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Antioxidant Properties of Prepared Blueberry (*Vaccinium myrtillus*) Extracts

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A blueberry extract (A) and two anthocyanin-derived extracts (B and C) were prepared. The contents of polyphenols, flavonoids, anthocyanins, and anthocyanin-derived pigments of the extracts were determined for the first time. The pigment profile of blueberry extract A corresponded to 15 anthocyanins, whereas extract B was mainly composed of anthocyanin−pyruvic acid adducts of the blueberry original anthocyanins and extract C was mainly composed of the respective vinylpyrano-anthocyanin−catechins (portisins). The extracts' abilities to inhibit lipid peroxidation, induced by 2,2′-azobis(2-methyl-propanimidamide) dihydrochloride in a liposomal membrane system were examined. The antioxidant capacities of the extracts were evaluated through monitoring oxygen consumption and by measuring the formation of conjugated dienes. All of the extracts provided protection of membranes against peroxyl radicals by increasing the induction time of oxidation. This effect increased with the polyphenol content and with the structural complexity of the anthocyanin-derived pigments of the extracts. The pigments present in extract C seemed to induce a higher protection of the liposome membranes toward oxidation. In addition, the antiradical properties and the reducing power of the extracts were determined by using DPPH and FRAP methods, respectively. The results from these assays were in agreement with those obtained with the liposome membranes.

KEYWORDS: Blueberry; anthocyanin; pyruvic acid; portisin; liposome; antioxidant

INTRODUCTION

Blueberries are among the fruits that are best recognized for their potential health benefits. Many of the heath-promoting properties of blueberries are thought to be attributable to their bioactive compounds (namely, proanthocyanidins and anthocyanins) (*1*, *2*). Anthocyanins are one of the most important groups of plant pigments and are present in blueberries at high concentrations (*3*). These natural pigments have been thoroughly investigated for technological applications, especially by the food industry. Indeed, replacing synthetic dyes by natural colorants has become a major issue in recent years. Pigments from natural sources are very attractive as they are usually safe and may display a wide range of colors. A great number of research projects are currently directed toward the identification and characterization of pigments from different natural sources, especially red fruit extracts, with the aiming of using them as colorants. Several investigations are being carried out especially focused on anthocyanin-derived pigments. The stability of anthocyanins (or other derived pigments) with regard to pH and temperature variations is one of the major obstacles for their application in food matrixes (*4*). Other factors such as light exposure, oxidation, and the presence of metals and other substances may also limit the application of these compounds in foodstuffs.

Recently, more attention has been paid to the antioxidants contained in fruits because epidemiological studies have revealed that a high fruit intake appears to be positively correlated with reduced mortality by cardiovascular disease and some types of cancer (*5*). One possible mechanisms responsible for these effects was attributed to the antioxidant activity presented by the fruits. Consequently, it would be of great importance if anthocyanins or anthocyanin-derived pigments could be used in the food industry, thereby guaranteeing the antioxidant effect and the color of the product.

A number of methods have been developed to measure the efficiency of dietary antioxidants either as pure compounds or in food extracts, as well as to determine the antioxidant activity of plasma as an index of the antioxidant status in vivo. These methods focus on different mechanisms of the antioxidant defense system, such as scavenging of oxygen and hydroxyl radicals, reduction of lipid peroxyl radicals, and inhibition of

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lipid peroxidation or chelation of metal ions (*6*, *7*). Thus, methods such as those using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (*8*) or *N*,*N*-dimethyl-*p*-phenylenediamine (DMPD) (*9*) measure the scavenging of stable radical species by antioxidants. The FRAP and TEAC assays measure the reducing capabilities of antioxidants (*10*, *11*). Other methods evaluate the inhibition of lipid peroxidation by antioxidants, quantifying products such as conjugated dienes (*12*) and lipid hydroperoxides as well as products resulting from the decomposition of lipid peroxides such as malonaldehyde. However, the use of LDL or liposomes appears to be a more promising method of assessing antioxidant properties relevant to human nutrition (*13*), because these systems allow investigation of the protection of a substrate by an antioxidant in a model biological membrane model or a lipoprotein.

The aim of this work was to study the potential antioxidant properties of a blueberry (*Vaccinium myrtillus*) extract and two respective anthocyanin-derived extracts. These two extracts were obtained after chemical reaction with pyruvic acid and catechin and are mainly composed of anthocyanin−pyruvic acid adducts (extract B) (*14*, *15*) and vinylpyranoanthocyanin−catechins (portisins) (extract C) (*16*, *17*). This latter extract (C) is of great interest for putative applications in the food industry because it presents a bluish hue even in acidic conditions. These anthocyanin-derived compounds display unusual colors and are currently being explored for further use in several food matrixes (beverages, dairy products, etc.). In this work, several colorimetric methods were used to characterize the content in polyphenols and flavonoids of the extracts, and HPLC was used to determine the content of total pigments. The antiradical properties and the reducing power of these samples were assessed using DPPH and FRAP assays, respectively. The activity against lipid peroxidation was determined using soybean phosphatidylcholine liposomes as a membrane model system, induced by 2,2′-azobis(2-methylpropanimidamide) dihydrochloride (AAPH). The extension of membrane lipid oxidation was followed by measuring the oxygen consumption and conjugated diene hydroperoxide production.

MATERIALS AND METHODS

Reagents. Toyopearl gel was purchased from Tosoh (Tokyo, Japan); AAPH, DPPH, $FeCl_3$, DMSO, pyruvic acid, quercetin, $AlCl_3$, NaOH, $NaNO_2$, Na_2CO_3, Trolox, catechin, Hepes, NaCl, and soybean L-α-phosphatidylcholine were purchased from Sigma-Aldrich (Madrid, Spain); 2,4,6-tripyridyl-*s*-triazine (TPTZ) was purchased from Fluka (Madrid, Spain), and Folin−Ciocalteu reagent was purchased from Merck (Darmstadt, Germany).

Extract Preparation. Two hundred grams of blueberries (*Vaccinium myrtillus*) picked in the field was subjected to extraction with 500 mL of 50% aqueous ethanol (pH 1.5) for 30 min at room temperature. The blueberry anthocyanin extract (A) was filtered in a 50 μm nylon membrane and then purified by Toyopearl gel column chromatography according to the procedure described previously (*18*).

Anthocyanin−pyruvic acid adducts (extract B) were prepared through the reaction of the genuine anthocyanin extract with pyruvic acid in water (pH 2.6, 35 °C) at an approximate molar ratio of pyruvic acid to anthocyanin of 50:1 during 5 days. The resulting extract was purified by Toyopearl gel column chromatography with the anthocyanin−pyruvic acid adducts fraction eluted with water/ethanol 20% (v/v).

Vinylpyranoanthocyanin−catechins (portisins) (extract C) were prepared through the reaction of anthocyanin−pyruvic acid adducts (extract B) with catechin and acetaldehyde at 35 °C in 20% aqueous ethanol (pH 1.5) at an approximate molar ratio of catechin to acetaldehyde to anthocyanin−pyruvate of 50:25:1. After 10 days of reaction, the solution was analyzed by HPLC-DAD, and the portisin extract was isolated by Toyopearl gel column chromatography by elution with water/ethanol 85% (v/v), following the procedure previously described (*17*). All of the extracts were freeze-dried and stored at −20° C until use. Two milligrams of each extract was redissolved in 10 mL of methanol for further analysis.

HPLC Conditions. All of the extracts were analyzed by HPLC (Knauer K-1001) on a 250 × 4.6 mm i.d. reversed-phase C18 column (Merck); detection was carried out at 511, 528, and 570 nm using a diode array detector (Knauer K-2800). The solvents were (A) H_2O/HCOOH (9:1) and (B) H_2O/CH_3CN/HCOOH (6:3:1). The gradient consisted of 20−85% B for 70 min at a flow rate of 1.0 mL/min. The column was washed with 100% B for 20 min and then stabilized at the initial conditions for another 20 min. Extract C was also analyzed by HPLC using the same conditions with a different solvent B [CH_3CN/H_2O/CH_3COOH (8:1.95:0.05)].

LC-MS Conditions. A liquid chromatograph (Hewlett-Packard 1100 series) equipped with an AQUA (Phenomenex, Torrance, CA) reversed-phase column (150 × 4.6 mm, 5 μm, C18) thermostated at 35°C was used. Solvents were (A) aqueous 0.1% trifluoroacetic acid and (B) acetonitrile, establishing the gradient as reported elsewhere (*18*). Double-online detection was made in a photodiode spectrophotometer and by mass spectrometry. The mass detector was a Finnigan LCQ (Finnigan Corp., San Jose, CA) equipped with an API source, using an electrospray ionization (ESI) interface. Both the auxiliary and the sheath gases were a mixture of nitrogen and helium. The capillary voltage was 3 V and the capillary temperature, 190 °C. Spectra were recorded in positive ion mode between *m*/*z* 120 and 1500. The mass spectrometer was programmed to do a series of three scans: a full mass, a zoom scan of the most intense ion in the first scan, and an MS-MS of the most intense ion using relative collision energies of 30 and 60.

Total Phenolics. The total polyphenol content of the extracts was determined following the Folin−Ciocalteu method adjusted to a microscale (*19*). In an Eppendorf tube, 790 μL of distilled water, 10 μL of sample dissolved in methanol, and 50 μL of Folin−Ciocalteu reagent were mixed. After 1 min, 150 μL of aqueous 20% Na_2CO_3 was added, and the mixture was mixed and allowed to stand at room temperature in the dark for 120 min. The absorbance was read at 750 nm, and the total polyphenol concentration was calculated from a calibration curve, using Trolox (a water-soluble analogue of vitamin E) as standard. The results were expressed as milligrams per liter of Trolox equivalents.

Total Flavonoids. Total flavonoid content of the extracts was determined according to a colorimetric method with some modifications (*20*). Aliquots of appropriately diluted solutions in methanol were transferred into an Eppendorf tube containing 1 mL of distilled water and mixed with 75 μL of 5% $NaNO_2$. After 5 min, 75 μL of 10% $AlCl_3$ solution was added. The mixture was allowed to stand for another 5 min, and then 0.5 mL of 1 M NaOH was added. The reaction solution was mixed and kept for 15 min. The increase in absorbance was measured at 510 nm. Total flavonoid content was calculated using a standard quercetin calibration curve. The results were expressed as milligrams per liter of quercetin equivalents.

Total Pigments. The blueberry extract (A) and the anthocyanin−pyruvic acid adduct extract (B) samples were analyzed by HPLC using the conditions described above. The concentrations of the anthocyanins and anthocyanin-derived pigments of the extracts were determined using calibration curves, and the results were expressed as milligrams per liter of malvidin 3-glucoside, milligrams per liter of malvidin−pyruvic acid adduct, and milligrams per liter of vinylpyranomalvidin−catechin for extracts A, B, and C, respectively. The standards malvidin−pyruvic acid adduct and vinylpyranomalvidin−catechin were obtained by semipreparative HPLC (*17*).

Radical DPPH Scavenging Activity. Following the method described in the literature (*8*) with some modifications, radical activities were determined by using DPPH as a free radical. The reaction for scavenging DPPH radicals was performed in polypropylene tubes at room temperature (22−23 °C). For each tube, an aliquot of 7.5 μg of extract was added to 3 mL of DPPH solution (60 μM in methanol). The decrease in absorbance was measured at 515 nm, at $t = 0$ and every 10 min, for 30 min. Methanol was used as blank solution, and DPPH solution without any sample extract served as control. The antiradical activity was calculated from the equation determined from

linear regression after plotting known solutions of Trolox with different concentrations (2.5−50 μM) (*8*, *21*). For the final results, the 0−20 min reaction time window was used. Antiradical activity was expressed as micromolar Trolox equivalents.

Ferric Reducing/Antioxidant Power (FRAP). The FRAP assay developed by Benzie and Strain (*10*) was performed with some modifications. In short, FRAP reagent (10 vol of 300 mM acetate buffer, pH 3.6, + 1 vol of 10 mM TPTZ in 40 mM HCl + 1 vol of 20 mM $FeCl_3$) was diluted to one-third with methanol and prewarmed at 37 °C. Three milliliters of this reagent was mixed with 7.5 μg of extract. This mixture was shaken, and the absorbance was read at 593 nm. The test was performed at 37 °C, and the 0−4 min reaction time window was used. Results are expressed as Trolox equivalents determined using a calibration curve.

Liposome Preparation. Liposomes were prepared by evaporation to dryness of l-α-phosphatidylcholine (PC) from soybean solution in chloroform with a stream of argon; the film was then left under vacuum over 3 h to remove all traces of the organic solvent. The resultant dried lipid film was dispersed with Hepes buffer (10 mM Hepes, 0.1 M NaCl, pH 7.4), and then the mixture was shaken above the phase transition temperature to produce multilamellar liposomes (MLV). Frozen and thawed MLVs were obtained by repeating five times the following cycle: freezing the vesicles in liquid nitrogen and thawing the sample in a water bath at 37 °C. Lipid suspensions were equilibrated at 37 °C for 30 min and extruded 10 times through polycarbonate filters of 100 nm pore size in a 10 mL stainless steel extruder to form large unilamellar vesicles (LUV) (*22*).

Lipid Peroxidation Procedures. Lipid peroxidation of soybean LUVs was induced by peroxyl radicals, generated at a constant rate, by thermal degradation of the azo compound AAPH in the presence or absence of antioxidants and followed by measuring the oxygen consumption and conjugated diene production.

Oxygen Consumption. The rate of oxygen consumption was measured continuously with a Clark-type oxygen electrode (Hansatech) provided with an automatic recording apparatus. The reaction mixture containing 1.3 mL of Hepes buffer, 200 μL of LUV (340 μM final concentration), and 1 μg of the antioxidant tested, dissolved in a volume of dimethyl sulfoxide (DMSO) that never exceeded 0.3% of the total volume, was left in a 37 °C thermostated bath for 1 h. This mixture was introduced in a closed glass vessel, protected from light, thermostated at 37 °C, and provided with a stirrer, and the reaction was started by the addition of AAPH (10 mM final concentration) (*23*). The induction periods in the presence of extracts were determined graphically from the profiles of oxygen consumption by the coordinates of the interception of the tangents to the inhibited and uninhibited rates of oxidation. Results were expressed relative to the ones obtained with Trolox.

Conjugated Diene Formation. Soybean liposomes (500 μM final concentration) were incubated in a water bath at 37 °C with the extract to test (313 ng/mL). Reaction was started with the addition of 10 μL the azo initiator (5 mM final concentration). Aliquots of liposomes (60 μL) were taken at 15 min intervals and dissolved with 940 μL of absolute ethanol directly in a 1 cm quartz cell (*24*). The formation of conjugated dienes was followed by recording the absorbance at 233 nm. The induction periods in the presence of extracts were determined graphically from the profiles of conjugated dienes formation by the coordinates of the interception of the tangents to the inhibited and uninhibited rates of oxidation. Results were expressed relative to the ones obtained with Trolox.

Statistical Analysis. All of the assays were performed in $n \geq 4$ assays. The mean values, standard deviations, and statistical differences evaluated through analysis of variance (ANOVA) were obtained using the SPSS computer package.

RESULTS AND DISCUSSION

Pigment Extract Characterization. Flavonoids, including anthocyanins, are considered to be the most important family of phenolic compounds in fruits. A good correlation between the concentration of fruit phenolics and the total antioxidant capacity has been reported (*25*, *26*). The present work aimed to study a blueberry (*V. myrtillus*) extract and its anthocyanin-derived extracts that are being developed to be further applied in the food industry. The major interest in the putative use of these extracts lies in their unusual colors in acidic conditions (more orange and bluish hues) even though arising from natural precursors. The overall color of these extracts can easily be perceived from their visible spectra displayed in **Figure 1**. Extract C is the most interesting one due to its unusual bluish color (λ_{max} = 586 nm). These extracts were characterized using spectrophotometric methods for total phenolics and total flavonoid compounds and HPLC analysis for total pigments. Blueberry extract A is composed of anthocyanins (**Figure 2A**), whereas extract B is mainly composed of anthocyanin−pyruvic acid adducts (**Figure 2B**) and extract C is composed of bluish anthocyanin-derived pigments named portisins (*27*) (**Figure 2C**). The pigment profiles of the extracts, which are indicated in the legends of Figure 2, were determined through LC/DAD-MS (**Tables 1−3**). From the analysis of the MS data it can be seen that all of the pigments yielded fragment ions after the loss of the respective sugar moiety ($[M - 162]^+$ for glucosides and galactosides and $[M - 132]^+$ for arabinosides). Furthermore, the pigments present in extract C yielded a fragment ion that corresponded to the loss of a sugar moiety and a retro-Diels−Alder fission of the catechin moiety ($[M - 314]^+$ for glucosides and $[M - 284]^+$ for arabinosides). The general structure of the compounds present in the different extracts is illustrated in **Figure 5**. The structural complexity of the pigments present in the different extracts increases in the following order: A < B < C. The levels of anthocyanins, anthocyanin−pyruvic acid adducts, and vinylpyranoanthocyanin−catechins (portisins) were determined by HPLC using calibration curves obtained for malvidin 3-glucoside, malvidin 3-glucoside−pyruvic acid adduct, and vinylpyranomalvidin 3-glucoside−catechin, respectively. As seen from the results presented in **Table 4**, the total pigment and flavonoid contents in extract C are much higher than in the other two extracts. This outcome could be anticipated as the molecular weights of the pigments increase in the order anthocyanins (A) < anthocyanin−pyruvic acid adducts (B) < vinylpyranoanthocyanin−catechins (C), which leads to higher amounts (grams) of product. On the other hand, this extract was obtained after more purification steps than the others, thereby contributing to a more concentrated pigment extract.

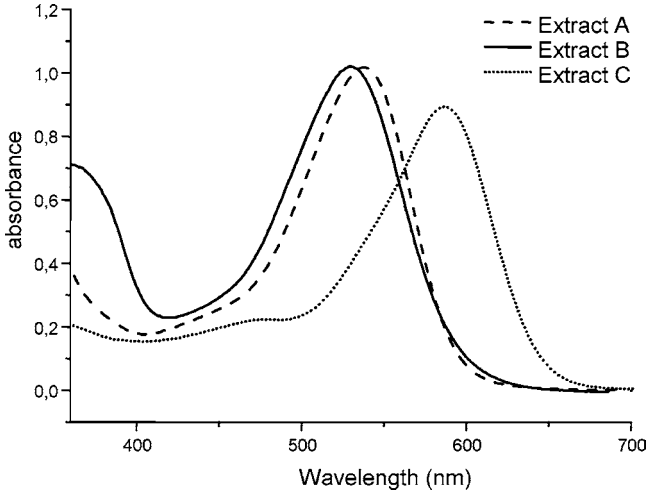

Figure 1. Visible spectra of blueberry (*V. myrtillus*) extract A (λ_{max} = 538 nm), anthocyanin–pyruvic acid extract B (λ_{max} = 531 nm), and vinylpyranoanthocyanin–catechin extract C (λ_{max} = 586 nm).

DPPH and FRAP. The free radical scavenging activity of the extracts was tested using the DPPH method. The tested substances react with DPPH, which is a stable free radical, and

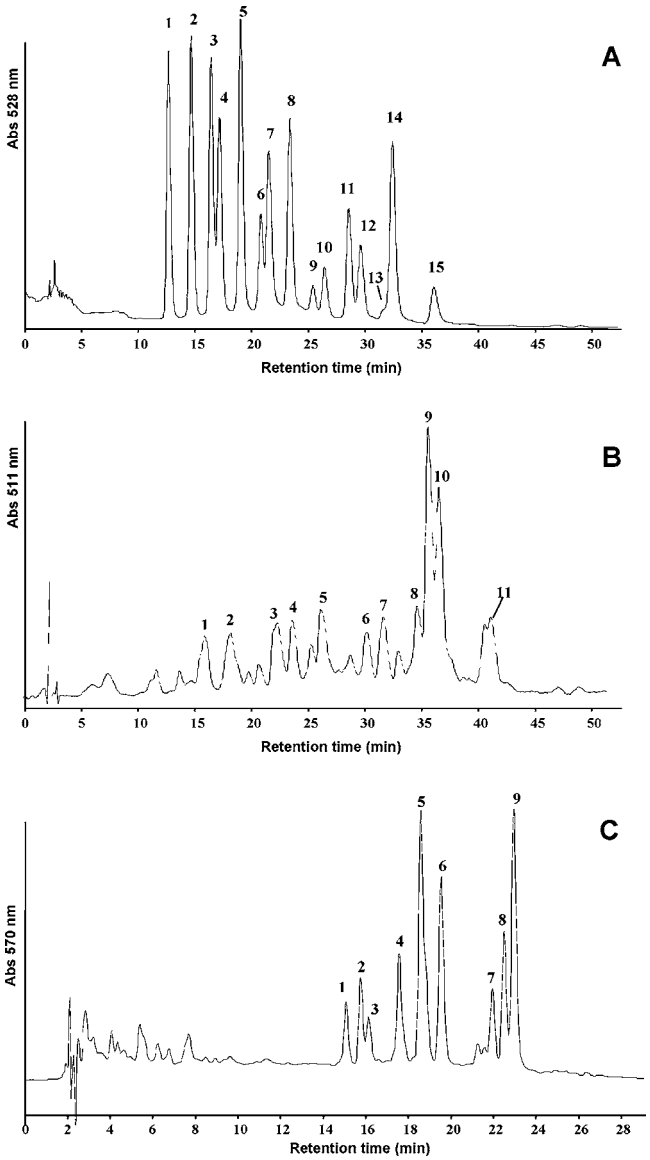

Figure 2. (**A**) HPLC chromatogram of blueberry (*V. myrtillus*) extract A recorded at 528 nm: (1) delphinidin-3-galactose, (2) delphinidin-3-glucose, (3) cyanidin-3-galactose, (4) delphinidin-3-arabinose, (5) cyanidin-3-glucose, (6) petunidin-3-galactose, (7) cyanidin-3-arabinose, (8) petunidin-3-glucose, (9) peonidin-3-galactose, (10) petunidin-3-arabinose, (11) peonidin-3-glucose, (12) malvidin-3-galactose, (13) peonidin-3-arabinose, (14) malvidin-3-glucose, (15) malvidin-3-arabinose. (**B**) HPLC chromatogram of blueberry extract B after 5 days of reaction with pyruvic acid recorded at 511 nm: (1) delphinidin-3-galactose(or glucose)–pyruvic acid adduct, (2) delphinidin-3-arabinose–pyruvic acid adduct, (3) cyanidin-3-galactose(or glucose)–pyruvic acid adduct, (4) cyanidin-3-arabinose–pyruvic acid adduct, (5) petunidin-3-galactose(or glucose)–pyruvic acid adduct, (6) petunidin-3-arabinose–pyruvic acid adduct, (7) peonidin-3-galactose(or glucose)–pyruvic acid adduct, (8) peonidin-3-arabinose–pyruvic acid adduct, (9) malvidin-3-galactose–pyruvic acid adduct, (10) malvidin-3-glucose–pyruvic acid adduct, (11) malvidin-3-arabinose–pyruvic acid adduct. (**C**) HPLC chromatogram of blueberry extract C recorded at 570 nm: (1) vinylpyrano–delphinidin-3-galactose(or glucose)–catechin, (2) vinylpyrano–delphinidin-3-glucose(or galactose)–catechin, (3) vinylpyrano–cyanidin-3-galactose(or glucose)–catechin, (4) vinylpyrano–cyanidin-3-arabinose–catechin, (5) vinylpyrano–petunidin-3-glucose(or galactose)–catechin, (6) vinylpyrano–petunidin-3-arabinose–catechin, (7) vinylpyrano–peonidin-3-galactose(or glucose)–catechin, (8) vinylpyrano–malvidin-3-arabinose–catechin, (9) vinylpyrano–malvidin-3-glucose(or galactose)–catechin.

Table 1. LC-MS Analysis of the Anthocyanins Present in Extract A

peak	anthocyanin	*m*/*z* (M^+)	fragments (*m*/*z*)
1	delphinidin-3-galactose	465	303; 162
2	delphinidin-3-glucose	465	303; 162
3	cyanidin-3-galactose	449	287; 162
4	delphinidin-3-arabinose	435	303; 132
5	cyanidin-3-glucose	449	287; 162
6	petunidin-3-galactose	479	317; 162
7	cyanidin-3-arabinose	419	287; 132
8	petunidin-3-glucose	479	317; 162
9	peonidin-3-galactose	463	301; 162
10	petunidin-3-arabinose	449	331; 162
11	peonidin-3-glucose	463	301; 162
12	malvidin-3-galactose	493	331; 162
13	peonidin-3-arabinose	433	301; 132
14	malvidin-3-glucose	493	331; 162
15	malvidin-3-arabinose	463	331; 132

Table 2. LC-MS Analysis of the Anthocyanin–Pyruvic Acid Adducts Present in Extract B

peak	anthocyanin–pyruvic acid (py) adduct	*m*/*z* (M^+)	fragments (*m*/*z*)
1	delphinidin-3-galactose(or glucose)–py	533	371; 162
2	delphinidin-3-arabinose–py	503	371; 132
3	cyanidin-3-galactose(or glucose)–py	517	355; 162
4	cyanidin-3-arabinose–py	487	355; 132
5	petunidin-3-galactose(or glucose)–py	547	415; 162
6	petunidin-3-arabinose–py	517	415; 132
7	peonidin-3- galactose(or glucose)–py	531	369; 162
8	peonidin-3-arabinose–py	501	369; 132
9	malvidin-3-galactose–py	561	399; 162
10	malvidin-3-glucose–py	561	399; 162
11	malvidin-3-arabinose–py	531	399; 132

induce a decrease of the absorbance measured at 515 nm, which indicates the scavenging potential of the extracts. It can be seen from the results shown in **Table 5** that there is an increasing trend of the radical scavenging capacity with the increase of the flavonoid and anthocyanin contents of the extracts (C > A > B). This is in agreement with the fact that these substances are responsible for much of the antioxidant capacity of fruits. Furthermore, the reducing power of the extracts was assessed using the FRAP method. Extracts A and B did not show any relevant differences between them, whereas extract C was found to have the highest reducing capacity. Overall, among the extracts studied with these in vitro techniques, extract C was found to have the highest scavenging activity and antioxidant capacity (**Table 5**). Besides the high levels of the pigments present in this extract, this feature could be attributed to the structure of the pigments (portisins) as a result of the extended conjugation of π electrons, which could easily stabilize the radical scavenged throughout the structure (**Figure 3**) (*27*). Concerning any structure−activity relationship, the *o*-dihydroxy groups in the B-ring and the hydroxyl group in the C-ring are usually related to the antioxidant properties of flavonoids (including anthocyanins). In light of this, the structure of the vinylpyranoanthocyanin−catechins present in extract C is thought to increase the overall antioxidant capacity of the extract due to those structural features. Indeed, these latter compounds possess more *o*-dihydroxy groups than the pigments present in extracts A and B, as well as an additional hydroxyl group in the C-ring of the catechin moiety.

Oxidation of Soybean PC Liposomes. Liposomes were used in this study as they are usually employed to mimic biological targets (e.g., cellular membranes). The evaluation of the antioxidant capacity of the extracts, against oxidation of soybean

Table 3. LC-MS Analysis of the Vinylpyrano–Anthocyanin–Catechins (Portisins) Present in Extract C

peak	vinylpyrano–anthocyanin–catechin (Cat)	*m*/*z* (M^+)	fragments (*m*/*z*)
1	vinylpyrano–delphinidin–3-galactose(or glucose)–Cat	803	641; 489
2	vinylpyrano–delphinidin–3-galactose(or glucose)–Cat	803	641; 489
3	vinylpyrano–cyanidin–3-galactose(or glucose)–Cat	787	625; 473
4	vinylpyrano–cyanidin–3-arabinose–Cat	757	625; 473
5	vinylpyrano–petunidin–3-galactose(or glucose)–Cat	817	655; 503
6	vinylpyrano–petunidin–3-arabinose–Cat	787	655; 503
7	vinylpyrano–peonidin–3-–galactose(or glucose)–Cat	801	639; 487
8	vinylpyrano–malvidin–3-arabinose–Cat	801	669; 517
9	vinylpyrano–malvidin–3-galactose(or glucose)–Cat	831	669; 517

Table 4. Contents of Total Phenolics, Total Flavonoids, and Total Pigments of a Blueberry (*V. myrtillus*) Extract (A) and Its Respective Anthocyanidin–Pyruvic Acid Adduct (B) and Vinylpyranoanthocyanidinanin–Catechin (C) Extracts[a]

extract	total phenolics (mg/L)	total flavonoids (mg/L)	total pigments (mg/L)
A	257.9 ± 8.5a	153.1 ± 9.3a	78.8 ± 4.0a
B	320.4 ± 7.5b	149.3 ± 7.1a	39.8 ± 2.0b
C	318.1 ± 13.8b	287.3 ± 8.7b	100.7 ± 5.0c

[a] Mean values followed by different letters in each column are significantly different at $P < 0.001$.

Table 5. Antiradical Activity and Reducing Power of the Blueberry (*V. myrtillus*) Extract A, Extract B, and Extract C Assessed by DPPH Method and FRAP Method, Respectively[a]

	extract A	extract B	extract C
DPPH	5.366 ± 0.803a	4.130 ± 0.390b	6.440 ± 0.756a
FRAP	0.736 ± 0.064a	0.840 ± 0.058a	1.766 ± 0.239b

[a] Mean values followed by different letters in each row are significantly different at $P < 0.001$ and at $P < 0.05$ if followed by the same letters.

PC liposomes, was performed using AAPH as a peroxidation initiator. Despite not being a relevant biomolecule, AAPH has been thoroughly used in this kind of study. Indeed, it undergoes thermal decomposition at 37 °C and generates peroxyl radicals at a known and constant rate, thereby allowing reproducible and quantitative analysis of the extracts' antioxidant capacity (*28*). The antioxidant/antiradical capacity of the extracts was assessed at the initial and propagation stages of oxidation through the measurement of oxygen consumption. Their influence in a further stage of oxidation was evaluated by measuring the conjugated diene formation. The results are expressed relative to the ones obtained with Trolox. The generation of peroxyl radicals from AAPH induces a significant oxidation of phosphatidylcholine, because they are able to subtract hydrogen atoms from polyunsaturated acyl chains, yielding lipid radicals that lead to the propagation chain reaction (*29*).

The data obtained from the oxygen consumption assays showed that all of the extracts scavenged efficiently the peroxyl radicals generated in the aqueous phase compared to the control (without any extract), as can be seen through the lag phase produced in the oxygen consumption graph (**Figure 4**). Nevertheless, it should be noted that the induction times of the lag phase for the blueberry extracts A and B are similar and much lower than the one for extract C. This effect is easily perceived in **Figure 5**, which displays the relevance of this feature relative to Trolox.

The formation of conjugated dienes, composed of hydroperoxides and respective degradation products, was monitored by measuring their characteristic absorbance at 233 nm. When lipids are protected in the presence of antioxidant, the oxidation induction time will vary with the antioxidative capacity of the compound tested. The phenolic compounds present in the extracts are thought to trap AAPH-derived peroxyl radicals and inhibit the initiation of lipid peroxidation, but, if located at the surface of the liposome, they may also quench liposome-derived peroxyl radicals, inhibiting the chain propagation (*30*). Consequently, the formation of conjugated diene compounds was inhibited until exhaustion of the antioxidant compounds. Extract C was shown to possess the highest capacity to inhibit

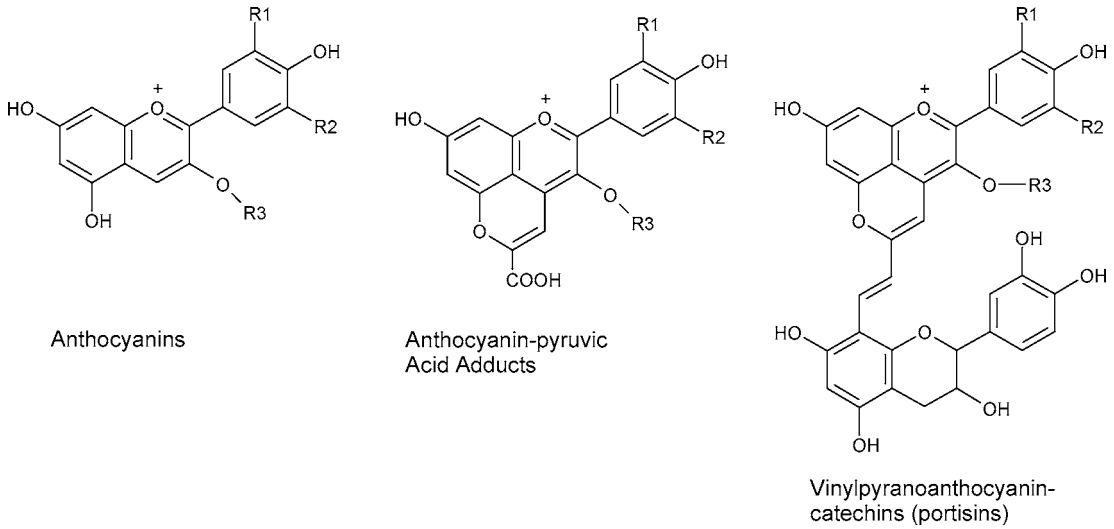

Figure 3. General structure of blueberry (*V. myrtillus*) anthocyanidin, anthocyanidinanin–pyruvic acid adducts, and portisins present in extracts A, B, and C, respectively. R1 and R2, independently of each other, are H, OH, or OMe. R3 is glucoseose, galactoseactose, or arabinosebinose.

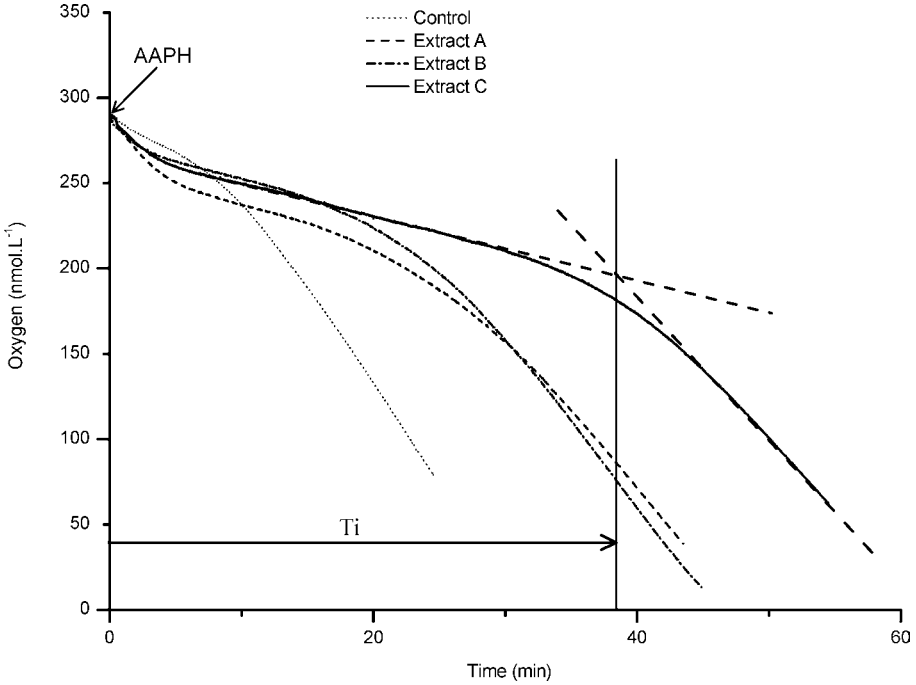

Figure 4. Oxygen consumption during the oxidation of soybean PC liposome membranes (LUV) (340 μM) induced by AAPH (10 mM) in the absence of antioxidants (control assay) and in the presence of the prepared blueberry (*V. myrtillus*) extracts A–C. Ti, induction time.

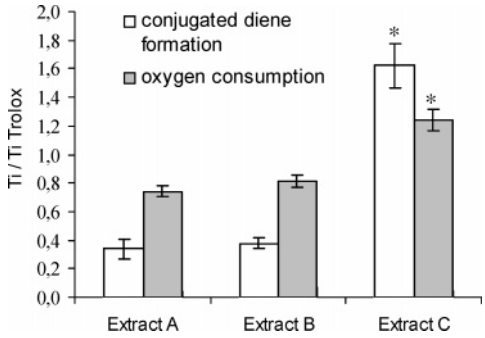

Figure 5. Inhibition of AAPH-initiated oxidation in soybean PC liposome membranes (LUV) by blueberry (*V. myrtillus*) extracts A–C measured by oxygen consumption and conjugated diene formation. Columns represent mean values ± standard deviation (SD). *, $P < 0.001$ versus extracts A and B.

conjugated diene formation by extending the initial induction time (**Figure 5**). The other two extracts displayed an increase of the induction time compared to the control (without any extract), but not as significant as that of extract C. These results agree with the ones yielded from the oxygen consumption assay. A structure−activity relationship could also be hypothesized as described above for the DPPH and FRAP assays.

The anthocyanin-derived blueberry extracts (especially extract C) revealed a higher antioxidant/antiradical capacity than the original blueberry extract (A). This trend is closely related to the flavonoid composition of the extracts, especially regarding the anthocyanin-derived pigment type and amounts. The structures of the pigments present in extracts B and C are thought to highly contribute to the increase of the overall antiradical and antioxidant capacities of the extracts. Studies involving the structure−activity relationship of pure individual anthocyanin-derived pigments and the influence of stereochemical conformation in their antioxidant activities will be performed with the aim to better understand the results obtained herein, especially at a molecular level. The purpose of the synthesis of these extracts is to possibly use them as colorants in the food industry as they present interesting and unusual colors (namely, orange and bluish hues). The in vitro antioxidant properties displayed by these extracts emphasize the interest of their putative application. Indeed, lipid oxidation remains an important issue for the food industry and human health. The oxidative damage induced by free radicals or other reactive oxygen species (ROS) toward biomolecules such as lipids and cell membranes is thought to be correlated with several degenerative diseases (cancer, cardiovascular diseases, immune system deficiency, inflammation, etc.) (*31*−*33*). Nevertheless, the conclusions drawn from this work cannot be extended to in vivo conditions due to the scarce data regarding the bioavailability of these phenolic compounds in the organism.

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