures through the Storage Period[a]

	total anthocyanins			
	LJ-AC5%	LJ-AC2.5%	AC%	AC2.5%
CIEa^*	0.6721***	0.8688***	0.7205***	0.9597***
Croma	0.8991***	0.9377***	0.9681***	0.9603***

[a] Correlations at $p < 0.05$ (∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$).

approximately the same as the addition of total phenolics in pure LJ plus AC.

These values were about 3 times higher than the sum of the individual ones obtained by HPLC, as previously determined by other authors (*8*). This can be explained by the fact that the Folin−Ciocalteau method overestimates the real phenolic content, because it also quantifies polymeric phenols and other nonphenolics metabolites (*8*). Nevertheless, the combination of both assays is useful to screen for the relative quality and quantity of polyphenols.

***In Vitro* Antioxidant Activity (DPPH Test/Assay).** The antioxidant activity of the different controls showed initial values lower for LJC (4.60 ± 0.07 mM Trolox equivalents) than for aronia dilutions (7.10 ± 0.34 mM AC2.5% and 11.91 ± 0.07 mM Trolox AC5%). Nevertheless, the beverages with LJ plus AC resulted in increased *in vitro* antioxidant activity, at least 2 times higher than LJC (8.74 ± 0.21 mM LJ-AC2.5% and 10.96 ± 0.37 mM LJ-AC5%). These values revealed higher levels than those of orange juice, red wine, or green tea (analyzed in our laboratory; data not shown). However, because LJ plus AC mixtures presented lower values of antioxidant activity than the ones expected after the addition of the individual compounds, a synergistic effect was ruled out. With the possibility of either polymerization or condensation reactions between monomeric anthocyanins, even additional or parallel complexation reactions could lead to the formation of new compounds with lower antioxidant activity (*32, 34*). Moreover, the presence of other antioxidant compounds from LJ or AC, such as proteins, which we have not analyzed, could react in the mixtures, decreasing the *in vitro* antioxidant values.

Main antioxidant activity losses were registered in all cases in the first 7 days of storage, remaining rather constant thereafter (**Figure 2C**). The highest loss was determined in LJC (40%), followed by LJ-AC2.5% (30%). This could be due to losses in vitamin C and hesperidin, but we could not exclude the effect of other compounds occurring in this mixtures because the antioxidant activity in LJ-AC5% was lower than 20%. Finally, losses in AC control solutions were always lower than 20% (**Figure 2**).

Relationship between Antioxidant Activity and Phytochemical Composition. In LJ-AC mixtures, vitamin C was not the main compound responsible for antioxidant activity, because after the first 14 days of storage, over 80% of the AA and DHAA was degraded. A good correlation ($R^2 > 0.80$) was found between *in vitro* antioxidant activity and the total anthocyanins in the mixtures with LJ during the first month,

Table 5. Analysis of Correlations between Antioxidant Activity (Measured by DPPH• Test) and Total Phenol Content (Measured by FCR), Total Anthocyanin, and Vitamin C in the Prepared Mixtures through the Storage Period[a]

	antioxidant activity				
	LJ-AC5%	LJ-AC2.5%	AC5%	AC2.5%	LJC
FCR	0.6522**	0.6117**	0.5874**		0.8936***
total anthocyanin	0.7898***	0.9370***	0.5535**		
vitamin C	0.7758***	0.9346***			0.9191***

[a] Correlations at $p < 0.05$ (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

and only the LJC presented a high correlation between the activity and the total phenolic composition ($R^2 > 0.90$) (**Table 5**).

In accordance with previous reports (*26*), the *in vitro* activity could be due to vitamin C, phenolic compounds, and total free anthocyanins in the mixtures during the first storage period (~15 days); thereafter, other compounds in the mixture (e.g., diketogulonic acid and 2-hydroxyfurfural) derived from the analytes could also influence this activity.

On the other hand, AC control solutions did not show any good correlation between antioxidant activity and anthocyanins (**Table 5**) or total phenolics-FCR, despite the fact that authors reported good correlations between total phenolics-FCR and antioxidant activity measured by different *in vitro* assays (e.g., FRAP and ORAC) (*12*).

It has been described that the major contributors to the antioxidant activity in aronia concentrates are the anthocyanins; however, this fruit has flavonoids, such as quercetin, which has demonstrated stronger *in vitro* antioxidant activity (*35*) because of its molecular structure. Nevertheless, there was not a good correlation between quercetins and antioxidant assays in the mixtures ($R^2 < 0.10$).

As concluding remarks of the presented results, the newly designed beverage of LJ-AC5% presented the highest retention of anthocyanins and the highest *in vitro* antioxidant activity over the 60 days of storage at room temperature in the dark, showing an attractive red color (CIEa^* and Croma values more consistent than that of the LJ-AC2.5%). Thus, the addition of 5%AC to a lemon juice could effectively increase the antioxidant properties of the final beverage/mixture as well as improve certain organoleptical properties of the mixture. Therefore, the new drink based on lemon juice plus aronia concentrate showed potential for the development of interesting functional products. Further studies of bioavailability are guaranteed to evaluate the benefits of such a product on human health.

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LITERATURE CITED

(1) Khan, N.; Afaq, F.; Mukhtar, H. Cancer chemoprevention through dietary antioxidants: Progress and promise. *Antioxid. Redox Signaling* **2008**, *10*, 475–510.

(2) Del Río, J. A.; Fuster, M. D.; Gomez, P.; Porras, I.; Garcia-Lidon, A.; Ortuño, A. Citrus limon: A source of flavonoids of pharmaceutical interest. *Food Chem.* **2004**, *84*, 547–461.

(3) Skoczynìska, A.; Jedrychowska, I.; Poreba, R.; Affelska-Jercha, A.; Turczyn, B.; Wojakowska, A.; Andrzejak, R. Influence of chokeberry juice on arterial blood pressure and lipid parameters in men with mild hypercholesterolemia. *Pharmacol. Rep.* **2007**, *59*, 177–182.

(4) Sueiro, L.; Yousef, G. G.; Seigler, D.; De Mejia, E. G; Grace, M. H; Lila, M. A. Chemopreventive potential of flavonoid concentrates from plantation-bred and wild *Aronia melanocarpa* (black chokeberry) fruits. *J. Food Sci.* **2006**, *71*, C480–C488.

(5) González-Molina, E.; Moreno, D. A.; García-Viguera, C. Genotype and harvest time influence the phytochemical quality of fino lemon juice (*Citrus limon* (L.) Burm. f.) for industrial use. *J. Agric. Food Chem.* **2008**, *56*, 1669–1675.

(6) Miyake, Y.; Suzuki, E.; Ohya, S.; Fukumoto, S.; Hiramitsu, M.; Sakaida, K.; Osawa, T.; Furuichi, Y. Lipid-lowering effect of eriocitrin, the main flavonoid in lemon fruit, in rats on a high-fat and high-cholesterol diet. *J. Food Sci.* **2006**, *71*, S633–S637.

(7) Kähkönen, M. P.; Hopia, A. I.; Heinonen, M. Berry phenolics and their antioxidant activity. *J. Agric. Food Chem.* **2001**, *49*, 4076–4082.

(8) Bermúdez-Soto, M. J.; Tomás-Barberán, F. A. Evaluation of commercial red fruit juice concentrates as ingredients for antioxidant functional juices. *Eur. Food Res. Technol.* **2004**, *219*, 133–141.

(9) Wu, X.; Gu, L.; Prior, R.; Mckay, S. Characterization of anthocyanins and proanthocyanidins in some cultivars of ribes, aronia, and sambucus and their antioxidant capacity. *J. Agric Food. Chem.* **2004**, *52*, 7846–7856.

(10) Slimestad, R.; Torskangerpoll, K.; Nateland, H. S.; Johannessen, T.; Giske, N. H. Flavonoids from black chokeberries, *Aronia melanocarpa*. *J. Food Compos. Anal.* **2005**, *18*, 61–68.

(11) Jakobek, L.; Seruga, M.; Medvidovicì-Kosanovicì, M.; Novak, I. Anthocyanin content and antioxidant activity of various red fruit juices. *Deutsch. Lebensm.-Rundsch.* **2007**, *103*, 58–64.

(12) Zheng, W.; Wang, S. Y. Oxygen radical absorbing capacity of phenolics in blueberries, cranberries, chokeberries, and lingonberries. *J. Agric. Food. Chem.* **2003**, *51*, 502–509.

(13) Tanaka, T. Chemical components and characteristics of black chokeberry. *J. Jpn. Soc. Food Sci.* **2001**, *48*, 606–610.

(14) Strigl, A. W.; Leitner, E.; Pfannhauser, W. Black chokeberry (*Aronia melanocarpa*) a natural source of colorants. *Deutsch. Lebensm.-Rundsch.* **1995**, *91*, 177–180.

(15) Cao, G.; Muccitelli, H.; Sánchez-Moreno, C.; Prior, R. Anthocyanins are absorbed in glycated forms in elderly women: A pharmacokinetic study. *Am. J. Clin. Nutr.* **2001**, *73*, 920–926.

(16) Oszmianski, J.; Wojdylo, A. *Aronia melanocarpa* phenolics and their antioxidant activity. *Eur. Food Res. Technol.* **2005**, *221*, 809–813.

(17) Rein, M. J.; Heinonen, M. Stability and enhancement of berry juice color. *J. Agric. Food. Chem.* **2004**, *52*, 3106–3114.

(18) Mazza, G.; Miniati, E. *Anthocyanins in Fruits, Vegetables and Grains*; CRC Press: Boca Raton, FL, 1993; pp 1−23 and 85−118.

(19) Wicklund, T.; Rosenfeld, H. J.; Martinsen, B. K.; Sundfør, M. W.; Lea, P.; Bruun, T.; Blomhoff, R.; Haffner, K. Antioxidant capacity and colour of strawberry jam as influenced by cultivar and storage conditions. *Food Sci. Technol.* **2005**, *38*, 387–391.

(20) Espín, J. C.; Soler-Rivas, C.; Wichers, H. J.; García-Viguera, C. Anthocyanin-based colorants: A new source of antiradical activity for foodstuff. *J. Agric. Food. Chem.* **2000**, *48*, 1588–1592.

(21) García-Viguera, C.; Zafrilla, P.; Artés, F.; Romero, F.; Abellán, P.; Tomás-Barberán, F. A. Colour and anthocyanin stability of red raspberry jam. *J. Sci. Food Agric.* **1998**, *78*, 565–573.

(22) García-Alonso, F. J.; Periago, M. J.; Vidal-Guevara, M. L.; Cantos, E.; Ros, G.; Ferreres, F.; Abellán, P. Assessment of the antioxidant properties during storage of a dessert made from grape, cherry, and berries. *J. Food. Sci.* **2003**, *68*, 1525–1530.

(23) Taruscio, T. G.; Barney, D. L.; Exon, J. Content and profile of flavanoid and phenolic acid compounds in conjunction with the antioxidant capacity for a variety of northwest *Vaccinium* berries. *J. Agric. Food. Chem.* **2004**, *52*, 3169–3176.

(24) Rababah, T. M.; Ereifej, K. I.; Howard, L. Effect of ascorbic acid and dehydration on concentrations of total phenolics, antioxidant capacity, anthocyanins, and color in fruits. *J. Agric Food. Chem.* **2005**, *53*, 4444–4447.

(25) Wu, X.; Beecher, G. R.; Holden, J. M.; Haytowitz, D. B.; Gebhardt, S. E.; Prior, R. L. Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. *J. Agric. Food Chem.* **2006**, *54*, 4069–4075.

(26) Martí, N.; Pérez-Vicente, A.; García-Viguera, C. Influence of storage temperature and ascorbic acid on pomegranate juice. *J. Sci. Food Agric.* **2001**, *82*, 217–221.

(27) Association of Official Analytical Chemists (AOAC). *Official Methods of Analysis*, 14th ed.; AOAC: Arlington, VA, 1984; pp 414−420.

(28) Pérez-Vicente, A.; Serrano, P.; Abellán, P.; García-Viguera, C. Influence of packaging material on pomegranate juice colour and bioactive compounds, during storage. *J. Sci. Food Agric.* **2004**, *84*, 639–644.

(29) Arnous, A.; Makris, D. P.; Kefalas, P. Effect of principal polyphenol components in relation to antioxidant characteristics of aged red wines. *J. Agric. Food Chem.* **2001**, *49*, 5736–5742.

(30) Llorach, R.; Tomás-Barberán, F. A.; Ferreres, F. Lettuce and chicory byproducts as a source of antioxidant phenolic concentrates. *J. Agric. Food Chem.* **2004**, *52*, 5109–5116.

(31) Real Decreto 667/1983, de 2 de marzo, por el que se aprueba la reglamentacióntécnico-sanitaria para la elaboración y venta de zumos de frutas y de otros vegetales y de sus derivados. Real Decreto, BOE 77 of March 31, 1983; reference 1983/09198, pp 9196−9200 (in Spanish).

(32) Gil-Izquierdo, A.; Gil, M. I.; Ferreres, F.; Tomás-Barberán, F. A. In vitro availability of flavonoids and other phenolics in orange juice. *J. Agric. Food Chem.* **2001**, *49*, 1035–1041.

(33) Boulton, R. R. The copigmentation of anthocyanins and its role in the color of redwine: A critical review. *Am. J. Enol. Vitic.* **2001**, *52*, 67–86.

(34) Brenes, C. H.; Del Pozo-Insfran, D.; Talcott, S. T. Stability of copigmented anthocyanins and ascorbic acid in a grape juice model system. *J. Agric. Food Chem.* **2005**, *53*, 49–56.

(35) Bao, J.; Cai, Y.; Sun, M.; Wang, G.; Corke, H. Anthocyanins, flavonols, and free radical scavenging activity of chinese bayberry (*Myrica rubra*) concentrates and their color properties and stability. *J. Agric. Food Chem.* **2005**, *53*, 2327–2332.

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