Research Article

Polyphenolic Reductants in Cane Sugar

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Abstract

Limited information is available to understand the chemical structures of cane sugar extracts responsible for their redox reactivity. This study employed Fremy's salt to test the hypothesis that hydroquinone/catechol-semiquinone-quinone redox cycle is responsible for the redox reactivity of sugarcane extracts; grape seed was employed as a quebracho tannin reference compound. Polyphenols were analyzed by the liquid 1H and 13C NMR before and after the selective oxidation by the Fremy's salt. Antioxidant having greater amounts of polyaromatic phenols (sugarcane extract from Taiwan < sugarcane extract from Philippines << grape seed) exhibited greater ability to reduce Fe (III) and contained higher amounts of terminal (vanillin) and linking (acid-butanol) proanthocyanidins units. Sugarcane extract from Taiwan was characterized by the aliphatic fluorophores having low Ex/Em wavelengths and was enriched with ionizable functional groups. Small amount of polyphenols in cane sugar (compared to grape seed) engaging in one-electron transfer reaction could provide beneficial antioxidant properties to quench radicals.

Keywords: Proanthocyanidin; Colorimetric method; Transition metals; Tannin; Antioxidant

Introduction

Naturally-occurring (poly) phenolic antioxidant structures range from polyflavanols (procyanidins), anthocyanins, to phenolic acids [1,2]. The ability of (poly) phenols to form stable semiquinone radical [3] enables one-electron transfer reactions to quench radicals [1], rendering their antioxidant activity. Polyphenolic structures with aromatic rings attached to the α–β conjugated system of the quinine (fully oxidized structure) are (i) able to form resonance-stabilized semiquinone radicals, (ii) less susceptible towards nucleophilic (Michael) addition, (iii) have low reduction potential, (iv) more likely to engage in reversible electron transfer [4,5] and are therefore expected to be the stronger antioxidant. Fremy's salt and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) [6] are the inorganic and organic free radicals frequently employed to investigate the antioxidant activity of polyphenols. For example, the ability to quench DPPH decreased in the following order: hydroquinone >> ascorbic acid > vanillin > cinnamic acid ≈ citric acid ≈ salicylic acid [7]. Because of its polyaromatic structure, procyanidin extracted from sorghum showed 15-30 times greater peroxyl radical-quenching ability than phenols and Trolox [8].

Limited report is available to understand the chemical structures responsible for the redox reactivity of sugarcane extracts. Liquid chromatography-mass spectrometry analysis of commercial brown sugar showed phenolic acids such as vanillin, syringic acid and benzoic acid in a few mg kg^{-1} range; catechin and flavonoids were not detected [9]. However, others observed flavonoids in raw sugar [10]. Desugared sugarcane extract [11] showed higher solubility in water (73%) than n-butanol (22%) and ethyl acetate (5%). The n-butanol extract was composed of 12 phenolic glucosides and exhibited the highest tyrosinase inhibition [11]. Sugarcane molasses and kokuto (evaporated sugarcane juice without centrifugation) contained phenolic acids, phenyl glycosides, phenyl propanoid glycosides and phenyl propanoids [12]. The DPPH assay has been used to test antioxidant activity of phenolic structures in cane brown sugar [9]. The objective of this study was to understand the role of polyphenols in the electron-donating ability of sugarcane extracts; grape seed was employed as a proanthocyanidins reference material. Liquid-phase 1H and 13C NMR Fremy's salt oxidation was employed to first determine the existence of polyphenols in cane sugar. The NMR results were compared with the following quantitative chemical assays: proanthocyanidins contents by acid-butanol, flavonoids by vanillin, radical quenching by DPPH, Fe (III) reduction and UV/visible and fluorescence spectrophotometry.

Materials and Methods

Distilled, Deionized Water (DDW) with a resistivity of 18 MΩcm (APS Water Services, Van Nuys, CA) was used in all procedures. All chemical reagents were obtained from Sigma-Aldrich (Milwaukee, WI) with the highest purity available. Good's buffers were used to set pH: sodium acetate (pK$_a$ 4.8 for 3.0 ≤ pH < 5.5), 2-(N-Morpholino) ethanesulfonic acid monohydrate (MES; pK$_a$ 6.2 for 5.5 ≤ pH < 6.5) and 3-(N-Morpholino)-propanesulfonic acid (MOPS; pKa 7.2 for 6.5 ≤ pH < 8.0).

Naturally-occurring antioxidant samples

Sugarcane extracts from Taiwan (hereby denoted Taiwan) and Philippines (hereby denoted Philippines) were manufactured by heating cane juice at 90-100°C for 40min. The products were then spray-dried at > 90°C. Grape seed extracts were purchased off the market shelf and contained dicalcium phosphate and cellulose bulking agent. Each antioxidant (0.05g) was dissolved in 20mL acetone-water (70:30 vol%), a recommended solvent to minimize the decomposition of condensed tannins [13], or methanol in amber glass vials with Teflon lined screw cap (40mL nominal volume, Thermo Fisher Scientific, Waltham, MA) by rotating end-over-end at 70rpm for 5 d; sucrose crystals visibly settled in Taiwan and Philippines

stock solutions. Because n-butanol was used to extract phenolic antioxidants from sugarcane extracts [11], separate stock solutions were obtained by dissolving 0.05g sample in 35mL n-butanol. All stock solutions were filtered (0.45μm Millipore Millex-GS; Millipore, Billerica, MA) and immediately analyzed.

Ferrozine, acid-butanol, vanillin and DPPH assays

Electron-donating capacity of each sample was investigated by (i) reduction of Fe^{III} by acetone-water (70:30 v/v) extracts under acidic pH and (ii) quantification of Fe^{II} product using ferrozine colorimetric assay [14]. Acidic pH was utilized to (i) make Fe^{III} reduction by dihydroxybenzene thermodynamically favorable [15], (ii) minimize oxidation of Fe^{II} product by O_2 [16] and (iii) minimize Fe^{II} complexation by phenolic components of extracts [17]. Acetone-water extract (0.2mL) and $FeCl_3$ stock solution (1mM in 0.5M HCl) were added to DDW to yield 50 μM $FeCl_3$ in 5mL total volume. Reactors were allowed to stand for 30min and then 0.2mL of resulting solution was added to 5mL ferrozine stock solution (3-(2-Pyridyl)-5, 6-diphenyl-1, 2,4-triazine-p, p-disulfonic acid monosodium salt hydrate; 0.1 gL^{-1} in 0.5 M MOPS buffer at pH 7) [14]. Absorbance at 562nm [14] was recorded using diode-array UV/visible spectrophotometer (HP8452A, Hewlett-Packard, Palo Alto, CA) with DDW as the blank immediately. Five-point calibration was obtained using 1.0 M $FeCl_2$ stock solution prepared in 1.0 mM HCl daily to minimize autoxidation. Control experiments indicated negligible Fe^{II} loss by complexation and autoxidation.

Acid-butanol assay utilizes oxidative cleavage of proanthocyanidins at 4,8 linkages to form red anthocyanidin pigment having λ_{max} of 550nm [18]. Briefly, 6mL of n-butanol+conc. HCl (95:5 vol%) and 1mL acetone-water extract were added to a culture tube. After adding 0.2mL of 2% (w/v) $NH_4Fe^{III}(SO_4)_2$ (in 2M HCl), the reactor was vortexed and then placed in boiling water bath for 50min. Full spectrum (280-650nm) was taken before and after boiling and absorbance was recorded at 550nm. Blank spectra were obtained for each extract before boiling. Calibration was obtained by repeating above-described procedures for 1-5mg L^{-1} delphinidin chloride.

Vanillin assay for meta-substituted flavanoids was conducted following the literature protocol [19]. Briefly, 1mL of methanol extracts was reacted with 5mL of working reagent (2.5mL of 1% vanillin+2.5mL of 8% HCl) in 30°C water bath for 20min and the absorbance was recorded at 500nm. Above-described procedure was repeated to obtain (1) blanks (1mL of sample and 5mL of 4% HCl) and (2) five point calibration (1mL of catechin standards+5mL of working reagent).

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) is a persistent free radical stabilized by π conjugation and sterically [7] and is quenched by hydroquinone as well as (poly) phenolic extracts of sorghum seeds [1]. The DPPH radical quenching assay followed the literature protocol [20]. Briefly, 4mL of DPPH dissolved in methanol (0.01g L^{-1}) was added to 4mL of methanol extracts at successive dilutions to achieve 20-60% inhibition. Five point calibrations were obtained using trolox to determine the trolox equivalence (in μg trolox g^{-1} sample).

Vanillin and DPPH assays were performed in replicate and were statistically analyzed by one-way ANOVA using MATLAB version

8.5.0.197613 (R2015a) (Math works, Natick, MA) with PLS toolbox version 8.0.1 (Eigenvector Research, Manson, WA).

Fluorescence excitation-emission (EEM) spectrophotometry

Fluorescence EEM of each sample was prepared in 3 different solvents with dilutions to minimize the inner-filtering: (1) 5g L^{-1} acetone-water extracts diluted (1:20) in pH 5 acetate buffer (20mM); n-butanol (0.05g in 35mL without dilution); and 0.16g L^{-1} methanol extracts diluted (1:11) in methanol. Fluorescence EEM of each extract was obtained using F-7000 spectrofluorometer (Hitachi, San Jose, CA) set to 220-400nm excitation and 280-600nm emission wavelengths in 3nm intervals; 5nm excitation and emission slits; 0.5s response times; and 2400nm min^{-1} scan speed. The blank EEM of background solution solvents were obtained daily and subtracted from each sample to remove background EEM and the lower intensity Raman scattering [21].

Solid state ^{13}C cross polarization and magic angle spinning (CPMAS) NMR

The ^{13}C signals of finely ground powder in KEL-F spinning tube (6mm diameter, JEOL, Tokyo, Japan) were recorded at 75.45MHz with a magic angle spinning of 6kHz, 1 ms of contact time and 3 s of pulse interval with 100Hz broadening factor for the Fourier transform. The chemical shifts were interpreted with respect to tetramethylsilane (TMS) and adamantane external reference (29.50ppm). Resulting ^{13}C NMR spectra were interpreted based on the chemical shift regions (in ppm): aliphatic C (0-45), O/N alkyl C (45-110), aromatic C (110-160) and carbonyl C (160-190).
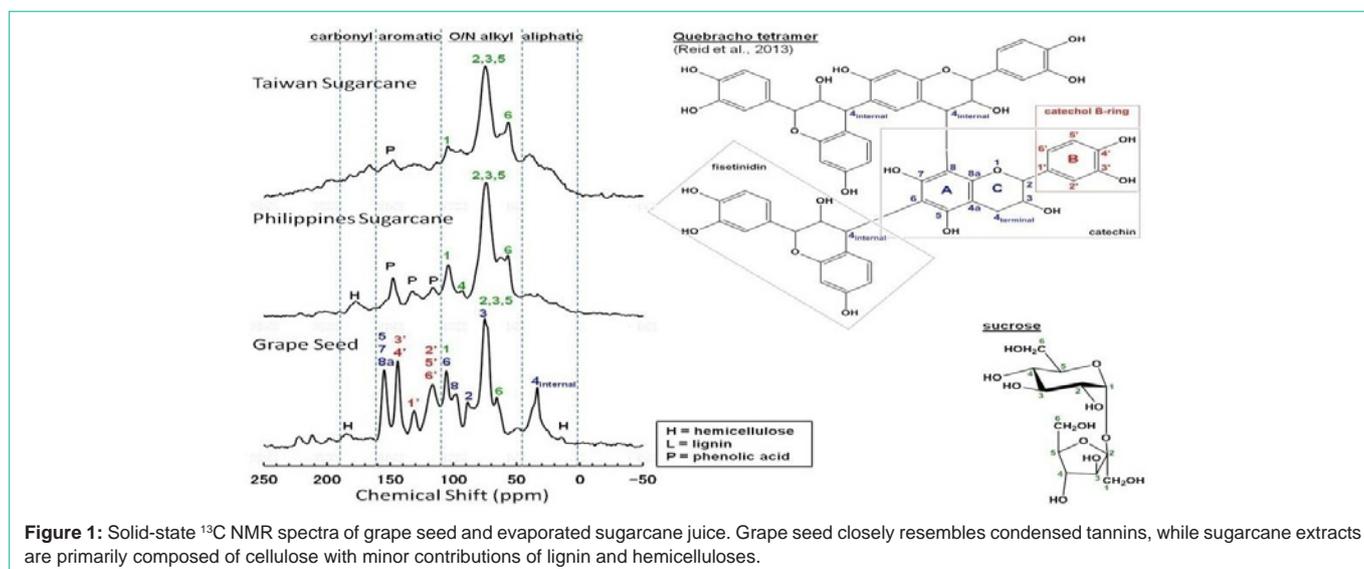
Oxidation of antioxidants by Fremy's salt

Following the literature protocol [22], few mg of the antioxidant sample was dissolved in 10mL D_2O. Fremy's salt (potassium nitrosodisulfonate, 50mg) was then added and the solution was magnetically stirred for 6h at 25°C. The oxidized samples were filtered (0.45μm) and then immediately analyzed by the solution-phase ^1H and ^{13}C NMR using ECA-600 FT NMR spectrometer (JEOL, Tokyo) with a 5mm probe. Spectra were recorded at 242.85MHz using a pulse width of 10.00μs (90°), an acquisition time of 0.4522 s and broadband proton decoupling at 30°C. The pulse delay time of 2s was employed. Each spectrum was scanned 30,000 times and a broadening factor of 5.00Hz was used in the Fourier transform procedure. Chemical shifts (ppm) were determined with respect to TMS (0ppm); HOD (4.7ppm in ^1H) from D_2O and methanol stabilizer of Fremy's salt (3.3ppm in ^1H, 49ppm in ^{13}C) was visible in all oxidized samples.

Results and Discussion

Solid-state ^{13}C CPMAS NMR

Figure 1 presents solid state ^{13}C CPMAS NMR spectra of grape seed and evaporated sugarcane juice samples from Taiwan and Philippines. Aromatic C peaks (110-160ppm) are distinctively more visible in grape seed than Taiwan and Philippines. The spectrum of grape seed closely resembles quebracho (condensed) tannins [23] having catechol B-ring, catechin and fisetindin units, as illustrated by the structure of quebracho tetramer in (Figure 1). Quebracho proanthocyanidins contain a fingerprint peak at 118ppm that is attributable to 2-, 5'- and 6'-C positions of the catechol B-ring (red numberings in quebracho tetramer structure, (Figure 1) [23].

Figure 1: Solid-state ^{13}C NMR spectra of grape seed and evaporated sugarcane juice. Grape seed closely resembles condensed tannins, while sugarcane extracts are primarily composed of cellulose with minor contributions of lignin and hemicelluloses.
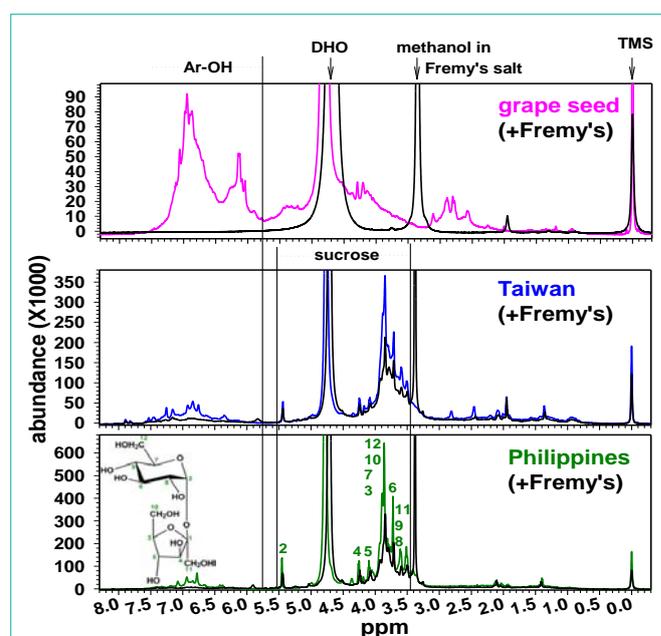
Additional aromatic C peaks in the 110-160ppm region of grape seed result from other C positions in the catechol B-ring as well as the A-ring of the catechin starter.

Previous solid state ^{13}C NMR analysis of commercial (food-grade) grape seed showed similar enrichment of aromatic C, compared to peanut shell; walnut shell, peach stone and Eucalyptus bark [24]. However, FTIR of grape seed contained sharper aliphatic C-H peaks (near 3000cm^{-1}) than other biomass, suggesting components other than proanthocyanidins [24]. Grape seed samples in the present study contained cellulose bulking agent. In (Figure 1), C1-C6 of cellulose appears within the O/N-alkyl region (50-100ppm) and overlap with catechin C of proanthocyanidins. As labeled on the grape seed spectrum, additional peaks from hemicelluloses and lignin [25] could overlap with proanthocyanidins. In particular, lignin gives broad peaks at 100-200ppm [26]. In summary, grape seed has the polyphenolic structural attributes of condensed tannins [23].

Compared to the grape seed, evaporated sugarcane juice from Taiwan and Philippines lacked phenolic peaks and resembled ^{13}C CPMAS-TOSS NMR spectrum of sugarcane bagasse [27]. Especially for Taiwan, peaks primarily exist in the O/N alkyl region and are representative of C-1, C-4, C-2,3,5 and C-6 of cellulose [25]. Untreated sugarcane bagasse had a spectrum similar to pure cellulose with intense amorphous/crystalline cellulose peaks at 50-120ppm [26] with minor contributions from lignin and hemicelluloses [27]. Peaks in aromatic regions of Philippine sugarcane (Figure 1) are attributable to phenolic acids.

Acid-butanol assay [18] indicated the highest (3.3 wt% delphinidin chloride equivalent) proanthocyanidins in grape seed (Table 1), in agreement with the ^{13}C NMR trends in (Figure 1). Only terminal units react in vanillin, while all except the terminal unit reacts in acid-butanol [28]. Acid butanol assay breaks interflavan bond to release flavanoid units and form anthocyanidin pigments. Vanillin (aromatic aldehyde) forms red adduct with the meta-substituted flavonoids. Based on vanillin and acid-butanol assays (Table 1), grape seed contained greater amount of terminal (vanillin) as well as linking (acid-butanol) units of proanthocyanidins.
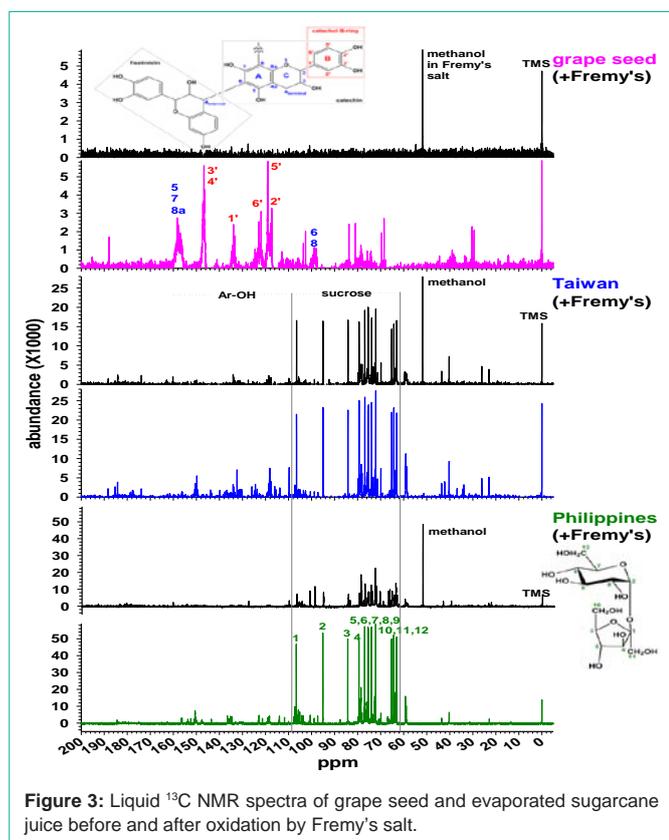

Figure 2: Liquid ^{1}H NMR spectra of grape seed and evaporated sugarcane juice before and after oxidation by Fremy are salt.

Solution-phase ^{13}C and ^{1}H NMR analyses of antioxidants before and after oxidation by Fremy's salt

In order to understand the chemical functionality responsible for the redox reactivity of sugarcane extracts, solution-phase ^{13}C and ^{1}H NMR analyses were undertaken before and after oxidation using Fremy's salt. Fremy's salt is widely employed to oxidize hydroquinones and catechus to quinines [6].

Figure 2 presents liquid ^{1}H NMR spectra of grape seed (pink), Taiwan (blue) and Philippines (green) before and after (black) oxidation using the Fremy's salt. (Figure 2) shows the following chemical shift regions: aliphatic protons of CH$_3$, CH$_2$ and CH groups (1.0-3.0ppm), alkoxy (mostly methoxy and ethoxy) groups and carbohydrates (4.0-6.0ppm) and phenolic protons (>6ppm) [29].

Figure 3: Liquid ^{13}C NMR spectra of grape seed and evaporated sugarcane juice before and after oxidation by Fremy's salt.

Figure 4: Fluorescence EEM in butanol (without dilution, a-c), and UV/v is spectra of acetone/water extracts set to pH3-8.5.
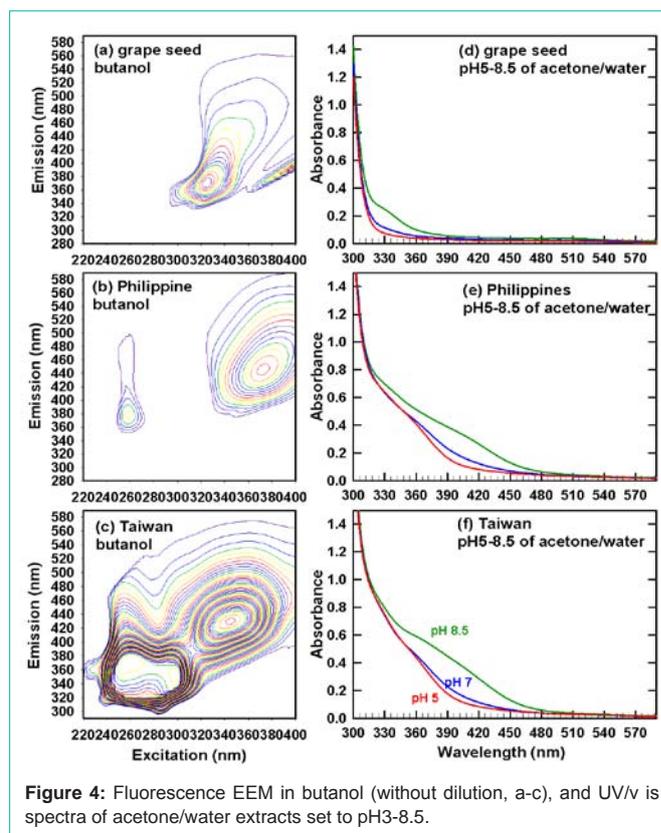
The alkoxy region of Taiwan and Philippine sugarcane extracts were dominated by sucrose [30] that was not oxidized by Fremy's salt. Phenolic groups (>6ppm) were the primary functionality oxidized by Fremy's salt (black spectra in (Figure 2) in each sample. In agreement with the solid-state ^{13}C NMR (Figure 1), grape seed has extensive polyphenolic peaks in (Figure 2). In conclusion, the phenolic peaks were distinctively removed after the oxidation by Fremy's salt in all samples in (Figure 2).

Figure 3 presents solution-phase ^{13}C NMR spectra of grape seed (pink), Taiwan (blue) and Philippines (green) before and after (black) oxidation using the Fremy's salt. In agreement with the solution-phase ^{1}H NMR (Figure 2), Taiwan and Philippines are dominated by the sucrose C1-12 (green numberings for Philippines before oxidation, (Figure 3), bottom) at 60-110ppm. The sucrose peaks [31] remained visible in Taiwan and Philippines after the oxidation. Much like ^{1}H NMR (Figure 2), grape seed is dominated by polyphenolic structures in (Figure 3) (>110ppm) [32,33]. The phenolic peak in

all 3 samples disappeared by Fremy's salt. In conclusion, (Figures 2,3) indicate that only polyphenols were oxidized by Fremy's salt; sugar peaks remained in Taiwan and Philippines after oxidation. Similar trends were observed for the oxidation by Fe (III) (Table 1): for butanol extracts, the electron-donating ability increased in the following order: Taiwan < Philippines < grape seed.

Conclusion

In conclusion, Fremy's salt results agreed with the trend in (Table 1) for the following assays: acid-butanol, ferrozine (for the butanol extract), vanillin and the absorbance of methanol extracts at 210nm. (Table 1) presents UV (360nm) absorbance of acetone-water extracts at pH 5, 7 and 8.5. At each pH, the following sample-dependent trend was observed: grape seed <<< Philippines < Taiwan. For each sample, the following pH-dependent trend was observed: pH5 < pH7 << pH8.5. The pH dependence indicates the deprotonation of phenolic acids and proanthocyanidins (Figure 1). As shown in (Figure 4), pH-dependent changes in UV/visible spectra (Figures 4d-4f) are greatest

Table 1: Characteristics of grape seed and sugarcane (from Philippines and Taiwan) extracts: DPPH radical quenching (in µg trolox g-1 seed), acid-butanol assay (in µg delphinidin g-1 seed), and reduction of 50 µM Fe (III), vanillin assay (in mg catehin g-1 seed). UV absorbance (360nm) is given for acetone-water extracts adjusted to pH 5, 7, and 8.5 and for pure butanol extract, and at λmax in methanol (210nm).
Mean±s.d. for duplicatea and triplicateb assays; different letters within columns indicate statistical difference (Turkey, P < 0.05).

Sample	absorbance (360nm)			λ_{max} (210nm) methanol	acid-butanol assay µg g^{-1} delphinidin Cl	Ferrozine assay Fe (II) formed (µM)		Vanillin[a] [catechin] Equiv. mg g^1	DPPH[b] [trolox] Equiv. µg g^{-1}
	acetone-water		Pure butanol						
	pH 5	pH 7	pH 8.5			Acetone-water	butanol		
Grape seed	0.04	0.06	0.10	0.47	0.97	33,165	35	37	320±27 A	542±22 A
Philippines	0.41	0.43	0.51	1.60	0.75	1,307	36	21	0 B	11±5 B
Taiwan	0.44	0.46	0.59	0.44	0.50	843	39	9	0 B	139±7 C

for Taiwan, followed by Philippines and grape seed. In addition, fluorescence EEM of Taiwan indicates distinctively more aliphatic structure (lower Ex/Em wavelengths) compared to the grape seed and Philippines (Figures 4a-4c). Therefore, Taiwan is likely to be most aliphatic and enriched with ionizable functional groups.

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