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Antimutagenic and Antioxidant Properties of Phenolic Fractions from Andean Purple Corn (*Zea mays* L.)

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The antimutagenic and antioxidant properties of various phenolic fractions obtained from Andean purple corn were examined by the Ames test and the DPPH antiradical assay. An anthocyanin-rich water fraction (WF) and an ethyl acetate fraction (EAF) showed a dose-dependent antimutagenic behavior against the food mutagen Trp-P-1 with IC_{50} values of 321.7 ± 21.36 and 95.2 ± 10.95 μg of chlorogenic acid equiv/plate, respectively, indicating that EAF was a more potent antimutagen. The antioxidant activities for WF and EAF were 1.019 ± 0.05 and 0.838 ± 0.11 μg of Trolox equiv/μg of phenolics, respectively. Further fractionation of WF and EAF revealed an ethyl acetate subfraction, EA-IV, with high antimutagen potency that contained a quercetin derivative. The mechanism of antimutagenic action of the WF is predominantly a blocking effect on the S-9 Mix activation system of the mutagen, whereas for the EAF, it is a dual mechanism involving blocking of the S-9 Mix and a scavenging action on Trp-P-1 electrophiles.

KEYWORDS: Purple corn extract; phenolic compounds; antimutagenic activity; antioxidant activity; mechanism of antimutagenic action

INTRODUCTION

The generation of heterocyclic aromatic amines that are highly mutagenic in the *salmonella*/reversion assay or Ames test during cooking of proteinaceous foods is very well documented (*1*), including IQ (2 amino-3-methylimidazo [4,5-f] quinoline), MeIQx (2 amino-3,8-dimethylimidazo [4,5-f] quinoxaline), PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine), and Trp-P-1 (3-amino-1,4-dimethyl-5h-pyrido[4,3-b]indole). The heterocyclic amine Trp-P-1 has been involved in several types of DNA damage that lead to genetic alterations. The accumulation of genetic alterations can lead normal cells to become cancer cells (*3*). Trp-P-1 is a direct acting mutagen toward *Salmonella* Typhimurium. The *N*-hydroxy form of Trp-P-1 is metabolically activated to N-O-acetyl-Trp-P-1, which damages DNA by the formation of DNA adducts that can end up in genetic mutations. Trp-P-1 can also produce reactive oxygen species (ROS) that can cause oxidative DNA damage (*4*).

Food is a complex mixture of different components, some of which act as antimutagens (*5*). The ability of different vegetable and fruit juices to act as antimutagens has been tested (*1*, *5*), and phenolic compounds present in such juices were considered responsible for the antimutagenic activity (*6*−*8*). The antimutagenic action of phenolic compounds against the food mutagen Trp-P-1 has been reported for sweet potato (*9*).

Purple corn (maiz morado in Spanish) has been used by people from the Andes to color foods and beverages for centuries. In addition, a refreshing drink called "chicha morada" is prepared by immersing the cobs in boiling water. The already-known antioxidant and anticarcinogenic properties of purple corn, in addition to its coloring attributes, make it an attractive crop for the Nutraceutical and Functional Food Market (*10*−*12*). More recently, purple corn extracts were tested for anti-obesity activity and amelioration of hyperglycemia (*13*). In addition, purple corn color did not show any hepatotoxicity or nephrotoxicity in mice depleted of glutathione by pretreatment with buthionine sulfoximine at a dose of 4500 mg/kg (*14*).

Previous investigations of purple corn bioactivities have mainly been focused on its anthocyanins. A purple corn color extract has been shown to inhibit colorectal carcinogenesis in male F344 rats pretreated with 1,2-dimethylhydrazine and PhIP (*10*). The inhibition was attributed only to anthocyanins present in the purple corn color. However, purple corn has a significant amount of phenolic compounds other than anthocyanins, including mainly phenolic acids and flavonols (*15*). The roles of other phenolic compounds present in the purple corn color extract have been ignored up to now.

The objective of this study was to fractionate the different phenolic compounds present in Andean purple corn and to determine their specific roles as antimutagens and antioxidants. The results from this work will provide important information for the food industry with respect to the use of the purple corn extracts not only as a colorant but also as a source of health-promoting compounds.

MATERIALS AND METHODS

Sample Material, Standards, and Reagents. Purple corn extract (PCE) was kindly provided by Fitofarma (Lima, Peru). The PCE was

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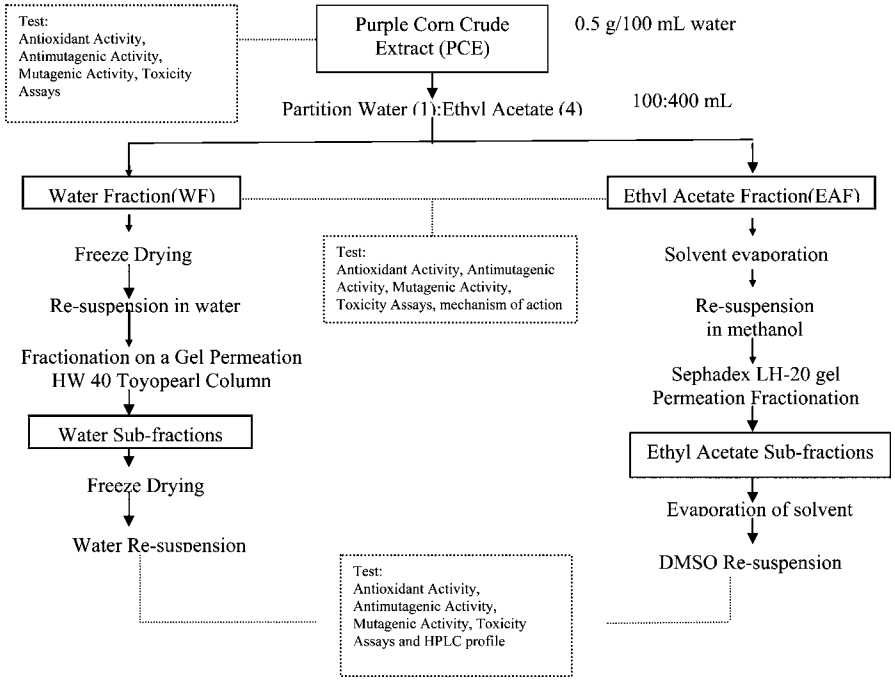

Figure 1. Fractionation of phenolic compounds from a purple corn crude extract (PCE).

obtained from purple corn grown in Arequipa, Peru. The powder extract was prepared by extraction of ground cobs (mesh 60) in a 60% aqueous ethanol solution at room temperature for 48 h (1 kg of cobs/7 L of solvent). The obtained extract was filtered and spray-dried (180 °C inlet and 85 °C outlet temperatures) using maltodextrins as carrier (0.5 kg of maltodextrin/100 L of extract). One kilogram of dried PCE contained ~40% maltodextrin.

For the spectrophotometric procedures, Folin−Ciocalteu reagent, sodium carbonate (Na_2CO_3), chlorogenic acid, Trolox, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO). Methanol was reagent grade, and Nanopure water was used. For the Ames test, *Salmonella* Typhimurium TA98 (TA98) and the S-9 Mix activation system were purchased from Molecular Toxicology Inc. (Boone, NC). The S-9 Mix was composed of liophilized Aroclor 1254 induced-male Sprague Dawley rat liver fraction S9 (40 mg of protein/mL) at a concentration of 0.04 mL of fraction S9/mL of S-9 Mix, NADPH Regenesys A (composed of 5 mM glucose-6-phosphate, 8 mM $MgCl_2$, and 33 mM KCl in 100 mM sodium phosphate buffer, pH 7.4), and NADPH Regenesys B (composed of 4 mM NAD). $MgSO_4 \cdot 7H_2O$, citric acid$\cdot H_2O$, K_2HPO_4, $NaNH_4HPO_4 \cdot 4H_2O$, sodium chloride, ampicillin, and glucose were purchased from Fisher Scientific (Fair Lawn, NJ). D-Biotin and L-histidine$\cdot$HCl$\cdot H_2O$ were obtained from Sigma Chemical Co. (St. Louis, MO). Agar was purchased from Difco (Kansas, MO), and Oxoid no. 2 nutrient broth was obtained from Oxoid (Ogdensburg, NY). Reagent-grade DMSO was used. The food mutagen Trp-P-1 was purchased from Toronto Research Chemicals Inc. (Downsview, Canada).

For the phenolic compound fractionation, Toyopearl HW-40 and Sephadex LH-20 were purchased from Sigma Chemical Co. (St. Louis, MO). Reagent-grade methanol and ethyl acetate were used. For the HPLC procedure, cyanidin-3-glucoside, pelargonidin-3-glucoside, peonidin-3-glucoside, delphinidin-3-glucoside, petunidin-3-glucoside, and malvidin-3-glucoside were purchased from Polyphenols Laboratories AS (Sandnes, Norway). Cyanidin, pelargonidin, and peonidin were obtained from ChromaDex (Santa Clara, CA). Phenolic acids (vanillic, *p*-coumaric, protocatechuic, ferulic, and benzoic acids), flavonols (quercetin, rutin, myricetin, kaempferol), flavanone (hesperidin), and flavones (apigenin, luteolin) were purchased from Sigma Chemical Co. (St. Louis, MO).

Isolation of a Total Phenolic Fraction (TPF). A TPF from a PCE was obtained by using reverse-phase C_{18} cartridges (Sep-Pak cartridges) (Waters Corp., Milford, MA). A 0.2-g sample of PCE was dissolved in 500 mL of distilled water. The Sep-Pak cartridge was conditioned with 50 mL of methanol followed by the addition of 50 mL of distilled water. The sample was loaded, and the phenolic compounds already attached in the matrix were eluted with 100 mL of methanol containing 0.1% TFA. Water-soluble impurities were eluted during loading of the cartridge. Methanol was evaporated in a rotoevaporator (Büchi, Switzerland) under vacuum at 40 °C and 240 mbar of pressure and then resuspended in distilled water, frozen at −80 °C, and freeze-dried in a freeze-dryer from FTS Systems, Inc. (Stone Ridge, NY) at −50 °C and 200 μmHg of pressure.

Fractionation of Phenolic Compounds. A detailed diagram of the fractionation of the PCE phenolic compounds is presented in **Figure 1**. Fractionation of 0.5 g of powder PCE was accomplished following dissolution in 100 mL of distilled water. The PCE solution was combined with 400 mL of ethyl acetate in a separation funnel. The mixture was agitated and left to stand until two phases were observed. The anthocyanin-rich phase was freeze-dried (at −50 °C and 200 μmHg of pressure), and the ethyl acetate phase was concentrated under vacuum (at 40 °C and 240 mbar of pressure) and resuspended in DMSO or methanol.

The anthocyanin-rich water fraction (WF) was further fractionated using a 50 cm × 2.5 cm Toyopearl HW40 gel permeation column preequilibrated with 20% methanol containing 0.1% TFA (20 h at 2 mL/min). A total of 0.1 g of the powdered WF was dissolved in 5 mL of 20% methanol containing 0.1% TFA. Compounds present on the column were eluted with 300 mL of 20% methanol, 600 mL of 40% methanol, 300 mL of 60% methanol, 300 mL of 80% methanol, and 750 mL of 100% methanol at a flow rate of 1 mL/min, and 180 fractions (12.5 mL/each) were collected. Compound elution was monitored at 280 nm and 520 nm using a Hewlett-Packard 8452A diode-array spectrophotometer. The subfractions obtained were concentrated to evaporate the solvent and then resuspended in water and freeze-dried.

Following solvent removal, the ethyl acetate fraction (EAF) was resuspended in methanol and loaded on a 50 × 2.5 cm Sephadex LH-20 column previously equilibrated with methanol for 12−20 h at 1.0 mL/min. Individual compounds were eluted at a flow rate of 0.5 mL/min, and 200 fractions (5 mL/each) were collected. Compound elution was monitored in each individual fraction at several wavelengths, namely, 280, 320, 360, and 520 nm, using a Hewlett-Packard 8452A diode-array spectrophotometer. The subfractions obtained were concentrated by evaporating the solvent and were then resuspended in DMSO. Total phenolics were quantified in each fraction by an adaptation of the Folin−Ciocalteau method and are expressed as chlorogenic equivalents (*16*). Antioxidant activity was quantified by

the 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method and are expressed as Trolox equivalents (*17*). The Folin−Ciocalteau phenolic contents of PCE and the C-18-purified TPF were 21.3 ± 0.5 and 51.7 ± 0.9 g of chlorogenic acid equiv/100 g of PCE or TPF powder, respectively, and the phenolic contents of WF and EAF obtained from a purified TPF were 59.7 ± 0.8 and 34.0 ± 0.3 g of chlorogenic acid equiv/100 g of WF or EAF fraction, respectively.

Toxicity and Mutagenic and Antimutagenic Activities. The Ames test (*18*) was used to test the mutagenic and antimutagenic activities of the PCE, TPF, WF, and EAF and their respective subfractions. *Salmonella* Typhimurium TA98, "S-9 Mix activation system", and the Trp-P-1 food mutagen were included in the test. Toxicity assays under the same conditions as used for the Ames test were performed to determine the maximum concentrations of chemical compounds (expressed as micrograms of chlorogenic acid equivalents per plate) that could be added without exerting any toxic effect on the bacteria used in the Ames test. A lyophilized disk of TA98 was added to 25 mL of nutrient broth (Oxoid no. 2) and incubated for 10−16 h at 37 °C and under agitation at 200 rpm in a shaking incubator. Optimal assay conditions existed when the cultures exhibited optical densities of 1.2−1.4 at 660 nm. The procedure included incubation in a sterile 12 × 75 mm tube with 0.1 mL of DMSO, 0.1 mL of extract or chemical substance to be tested, 0.5 mL of S-9 Mix activation system (20 μL/plate), and 0.1 mL of TA98. The mix was shaken gently and incubated for 20 min at 37 °C. Then, 2 mL of top agar was added with thorough mixing and serially diluted with peptone water to create logarithmic differences in cell concentrations. Diluted test suspensions were plated on nutrient agar plates using the drop surface technique and incubated for 12−15 h at 37 °C. Well-separated colonies on 20 μL drops were counted having colonies between 3 and 30 using a magnifier. Results are expressed as cfu/mL.

To test mutagenic activity, 0.1 mL of DMSO (mutagen solvent), 0.1 mL of compound to be tested, 0.5 mL of S-9 Mix activation system, and 0.1 mL of TA98 were incubated together for 20 min at 37 °C. Then, 2 mL of top agar was added, and the whole solution thoroughly mixed. The solution was poured on minimal-glucose agar plates, allowed to harden, and incubated at 37 °C for 48 h. Under these conditions, the *Salmonella* Typhimurium TA98, which carries a defective or mutant gene making it unable to synthesize histidine, can regain its gene function, and revertant cells will grow in the histidine-lacking medium. An assay blank was formed by combining 0.1 mL of DMSO with 0.1 mL of extract solvent (water or DMSO) prior to addition of 0.5 mL of S-9 Mix activation system and 0.1 mL TA98. Blank incubations were used to determine the spontaneous rate of bacterial mutation (revertants). Reversion rates in blank incubations were used as negative controls for the assay. The number of spontaneous revertants was expected to be between 20 and 60 colonies per plate. The spontaneous reversion rate was subtracted from the raw treatment values prior to statistical analysis. Mutagenic activity was tested for the PCE, TPF, WF, EAF and their respective subfractions.

To test antimutagenic activity, 0.1 mL of Trp-P-1 (0.75 μg/mL solution), 0.1 mL of the compound to be tested, 0.5 mL of S-9 Mix activation system, and 0.1 mL of TA98 were placed in a sterile 12 × 75 mm tube and preincubated for 20 min at 37 °C. Then, 2 mL of molten top agar was added, and the solution was thoroughly mixed and poured on minimal-glucose agar plates and incubated at 37 °C for 48 h, after which revertants were counted. A positive control consisting of 0.1 mL of Trp-P-1, 0.1 mL of extract solvent, 0.5 mL of S-9 Mix, and 0.1 mL of TA98 treated as above was included in the test. A control used 0.075 μg of Trp-P-1 per plate and normally showed 794 ± 189 revertants per plate.

Antimutagenic activity was calculated as percentage inhibition of mutagenic activity

$$\text{Inhibition (\%)} = 100 \times \left[1 - \left(\frac{\text{treatment} - \text{negative control}}{\text{positive control} - \text{negative control}}\right)\right]$$

For PCE, TPF, WF, and EAF, antimutagenic activity was tested in the range 0−850 μg of chlorogenic acid equiv/plate. However, for the WF and EAF subfractions, antimutagenic activity was assayed only at a specific concentration determined from the dose−response curves for the WF and EAF. IC_{50} values were determined from dose−response curves and corresponded to the concentration of the tested compound (micrograms of chlorogenic acid equivalents per plate) required for 50% inhibition of mutagenic activity.

Mechanism of Antimutagenic Action. To account for the mechanism of antimutagenic action, the approach described by Hour and others (*19*) was utilized for the WF, EAF, and the subfraction that showed the highest antimutagenic activity. The treatments tested were as follows:

(I) Blank. 0.1 mL Trp-P-1 (0.75 μg/mL) was incubated with 0.5 mL of S-9 Mix, 0.1 mL of TA 98, and 0.1 mL of DMSO at 37 °C for 30 min without the antimutagen.

(II) Mutagen and Then Antimutagen. S-9 Mix (0.5 mL) and TA98 (0.1 mL) were mixed with 0.1 mL of Trp-P-1 (0.75 μg/mL) at 37 °C for 15 min, and then 0.1 mL of tested compound was added and incubated at 37 °C for an additional 15 min.

(III) Antimutagen and Then Mutagen. S-9 Mix (0.5 mL) and TA98 (0.1 mL) were preincubated with 0.1 mL of tested compound at 37 °C for 15 min, and then 0.1 mL of Trp-P-1 (0.75 μg/mL) was added and incubated for an additional 15 min at the same temperature.

(IV) All Components and Then Bacteria. Trp-P-1 (0.1 mL), the tested compound (0.1 mL), and S-9 Mix (0.5 mL) were incubated for 15 min at 37 °C, and then 0.1 mL of TA98 was added and incubated at the same temperature for an additional 15 min.

(V) All Components Simultaneously. Trp-P-1 (0.1 mL), the tested compound (0.1 mL), TA98 (0.1 mL), and S-9 Mix (0.5 mL) were incubated at 37 °C for 30 min.

The WF was tested at a concentration equivalent to 200 μg of chlorogenic acid/plate. The EAF was tested at a concentration equivalent to 90 μg of chlorogenic acid/plate. The subfraction with the highest antimutagenic activity was tested at a concentration equivalent to 50 μg of chlorogenic acid/plate.

HPLC Analysis. Phenolic compounds were separated using a binary Waters 515 HPLC system, a Waters 717 plus autosampler automated gradient controller, an SP8792 temperature controller, and a Waters 996 photodiode-array detector. For peak integration, Millenium^{32} software from Waters (Milford, MA) was used. An Atlantis C_{18} 5-μm, 4.6 mm × 150 mm column and a 4.6 mm × 20 mm guard column were used for the separation of phenolic compounds. The mobile phase was composed of solvent A (Nanopure water adjusted to pH 2.3 with 2 N HCl) and solvent B (HPLC-grade acetonitrile). The elution was as follows: isocratic conditions from 0 to 5 min with 85% A and 15% B; linear gradient conditions from 5 to 30 min starting with 85% A and ending with 0% and starting with 15% B and ending with 100%; and then, isocratic conditions from 30 to 35 min with 0% A and 100% B. The flow rate was 1 mL/min, and a 10-μL sample was injected (*20*). The temperature of the column was kept at 35 °C. Phenolic compounds were identified on the basis of retention time and UV−visible spectral data compared to known standards as described by Pedreschi and Cisneros-Zevallos (*15*). Acylated forms were deduced by performing basic hydrolysis according to the method described by Pedreschi and Cisneros-Zevallos (*15*).

Statistical Analysis. In all cases, five replicates were used to test mutagenic and antimutagenic activities. For the other assays, six replicates were used. Replicates were obtained from several fractionations of the PCE. Results were processed by using the one-way variance analysis (ANOVA). Differences at $p < 0.05$ were considered to be significant. A Tukey for comparison of multiple means and a χ^2 test for comparison of dose−response curves were used. SPSS software (SPSS Inc., 2002) was used to run all specific statistical analysis.

RESULTS AND DISCUSSION

Purple Corn Extract (PCE) and the C-18 Purified Phenolic Fraction (TPF). The purple corn extract (PCE) and the C-18-purified phenolic fraction (TPF) showed antimutagenic activities with similar trends of dose−response inhibitions against the activated mutagen Trp-P-1 for a range of phenolic concentrations of 0−850 μg/plate (**Figure 2**a). The curves were

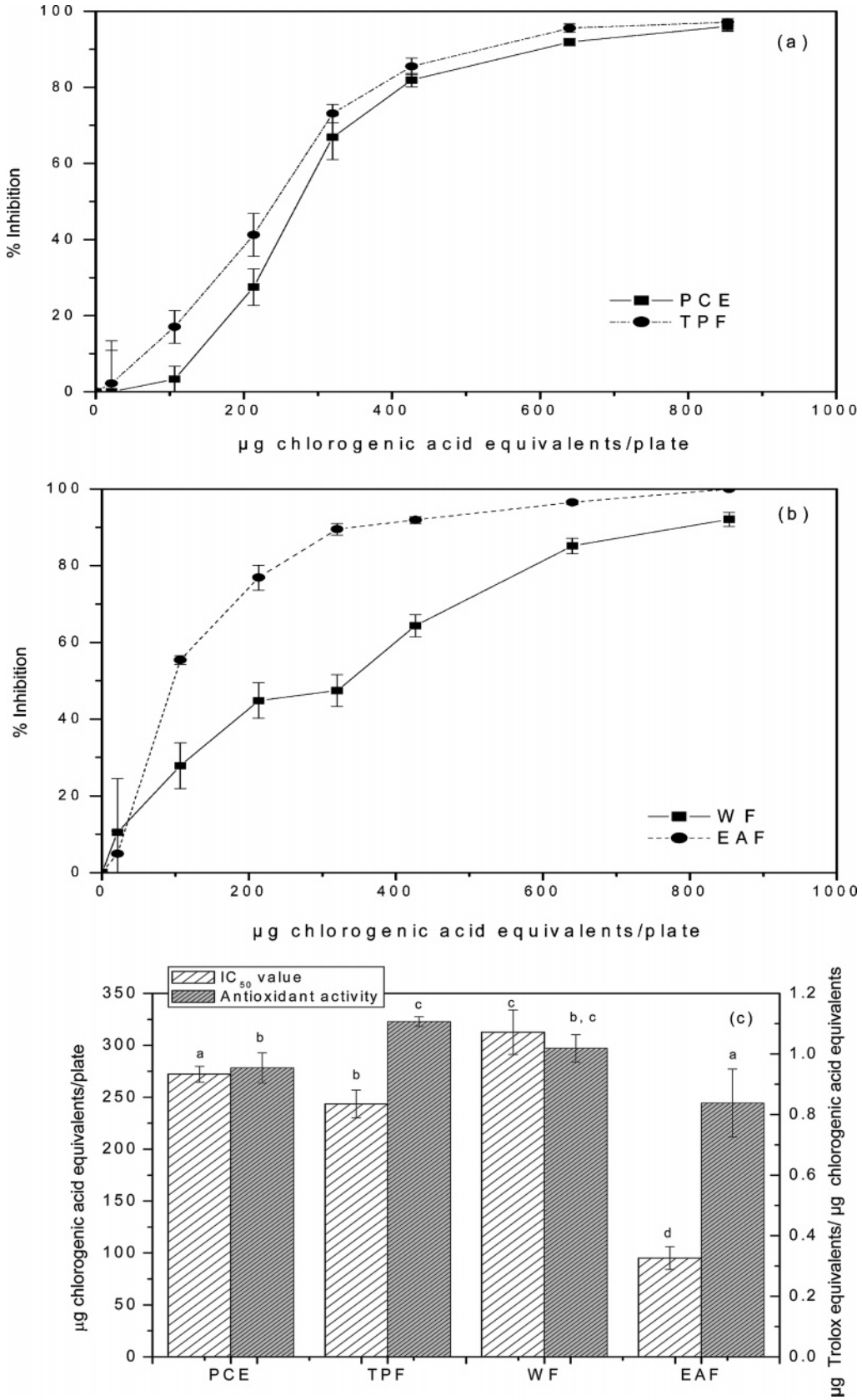

Figure 2. Inhibition of mutagenic activity on TA98 against the food mutagen Trp-P-1 by (a) PCE and TPF and (b) WF and EAF in the range 0–850 μg of chlorogenic acid equiv/plate. (c) IC_{50} and antioxidant values for PCE, TPF, WF, and EAF.

slightly different (χ^2 test, $p < 0.05$), and the gap observed could be due to a slight inhibitory effect of other compounds present in the PCE such as maltodextrins. The antimutagenic activities of PCE and TPF reached maxima of 96.02 ± 1.2% and 97.2 ± 0.2%, respectively, for a phenolic concentration of 850 μg/plate. No mutagenic (~20−35 revertants/plate) or cell toxicity effects (~10^6−10^7 cfu/mL microbial growth) were observed in the range of concentrations tested. The antimutagenic activity of phenolic compounds has been extensively reported (*7*−*9*, *21*−*24*); however, antimutagenic properties of purple corn due to

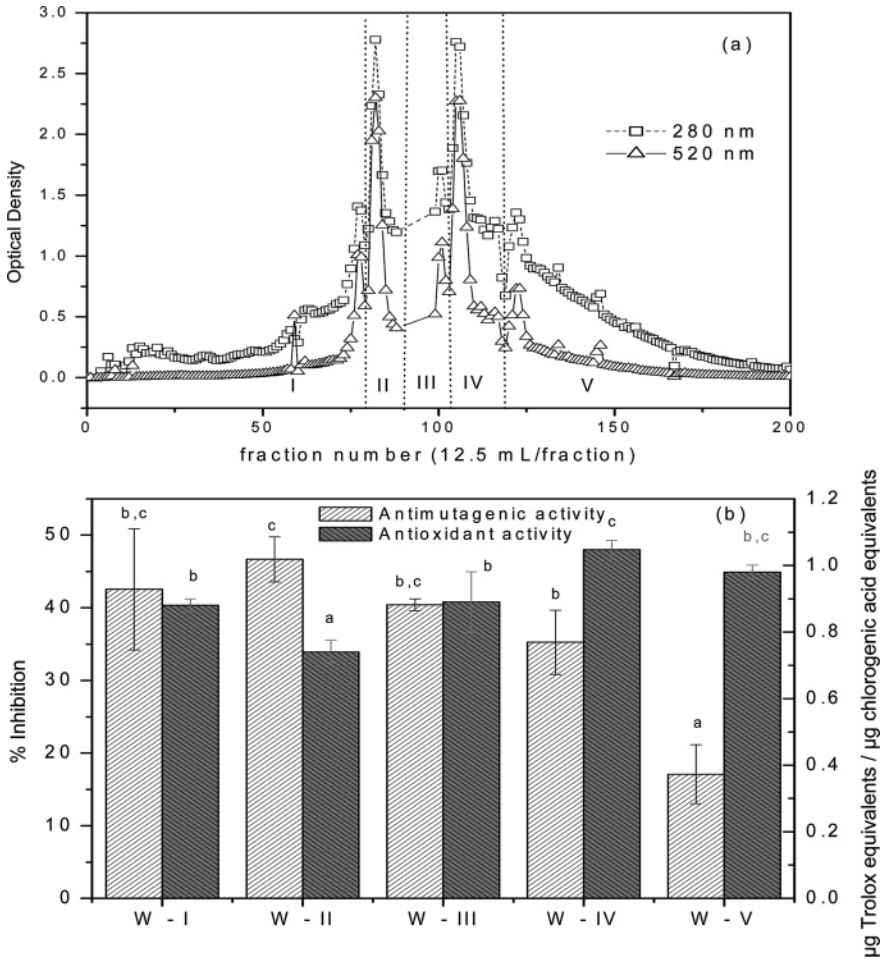

Figure 3. (a) Fractionation of the anthocyanin-rich WF on a Toyopearl HW-40 column and (b) antioxidant and percent inhibition of mutagenic activity for the water subfractions on TA98 against the food mutagen Trp-P-1. Antimutagenic activity was tested at a concentration of 200 μg of chlorogenic acid equiv/plate.

phenolic compounds have not been characterized before. Additionally, the specific antioxidant activities for PCE and TPF were determined as 0.954 ± 0.05 and 1.107 ± 0.016 μg of Trolox equiv/μg of phenolics, respectively. These antioxidant activities on a phenolic basis are similar to or higher than those of blueberries (*11*). There is a need to determine whether the antioxidant activity plays a role in the observed antimutagenic properties.

Water and Ethyl Acetate Phenolic Fractions from PCE. The two main fractions obtained by partition, the anthocyanin-rich WF and the EAF, showed dose-dependent antimutagenic activities in the range of concentrations used (**Figure 2b**). No mutagenic effects were observed for either fraction (~20−50 revertants/plate); however, toxic effects were seen for the EAF only at concentrations >320 μg/plate (a decrease from 10^7 to 10^5 cfu/mL). These results will restrict the use of the Ames test to a certain concentration range for the EAF, but they also suggest the possibility of investigating the type of phenolics present in the EAF as natural antimicrobials. Inhibitions of mutagenic activity against the food mutagen Trp-P-1 for WF and EAF were 92.09 ± 1.8% and 89.5 ± 1.6% for phenolic concentrations of 850 and 320 μg/plate, respectively.

The antioxidant activities for the WF and EAF was determined as 1.019 ± 0.05 and 0.838 ± 0.11 μg of Trolox/μg of phenolics, respectively. A higher antioxidant activity of the WF was expected, as this fraction is rich in anthocyanins and these types of phenolic compounds have been reported to have higher antioxidant activities than phenolic acids and flavonols mainly present in EAF (*25*, *26*).

The EAF seemed to contain the most bioactive antimutagens, requiring the lowest phenolic concentration to obtain a 50% inhibition in mutagenic activity (IC_{50} value of 95.2 ± 10.95 μg/plate) compared to PCE, TPF, and WF (Figure 2c). However, there was no clear relationship between antioxidant and antimutagenic activities. Further fractionation of the WF and EAF was performed to determine the specific phenolic compounds in the PCE associated with the responses.

WF Subfractionations. Fractionation of the anthocyanin-rich WF on a Toyopearl HW40 column yielded five subfractions (W-I, W-II, W-III, W-IV, and W-V), which were characterized at 280 and 520 nm (Figure 3a). The five subfractions showed no toxicity (~10^6 cfu/mL microbial growth) or mutagenic effects (~20−25 revertants/plate) when tested at 200 μg/plate. It was reported previously that anthocyanins do not exert toxic effects in the Ames test (*27*).

All of the water subfractions showed antimutagenic activities. The antimutagenic activities of subfractions W-I to W-IV were in similar ranges (~35−46%, not significantly different, $p > 0.05$) with fractions W-III and W-IV having slightly lower values. On the other hand, subfraction W-V was significantly lower ($p < 0.05$) and the least efficient antimutagen compared to the other subfractions (~17%, $p < 0.05$) (Figure 3b). The antimutagenic activity of anthocyanins using the Ames test has been reported previously for different crops and mutagens (*22*,

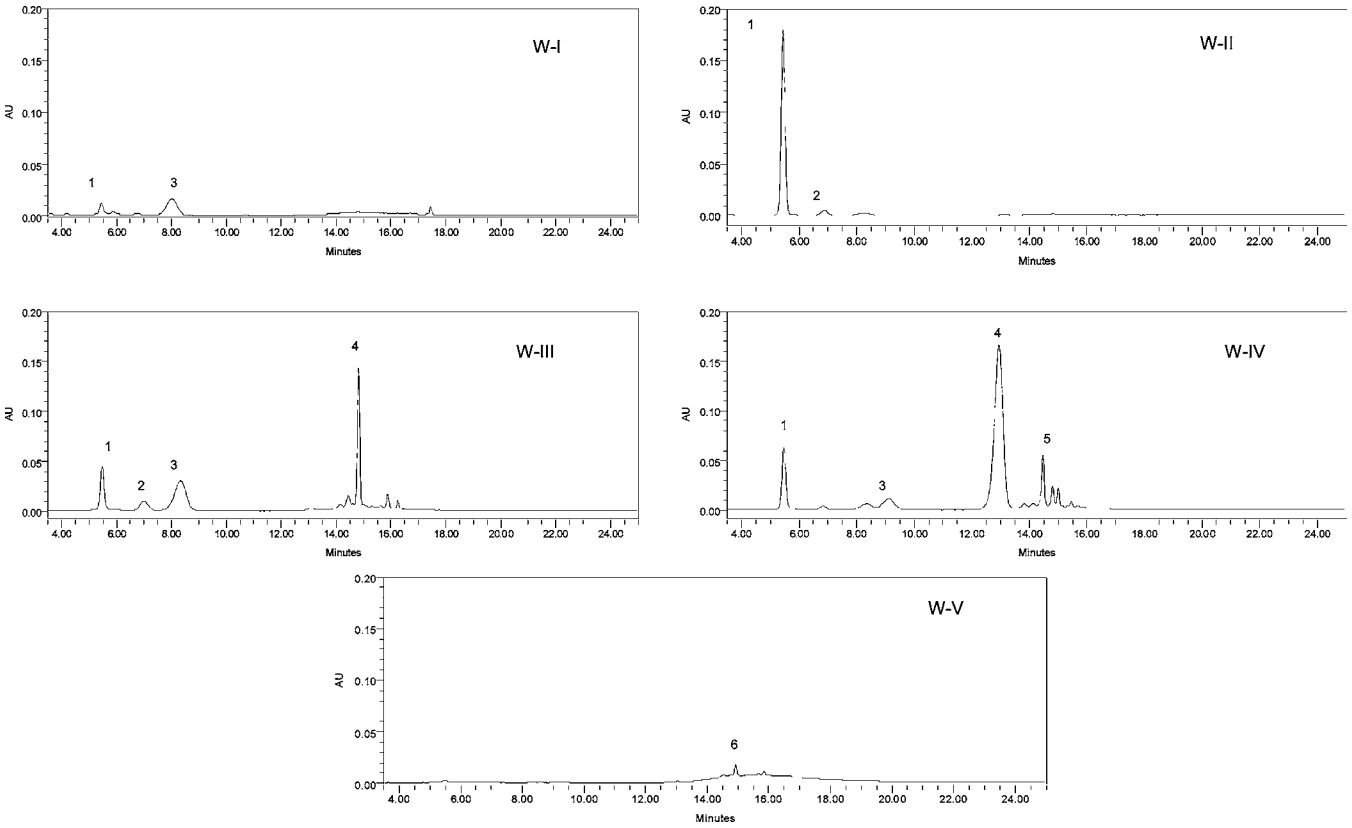

Figure 4. HPLC profile for the water subfractions at 520 nm. Samples were injected at a concentration of 200 μg of chlorogenic acid equiv/mL.

Table 1. Relative Percentage Contributions of the Different Peaks Obtained in the Water Subfractions

subfraction	peak no.	retention time (min)	λ_{max} (nm)	relative A_{520nm}[a] (%)	compound
W-I	1	5.45	202.4, 279.1, 515.8	18.93	cyanidin-3-glucoside
	3	8.033	198.9, 279.1, 515.8	81.07	peonidin-3-glucoside
W-II	1	5.45	202.4, 279.1, 515.8	95.49	cyanidin-3-glucoside
	2	6.891	201.3, 275.5, 508.5	4.51	pelargonidin3-glucoside
W-III	1	5.483	202.4, 279.1, 515.8	17.49	cyanidin-3-glucoside
	2	7.002	201.3, 275.5, 508.5	8.18	pelargonidin-3-glucoside
	3	8.335	198.9, 279.1, 515.8	37.89	peonidin-3-glucoside
	4	14.822	203.4, 279.1, 515.8	36.44	acylated cyanidin-3-glucoside
W-IV	1	5.478	202.4, 279.1, 515.8	12.49	cyanidin-3-glucoside
	3	9.114	198.9, 279.1, 515.8	4.41	peonidin-3-glucoside
	4	12.944	203.4, 279.1, 515.8	75.92	acylated cyanidin-3-glucoside
	5	14.476	202.4, 276.7, 508.5	7.18	acylated pelargonidin-3-glucoside
W-V	6	14.929	202.0, 279.1, 515.8	100	acylated peonidin-3-glucoside

[a] Percentage area is defined as the integrated area of a single peak divided by the total area of all integrated peaks.

24). In general, all five water subfractions showed similar antioxidant activities, ranging from ~0.880 to 1.050 μg of Trolox/μg of phenolics (**Figure 3b**), indicating that there was no clear relationship between anthocyanin antioxidant activities and antimutagenic properties.

The HPLC-DAD characterization revealed the presence of glucoside forms of cyanindin, peonidin, pelargonidin, and their respective acylated counterparts (Figure 4). Two or four anthocyanins were present in each subfraction with the exception of WF-V, which had only one type. WF subfractions showed mainly peonidin-3-glucoside in W-I (~81% relative A_{520nm}), cyanidin-3-glucoside in W-II (~95% relative A_{520nm}), peonidin-3-glucoside and acylated cyanidin-3-glucoside in W-III (~38% and 36% relative A_{520nm}, respectively), acylated cyanidin-3-glucoside in W-IV (~76% relative A_{520nm}), and acylated peonidin-3-glucoside in W-V (~100% relative A_{520nm}) (Table 1). These results suggest that the presence of acylated anthocyanins alone or in larger amounts in a mixture (e.g., 100% and 83% for W-V and W-IV, respectively) reduces the antimutagenic efficiency of the water subfractions in purple corn. On the other hand, the presence of only anthocyanin glucosides in a mixture (e.g., 100% for both W-I and W-II) can enhance the antimutagenic potency of the water subfractions. This enhanced potency is independent of the type of aglycons present in the glucoside forms of the W-I and W-II mixtures.

The obtained results differ from those reported for cyanidin- and peonidin-derived anthocyanins from purple-fleshed sweet potato (*9*). In that study, acylated anthocyanins showed similar or higher antimutagenic potencies compared to deacylated anthocyanins, relating them to the aglycons present and the acylating groups. For example, cyanindin-derived anthocyanins had a stronger potency than peonidin-derived anthocyanins, and the acylating groups, caffeic and ferulic acid, showed high antimutagenicity effects as well. It is likely that the observed

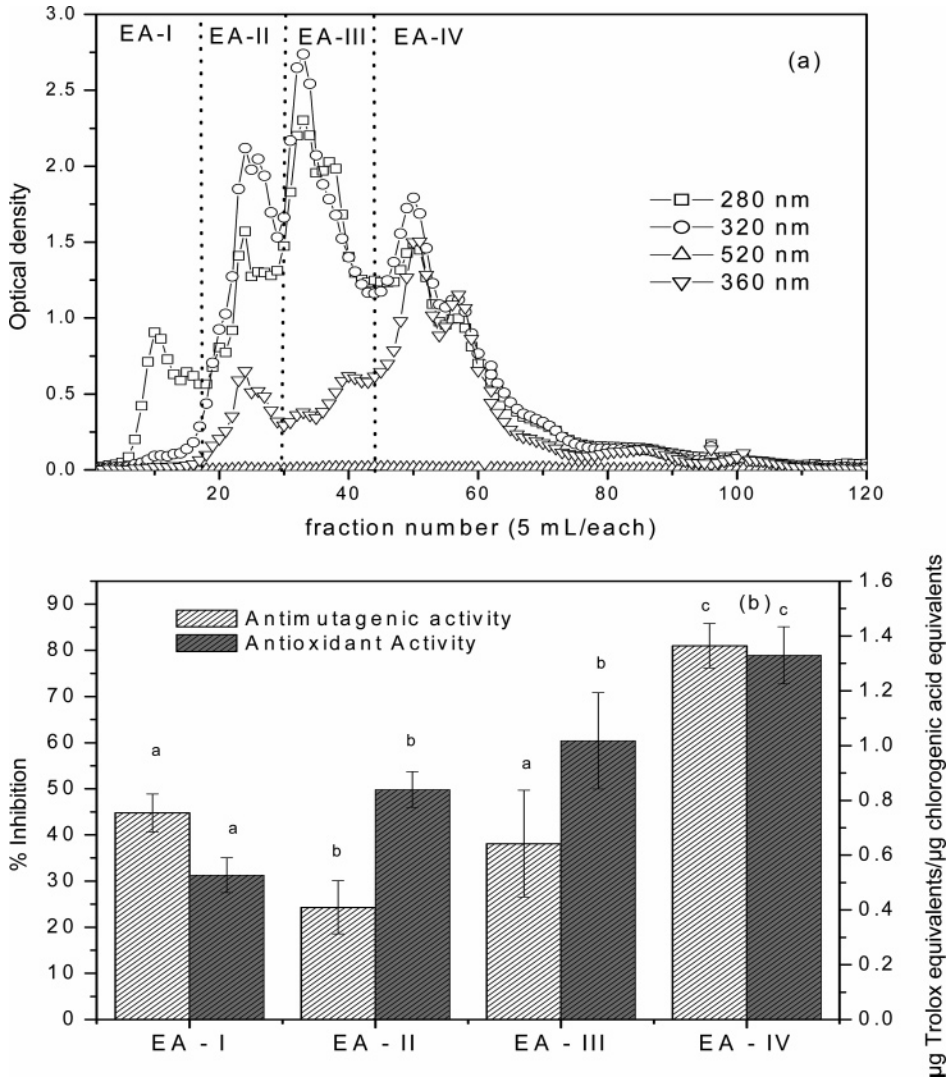

Figure 5. (a) Fractionation of the ethyl acetate fraction EAF on a Sephadex LH-20 column and (b) antioxidant and percent inhibition of mutagenic activity for the ethyl acetate subfractions on TA98 against the food mutagen Trp-P-1. Antimutagenic activity was tested at a concentration of 90 μg of chlorogenic acid equiv/plate.

differences are related to the structural forms of the anthocyanins in sweet potato, which have more complex glycoside groups and the presence of acylating phenolic acids, whereas purple corn anthocyanins have simple glucose attached and malonic acid as acylating groups (*16*, *28*, *29*).

More work is needed to understand the structure−bioactive property relationship of anthocyanins. Some features such as molecule polarity by the introduction of hydroxyl groups can reduce antimutagenic activity (*21*) while enhancing antioxidant activity (*30*, *31*).

EAF Subfractionations. Fractionation of EAF on a Sephadex LH-20 column yielded four subfractions (EA-I, EA-II, EA-III, and EA-IV), which were characterized at 280, 320, 360, and 520 nm (Figure 5a). The four subfractions showed no toxicity effects (~10^7 cfu/mL microbial growth) or mutagenic effects (~20−45 revertants/plate) at a phenolic concentration of 90 μg/plate. In general, all four EAF subfractions showed antimutagenic activity against the food mutagen Trp-P-1 (**Figure 4b**). The antimutagenic activities of the EAF subfractions were in the range of ~24−81% and presented the following descending order: EAF-IV > EAF-III ≈ EAF-I > EAF-II. Subfraction EA-IV was the most potent antimutagen compared to the other subfractions ($p < 0.05$), followed by EAF-III, and they showed inhibition of mutagenic activities of 81.0 ± 4.8% and 38.1 ± 11.6%, respectively.

The antioxidant activities for the EAF subfractions were in the range of ~0.530−1.330 μg of Trolox/μg of phenolics and decreased as follows: EAF-IV > EAF-III ≈ EAF-II > EAF-I. Once again, subfraction EA-IV was the highest in antioxidant activity among the different EAF subfractions ($p < 0.05$), followed by EAF-III, with values of 1.330 ± 0.104 and 1.020 ± 0.180 μg of Trolox/μg of phenolics, respectively.

The HPLC-DAD characterization revealed the presence of simple phenolic acids, flavonols, a flavanone, and bound hydroxycinnamic acid forms. These phenolic compounds were present in mixtures of three or six phenolic compounds in each subfraction with the exception of EAF-IV, which had only one type of phenolic compound (Figure 6). Results revealed the presence of mainly bound hydroxycinnamic acid forms in EA-I (100% relative A_{280nm}), phenolic acids in EA-II (~53% relative A_{280nm}), quercetin derivatives in EA-III (~80% relative A_{280nm}), and a quercetin derivative in W-IV (100% relative A_{280nm}) (Table 2). These results suggest that the presence of quercetin derivatives alone or in larger amounts in a mixture (e.g., 80−100% in EA-III and EA-IV) enhances the antimutagenic efficiency of the ethyl acetate subfractions from purple corn extracts. On

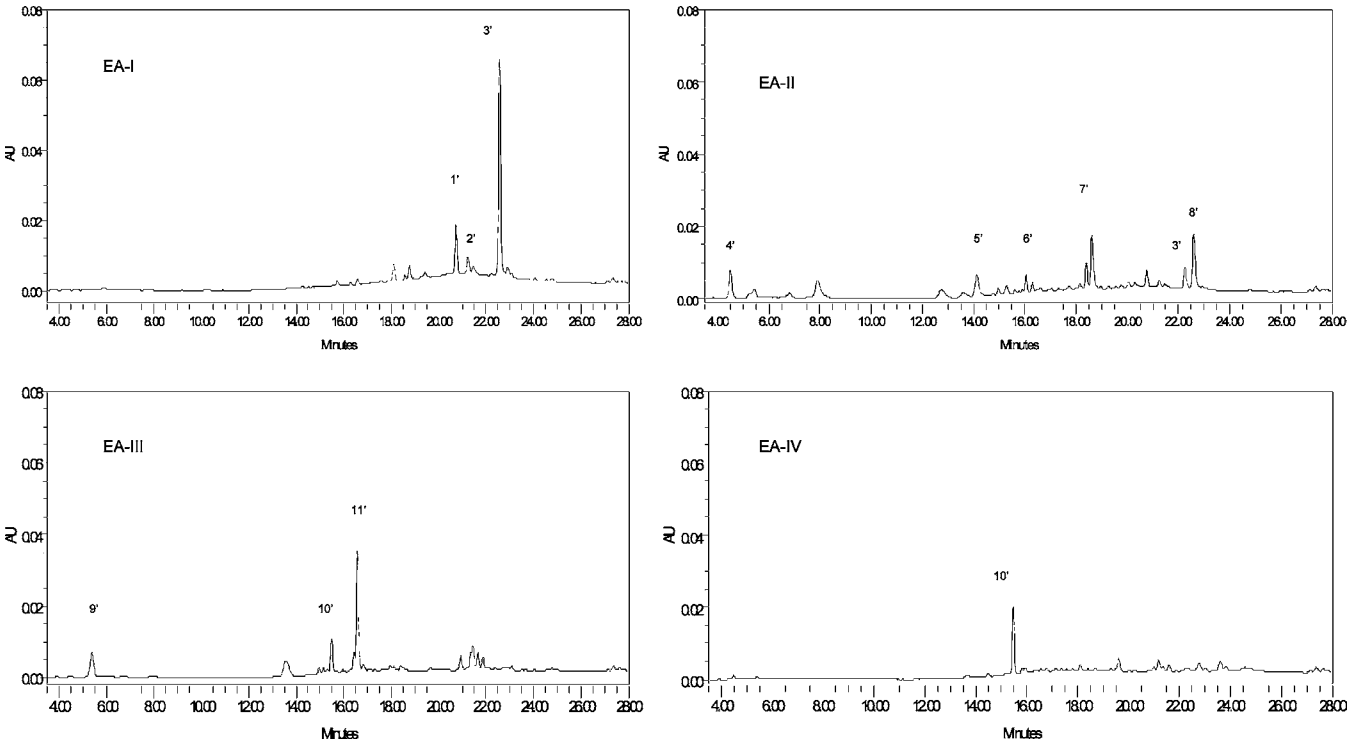

Figure 6. HPLC profile for the ethyl acetate subfractions at 280 nm. Samples were injected at a concentration of 90 μg of chlorogenic acid equiv/mL.

Table 2. Relative Percentage Contribution of the Different Peaks Obtained in the ethyl acetate subfractions

subfraction	peak no.	retention time (min)	λ_{max} (nm)	relative A_{280nm}[a] (%)	compound
EA-I	1′	20.658	198.9, 324.3	16.28	hydroxycinnamic derivative[b]
	2′	21.367	200.1, 315.9	5.36	hydroxycinnamic derivative[b]
	3′	22.495	198.9, 310	78.36	hydroxycinnamic derivative[b]
EA-II	4′	4.394	204.8, 259, 293.3	21.21	protocatechuic acid
	5′	13.78	202.4, 224.8, 308.8	15.79	*p*-coumaric acid
	6′	18.269	197.8, 288.6	14.43	hesperitin derivative
	7′	18.489	197.8, 291, 315.9	31.98	unknown
	3′	22.150	198.9, 310	11.35	hydroxycinnamic derivative[b]
	8′	22.538	197.8, 326.7	5.24	hydroxycinnamic derivative[b]
EA-III	9′	5.273	202.4, 279.1, 515.8	19.68	cyanidin-3-glucoside
	10′	15.278	202.4, 254.2, 353	16.15	quercetin derivative
	11′	16.409	198.9, 253.1, 351.8	64.18	quercetin derivative
EA-IV	10′	15.302	202.4, 254.2, 353	100	quercetin derivative

[a] Percentage area is defined as the integrated area of a single peak divided by the total area of all integrated peaks. Peak absorbances at other wavelengths were also measured but were not presented to simplify the analysis. [b] Bound hydroxycinnamic forms composed of ferulic and *p*-coumaric derivatives (*12*).

the other hand, the presence of phenolic acids in a mixture (e.g., 53−100% in EA-II and EA-I) reduces the antimutagenic potency of the ethyl acetate subfractions. EA-IV, the most bioactive subfraction, shows a quercetin derivative similar to a rutin-like molecule as determined from UV−visible spectral data (Figure 6, **Table 2**). Rutin has been reported as an excellent antimutagen against the mutagens IQ (2-amino-3-methyl-imidazo[4,5-*f*]-quinoline) and a type of benzo[*a*] pyrene (*21*, *32*). Furthermore, quercetin has been shown to have a higher DPPH antioxidant activity than several anthocyanidins and phenolic acids (*30*). The quercetin derivative in the present study showed both high antioxidant and antimutagenic properties.

To account for the relative contributions of the EAF and WF in terms of bioactivity, it is important to know the relative contribution of each fraction to the total phenolics present in the PCE. In this study, the C-18-purified TPF represented ∼21.86% of PCE by weight after recovery from the Sep-Pak cartridge. This TPF was partitioned in water/ethyl acetate, yielding purified WF and EAF that represented ∼74.6% and 22.3% of TPF by weight. According to this result, PCE is an important source of nonanthocyanin phenolic compounds that had been ignored in previous studies in that bioactivity was tested and attributed only to the anthocyanins (*10*, *13*).

Mechanism of Antimutagenic Action. The TA98 cells were subjected to five different treatments to determine the antimutagenic mechanism of action of the anthocyanin-rich WF, EAF, and EA-IV (**Figure 7**). Several mechanisms have been proposed for the action of phenolic compounds as antimutagens; however, two main mechanisms in the Ames test include the inhibition of enzyme systems such as the cytochrome-P450-dependent bioactivation of the various mutagens and the scavenging of metabolically generated mutagenic electrophiles (*26*). In addition, a third proposed mechanism might include the blocking of the mutagen transfer into the cytosol by phenolic binding or insertion into the transporters of the outer membrane of the cell (*19*).

The controls, which included a preincubation of bacteria TA98 cells and the activated mutagen Trp-P-1, showed mutagenic effects of ∼600 revertants/plate for all three experiments (treatment I). The WF showed antimutagenic activity when it was supplied after the preincubation of bacteria and the activated Trp-P-1 (treatment II), suggesting a scavenging action of the anthocyanins against the generated mutagenic electrophiles (**Figure 7**a). The WF also showed a higher antimutagenic effect ($p < 0.05$) when it was preincubated with the bacteria and the S-9 Mix before exposure to Trp-P-1, suggesting that anthocyanins can interfere with the S-9 Mix enzymes responsible for the mutagen activation (treatment III). A similar response was obtained ($p > 0.05$) when the WF, S-9 Mix, and Trp-P-1 were preincubated together before exposure to the bateria cells (treatment IV), indicating this time that anthocyanins might preferentially act on the enzyme systems. Treatment V, which corresponded to a 30-min incubation of all of the components together showed mutagenic suppression comparable to those

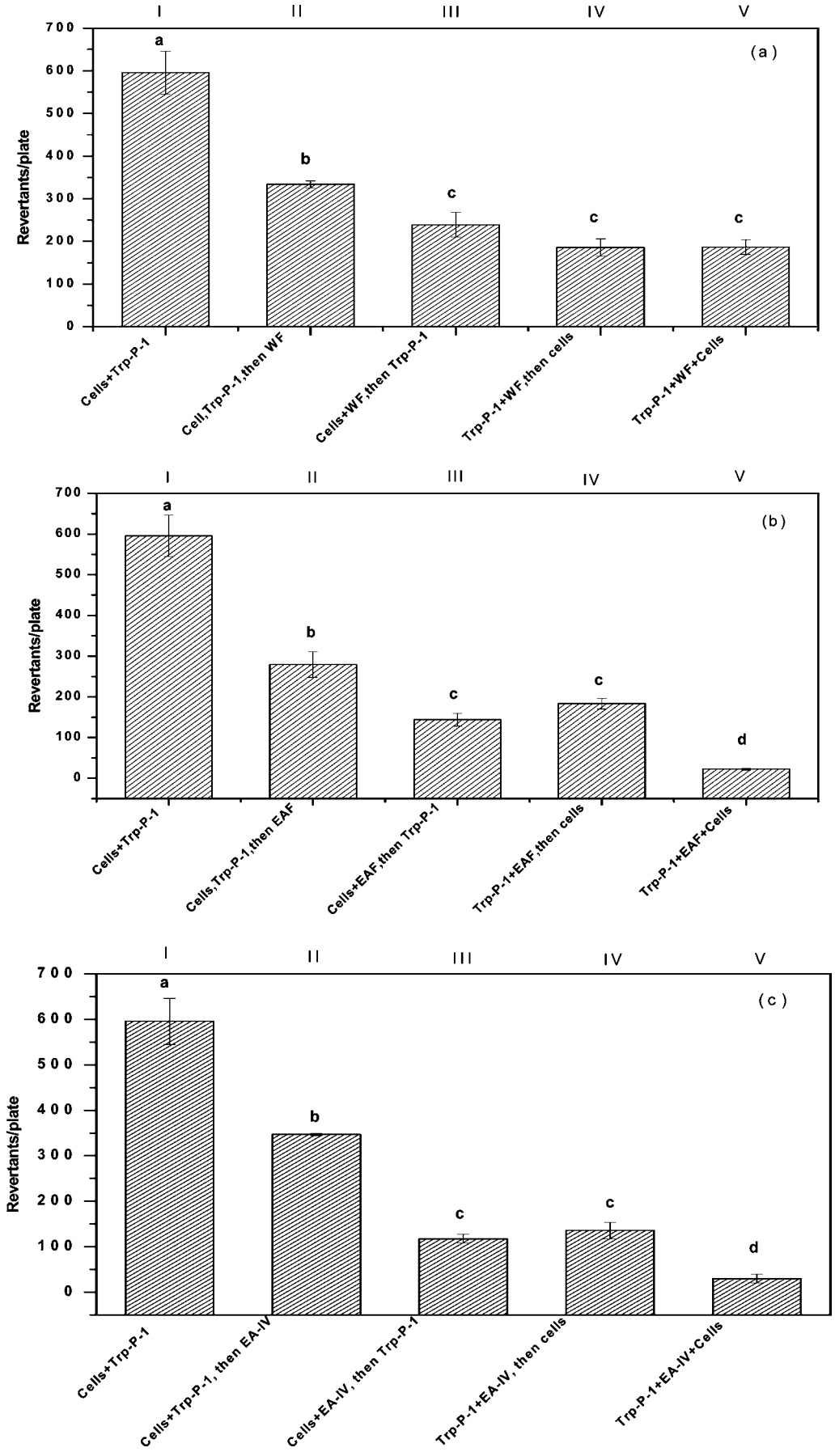

Figure 7. Mechanisms of antimutagenic action for (a) WF, (b) EAF, and (c) EA-IV. The concentrations used were 200, 90, and 50 μg of chlorogenic acid equiv/plate.

of treatments III and IV ($p > 0.05$). The latter treatment would confirm that the predominant mechanism of antimutagenic action by the WF is due to inhibition of the S-9 Mix enzymes responsible for the mutagen activation of Trp-P-1. This predominant mechanism would explain the lack of correlation between the antioxidant and antimutagenic activities observed before for purple corn anthocyanins.

On the other hand, both the EAF and the subfraction EA-IV showed similar trend behaviors in the inhibition of the mutagenic activity of Trp-P-1 (Figure 7b,**c**). Results for treatments II, III, and IV showed trends similar to those obtained for the WF. However, treatment V resulted in a further reduction of mutagenic activity ($p < 0.05$), indicating that the nonanthocyanin phenolic compounds in EAF and EA-IV were involved in further scavenging of mutagen electrophiles due most likely to the longer period of incubation of the different components of the assay. These results suggest that nonanthocyanin compounds from the EAF and EA-IV show a dual antimutagenic mechanism of action involving both enzyme inactivation and the scavenging of reactive electrophiles that take place in different proportions compared to the WF. In addition, this dual mechanism would also explain the enhanced antioxidant and antimutagenic activities observed before for the EA-IV subfraction.

The predominant mechanism of action would be dependent not only on the phenolic structures but also on the reaction kinetics taking place among the different components of the Ames test. This will include the reaction kinetics of Trp-P-1 and the S-9 Mix, the antimutagen with the S-9 Mix, the antimutagen and the generated electrophiles, and these with the cells. Further studies are needed to determine the real impact of reaction kinetics.

In general, this study has shown that phenolic compounds present in Andean purple corn have antimutagenic properties. The phenolic compounds present in the ethyl acetate fractions were mainly composed of phenolic acids and flavonols and were more potent antimutagens than the anthocyanins present in the water fraction. Quercetin derivatives are responsible for this enhanced activity. No toxic effects were seen at doses lower than 350 μg of chlorogenic acid equiv/plate. The antimutagenic mechanism of action of purple corn phenolic compounds involved enzyme inactivation and scavenging of electrophiles and depended on the phenolic fraction. This work states the importance of considering the phenolic compounds other than anthocyanins in biological studies when using Andean purple corn extracts.

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