Food Chemistry 115 (2009) 1299–1305

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Antioxidant activity of Sonoran Desert bee pollen

Blaise W. LeBlanc [a,*], Owen K. Davis [b], Stephen Boue [c], Anthony DeLucca [c], Thomas Deeby [a]

[a] Carl Hayden Bee Research Center, USDA ARS, 2000 E. Allen Rd., Tucson, AZ 85719, United States
[b] University of Arizona, Department of Geophysics, Tucson, AZ 85721, United States
[c] Southern Regional Research Center, USDA ARS, New Orleans, LA 70179, United States

ARTICLE INFO

Article history:
Received 15 August 2007
Received in revised form 23 December 2008
Accepted 19 January 2009

Keywords:
Bee pollen
Phenolics
Antioxidant
Radical
DPPH
FRAP
GC–MS
Solid phase micto-extraction
SPME

ABSTRACT

Bee pollen (pollen collected by honey bees) was collected in the high intensity ultraviolet (UV) Sonoran Desert and analyzed by the DPPH (radical 2,2-diphenyl-1-picryhydrazyl) assay and the FRAP (ferric reducing-antioxidant power) assay on six different pollen samples and in eight different water miscible solvents at 50 mg/ml. The bee pollen taxa were characterized for each pollen type by acetylization of the pollen extracts followed by microscopy and comparison with a library of samples native to the Sonoran Desert. The standards (R-(+)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), known as TROLOX, gallic acid and α-tocopherol (vitamin E) were analysed as standards to determine the potency of each pollen sample in the most efficient solvent. The Mimosa pollen sample displayed the highest antioxidant activity. Total polyphenolics, flavanols, flavones were determined, and the results are reported in milligrams of gallic acid, quercetin and naringenin per gram of pollen, respectively. There was good correlation between antioxidant activity and total phenolics. The order of effectiveness of the pollen samples in regard to antioxidant activity was determined and the most effective extraction solvents are discussed. Finally, solid phase micro-extraction, coupled with gas chromatography–mass spectroscopy was utilized to identify and quantify polyphenolic compounds known to have free radical scavenging activity in the pollen samples.

1. Introduction

Pollen is the reproductive cells of plants. Bees, other insects, wind and water pollinate plants by transferring pollen from the stamen to the stigma of another plant. Honey bees collect pollen by adding sugars from nectar to hold the grains together and then transfer them back to the colony by packing them into hairs on the corbiculae (hind legs) of bees (Snodgrass, 1975). The role of pollen to the sustenance of the bee colony cannot be overestimated. The bees consume pollen in their own diets and use it to feed larvae. The bees place the pollen in honeycombs with their legs and cover this pollen with honey. This pollen store is referred to by beekeepers as "bee bread" (Stanley & Linskens, 1974, p. 98). It was determined that an average value of 145 mg of pollen is required to rear just one worker bee. Results from survival studies conducted, on caged bees, where bees were fed pollen samples collected from the Sonoran Desert revealed that Mesquite pollen was most desirable to bees (Schmidt & Johnson, 1984), while Palm pollen was much less desirable. Bee pollen is also used as a dietary supplement for humans and reports have appeared in the literature regarding the antioxidant activity of bee collected pollen and the total phenolics (Campos, Webby, Markham, Mitchell, & Da Cunha, 2003; Leja, Mareczek, Wyzgolik, Klepacz-Baniak, & Czekonska, 2007; Rozema et al., 2001). Propolis, which is a resinous, high phenolic containing apiculture product has been well characterized for its health benefits related to its anti-microbial and antioxidant activities (Kosalec, Bakmaz, & Pepeljnjak, 2003; Russo et al., 2004).

Pollen serves several purposes in plant reproduction. An open reproductive system where the DNA of one plant is transferred to another plant is likely to have an efficient method of protection against environmental conditions, especially UV exposure. Evidence of this is that mountain plants have correspondingly higher levels of epoxy-carotenoids and xanthophylls with an increase in altitude (Asbek, 1958; Stanley & Linskens, 1974, pp. 228–229). The DNA in pollen does not have a metabolic repair mechanism found in other diploid cellular DNA. Therefore, it is likely that antioxidative compounds in pollen could offer protection against solar radiation. Previously it was reported that pine pollen could be harmed during 3–4 h exposure to UV radiation from light of the sun. Pine needles and pollen have been found to have a relatively high percentage of ascorbic acid. It was also shown that the flavonoid content and concentrations of outer pollen walls differ compared to the intracellular cytoplasmic concentrations, cell wall fractions, and extraction residues and that many of the phenolics are organic acids such as: p-hydroxybenzoic, p-coumaric, vanillic, gallic and ferulic acid (Rosema et al., 2001; Standifer, 1966; Strohl & Siekl, 1965). Many of the pigments are phenolic components and

* Corresponding author. Tel./fax: +1 (520) 792 1296.
E-mail address: blaise_LL@hotmail.com (B.W. LeBlanc).

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doi:10.1016/j.foodchem.2009.01.055

have been isolated as flavonoid glycosides (Heslop-Harrison, 1973; Tapi & Menziani, 1955).

There are very few reports characterizing the antioxidant activities of bee pollen. We are not aware of any previous reports on the antioxidant assays of Sonoran Desert bee pollen except those listed on the world wide web site (http://www.ccpollen.com/ORAC.shtml) that compares the oxygen radical absorbing capacity assay (ORAC) results on high desert bee pollens and reports that the bee pollen is more effective as an antioxidant than pomegranate extract or black raspberry extracts. Thus, the aim of this work was to evaluate the antioxidant activity of six pollen samples collected in the Sonoran Desert that are exposed to high levels of UV radiation from the sun. Also, since very little research has been reported on pollen extraction, we report our optimized extraction procedure. For the extraction, eight different solvents were used to determine the most effective solvents. Once the most facile solvent(s) were determined, the active antioxidant compounds were quantified by spectrophotometric assays and identified by solid phase micro-extraction and gas chromatography mass spectroscopy. Furthermore, this study evaluates the hypothesis that an early fall blooming taxa, such as the Yucca plant, does not have the reactive oxygen species (ROS) quenching ability as a taxa that blooms during a UV-intense period such as the Mesquite, Yucca, or especially the Chenopod and Mimosa pollen samples, which were collected during July through August.

2. Materials and methods

2.1. Materials

The pollen pellets were collected between March and November 2006 from beehives located in the Sonoran Desert, North of Tucson, AZ, USA and were purchased from Freddy T's Beeswax, Oracle, AZ, USA with the exception of the Palm pollen, which was collected at our facility. The six pollen samples analyzed in this study were characterized as: Mesquite, Yucca, Palm, Terpentine Bush, Mimosa and Chenopod. The pollen taxa and the occurrence of the pollen taxa in the samples are listed in Table 1. The pollen samples were dried at 50 °C and then kept at room temperature under a nitrogen atmosphere until the mass, measured on an analytical balance, was constant. The reagents and solvents were purchased from Sigma Aldrich and used as described.

2.2. Preparation of the extracts

2.2.1. Solvents comparisons

The pollen pellets (500 mg) were suspended in water miscible solvents (5 ml) (listed in Tables 2 and 3) in a screw cap centrifuge tube and the concentration maintained at 50 mg/ml volumetrically. The test tubes were vortexed and placed in an ultrasound bath at 41 °C for 90 min, with vortexing every 30 min. The samples were refrigerated at 3 °C overnight then centrifuged at 2500 RPM for 1 min. Any samples that remained turbid or opaque were refrigerated and centrifuged at 3200 RPM for 10 min and this step repeated until transparent. The supernatant was then pipetted into a 20 ml scintillation vial. Any samples that contained particulate were microfiltrated with a Pasteur pipet with a cotton filter. The samples were then capped and refrigerated at 3 °C. All antioxidant assays were performed within 7 days of extraction.

Methanolic extracts for ED_{50}, total polyphenolics, flavones, flavanols, flavonones and analysis by solid phase micro-extraction with gas chromatography–mass spectroscopy: The pollen pellets (2.000 g) were suspended in 7 ml methanol (MeOH) and placed in an ultrasound bath for 30 min followed by centrifugation at 3200 RPM for 5 min. The supernatant was transferred to a tared 100 ml round bottom flask. These steps were repeated 8 times. The MeOH was flash evaporated and dried under a gentle stream of nitrogen. To the residue was added 2 ml of water and the suspension was flash frozen and freeze dried overnight. The residue was dissolved in MeOH for a final concentration of 20 mg/ml.

2.3. Preparation for microscopic analysis

The centrifugants from the antioxidant assays were acetylated (9:1 solution of acetic anhydride and sulphuric acid), stained (safranin "O"), mounted in glycerin on microscope slides and the pollen grains counted (ca. 500 grains per sample) at 460× magnification.

2.4. Determination of antioxidant activity using the DPPH radical scavenging method

The 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assay is a robust, facile antioxidant assay. We used a modification of the assay conditions reported by Hatano, Kagawa, Yasuhara, & Okuda (1988). The method was scaled to a 96 well plate format (330 μl scale). The DPPH molecule is a stable radical that accepts an electron from the analyte. Ethyl alcohol (280 μl) was mixed with the solvent extract (72 μl) in a scintillation vial and DPPH solution (242.5 μl of a 51 mg DPPH/100 ml MeOH) added and the vial was capped and vortexed. The more active samples were readily apparent, as the purple colour becomes a light yellow colour. Immediately following, 160 μl was transferred to each well and the samples run in triplicate. The micro-plate was sealed (Thermal Seal, Excel Scientific, Wrightwood, CA) and incubated in the dark for 1 h at room temperature. After a programmed 30 s shake cycle, the absorbance was recorded at 517 nm. Exaction solvents (72 μl) volumes was used as controls for each solvent extract. The percent activity calculated by the following equation by substituting the mean of the absorbance values, which were recorded in triplicate:

$$\%\text{scavenging activity} = 100 \times [\text{control-sample}]/\text{control}$$

ED_{50} values (concentration required to quench 50% of the radicals under the experimental conditions in this assay) were calculated for several pollen MeOH and DMF extracts showing high levels of scavenging activity.

2.5. Determination of antixoxidant activity using ferric reducing-antioxidant power

The ferric reducing-antioxidant power (FRAP) assay was performed according to conditions reported by Benzie and Strain (1999). Water (735 μl; 18 M-Ohm; Barnstead) was mixed with 450 μl of FRAP solution. The FRAP solution was freshly prepared by mixing: 10 mM 2,4,6-tripyridyl-S-triazine (TPZ) in 40 mM HCl, 20 mM $FeCl_3$ solution and 0.3 M NaOAc/HOAc buffer at pH 3.6 at (1:1:10) parts per volume respectively. To a 20 μl scintillation vial was added 450 μl of the FRAP solution and 20 μl of the pollen extract and 735 μl water and the mixture was capped and vortexed; the more active samples turned a dark blue color spontaneously. Next, 100 μl was transferred the well of a 96 well, flat bottom micro-plate (Costar, Cambridge, MA). The samples were run in triplicate and the micro-plate sealed (Thermal Seal) and incubated in the dark in an oven maintained at 37 °C for 30 min. The temperature was maintained at 37 °C, and absorbance was recorded at 593 nm, after a 30 s shake cycle. The mean values for the blanks were subtracted from the means of the absorbance values. Extraction solvent (20 μl) was used as a control. The reported values are expressed in terms of the amount of the ferrous form of [Fe^{2+}] produced from a standard curve plot

Table 1
Pollen plant Taxa for each pollen analysed expressed as % Taxons.

Taxon	Pollen type					
	Mesquite (% Taxon)	Yucca (% Taxon)	Palm (% Taxon)	Terpentine Bush (% Taxon)	Mimosa (% Taxon)	Chenopod (% Taxon)
Acacia	0.0	2.7	0.0	0.2	11.8	4.6
Celtis	9.4	0.0	0.0	0.0	6.1	0.0
Cereus	2.9	11.3	0.0	0.0	0.4	0.0
Prosopis	54.8	0.0	0.0	0.0	18.5	0.0
Quercus	0.0	0.0	0.0	0.0	0.2	0.0
Washingtonia	0.0	0.0	100.0	0.0	0.0	0.0
Anisacanthus	0.0	0.0	0.0	11.0	0.0	7.0
Cylindropuntia	4.5	0.0	0.0	0.0	1.2	0.0
Larrea	28.3	0.0	0.0	0.0	10.8	0.0
Mimosa	0.0	0.0	0.0	0.0	26.1	0.0
Yucca	0.0	85.0	0.0	0.0	4.5	0.0
Ambrosia	0.0	0.0	0.0	14.5	0.0	15.8
Other Compositae	0.0	0.0	0.0	59.8	1.4	20.0
Chenopodiaceae – *Amar*	0.0	0.0	0.0	4.0	3.7	32.6
Gramineae	0.0	0.0	0.0	0.0	2.6	4.0
Boerhaavia	0.0	0.0	0.0	1.8	0.4	2.2
Boraginaceae	0.0	0.0	0.0	0.0	0.0	0.2
Datura	0.0	0.0	0.0	4.2	1.0	1.0
Eriogonim	0.0	0.0	0.0	3.2	0.0	0.0
Euphorbia	0.0	0.0	0.0	0.0	0.8	6.2
Hydrophyllum	0.0	0.0	0.0	0.2	2.4	0.0
Labiatae	0.0	0.0	0.0	0.0	0.2	0.0
Portulaca	0.0	0.0	0.0	0.0	3.1	0.0
Ranunculus	0.0	0.0	0.0	0.0	1.0	6.2
Solanum	0.0	0.0	0.0	0.8	3.9	0.0
Sphaeralcea	0.0	0.0	0.0	0.2	0.0	0.0
Tidestromia	0.0	0.0	0.0	0.0	0.0	0.2
Total # grains identified	509.0	512.0	523.0	498.0	509.0	500.0

Table 2
Diphenylpichydrazyl (DPPH) assay results for pollen solvent extracts expressed as% activity.

Solvent	Pollen type					
	Mesquite (% activity)	Yucca (% activity)	Palm (% activity)	Terpentine Bush (% activity)	Mimosa (% activity)	Chenopod (% activity)
Water	28.59 ± 0.36^{de}	0.00^{e}	5.68 ± 2.55^{bc}	10.58 ± 0.00^{d}	52.10 ± 3.03^{e}	34.93 ± 1.80^{d}
Methanol	85.46 ± 0.46^{a}	41.73 ± 2.03^{b}	19.76 ± 3.32^{a}	68.57 ± 1.03^{b}	90.45 ± 0.69^{a}	87.92 ± 0.46^{a}
Ethanol	57.70 ± 1.31^{b}	18.90 ± 0.69^{c}	11.35 ± 3.46^{b}	33.50 ± 1.26^{c}	$75.90 \pm 1.19b^{c}$	54.88 ± 1.36^{bc}
Propanol	47.34 ± 1.41^{cd}	18.32 ± 4.90^{c}	$6.49 \pm 1.89b^{c}$	35.92 ± 2.15^{c}	69.25 ± 1.16^{cd}	43.00 ± 2.17^{d}
2-Propanol	32.15 ± 1.48^{e}	10.87 ± 0.32^{cd}	6.34 ± 1.74^{bc}	38.93 ± 1.84^{c}	63.02 ± 1.15^{d}	47.72 ± 0.93^{cd}
Acetone	16.99 ± 2.41^{f}	15.28 ± 2.87^{c}	5.11 ± 2.35^{b}	14.53 ± 3.10^{d}	52.30 ± 1.53^{e}	23.30 ± 2.30^{e}
Dimethylformamide	80.17 ± 0.57^{a}	59.50 ± 0.95^{a}	24.46 ± 1.36^{a}	78.27 ± 0.59^{a}	89.05 ± 0.52^{a}	85.37 ± 0.26^{a}
Acetonitrile	6.99 ± 2.63^{g}	1.20 ± 3.35^{de}	0.90 ± 1.34^{c}	8.03 ± 2.41^{d}	14.40 ± 1.91^{f}	10.10 ± 2.44^{f}

Different letters indicate significant differences between solvents within each pollen type ($P < 0.05$ ANOVA).

Table 3
Ferric reducing-antioxidant power assay (FRAP) results for pollen solvent extracts expressed as $[Fe^{3+}]_{mM}$ reduced to $[Fe^{2+}]_{mM}$.

Solvent	Pollen Type					
	Mesquite ($[Fe^{2+}]_{mM}$)	Yucca ($[Fe^{2+}]_{mM}$)	Palm ($[Fe^{2+}]_{mM}$)	Terpentine Bush ($[Fe^{2+}]_{mM}$)	Mimosa ($[Fe^{2+}]_{mM}$)	Chenopod ($[Fe^{2+}]_{mM}$)
Water	1.56 ± 0.15^{d}	0.43 ± 0.03^{c}	0.47 ± 0.02^{b}	0.85 ± 0.02^{e}	2.56 ± 0.04^{de}	2.49 ± 0.07^{bc}
Methanol	2.89 ± 0.10^{b}	1.21 ± 0.02^{b}	0.93 ± 0.03^{a}	2.00 ± 003^{b}	3.96 ± 0.18^{ab}	2.70 ± 0.02^{b}
Ethanol	1.98 ± 0.02^{c}	0.38 ± 0.01^{c}	0.24 ± 0.02^{c}	1.11 ± 0.03^{d}	3.22 ± 0.17^{cd}	2.26 ± 0.01^{cd}
Propanol	1.59 ± 0.09^{d}	0.28 ± 0.06^{c}	0.05 ± 0.02^{d}	1.03 ± 0.00^{d}	3.38 ± 0.72^{bc}	1.97 ± 0.03^{e}
2-Propanol	1.26 ± 0.01^{e}	0.09 ± 0.02^{d}	0.15 ± 0.02^{cd}	1.51 ± 0.03^{c}	2.56 ± 0.04^{d}	2.10 ± 0.07^{de}
Acetone	0.52 ± 0.01^{f}	0.27 ± 0.01^{c}	0.05 ± 0.02^{d}	0.37 ± 0.02^{f}	1.75 ± 0.06^{e}	0.79 ± 0.01^{f}
Dimethylformamide	3.52 ± 0.09^{a}	1.66 ± 0.09^{a}	0.51 ± 0.03^{b}	2.58 ± 0.07^{a}	4.15 ± 0.21^{a}	3.09 ± 0.20^{a}
Acetonitrile	0.20 ± 0.02^{g}	0.0^{d}	0.07 ± 0.01^{d}	0.27 ± 0.03^{f}	0.53 ± 0.01^{f}	0.25 ± 1.72^{g}

Different letters indicate significant differences between solvents within each pollen type ($P < 0.05$ ANOVA).

of ferrous ascorbate, as the ferric form is reduced. Standards for the concentrations in mg/ml of the antioxidants gallic acid, α-tocopherol and TROLOX, versus $[Fe^{2+}]_{mM}$ are above so the entries from Table 2 for the FRAP assay, which are expressed in $[Fe^{2+}]_{mM}$ can be converted into standard equivalent by the following linear equations:

$$[Fe^{2+}]_{mM} = 11.266\text{Abs. @ 593 nm} + 0.03884; \quad R^2 = 0.9973$$

$$[\text{Trolox}]_{mg/ml} = 0.0343 \times [Fe^{2+}]_{mM} - 0.0222; \quad R^2 = 0.9921$$

$$[\text{Gallic acid}]_{mg/ml} = 0.0341 \times [Fe^{2+}]_{mM} - 0.0211; \quad R^2 = 0.9926$$

$$[\alpha\text{-Tocopherol}]_{mg/ml} = 0.1234 \times [Fe^{2+}]_{mM} - 0.0461; \quad R^2 = 0.9983$$

2.6. Determination of total polyphenolic compounds

The procedure is a modification of preparative method previously reported (Yu, Perret, Harris, Wilson, & Haley, 2003) and is scaled to the volumes of a micro-plate reader. In brief, 200 μl of the methanolic extract was transferred to a scintillation vial containing 300 μl of 3% HCl. The mixture is capped, vortexed and allowed to stand 3 min. Next, 100 μl of the acidified mixture is pipetted to 1000 μl of 3% sodium bicarbonate and the mixture is vortexed and allowed to stand 2 min; 20 μl Folin-Ciocalteu reagent were added and the solution vortexed and allowed to stand at room temperature for 30 min. The samples were run in triplicate by pipetting 200 μl to each well of a 96 well micro-plate. The results are calculated in mg/ml gallic acid equivalents (GAE), which is a reported on standard for this method (Miraliakbari & Shahidi, 2008; Moreira, Dias, Pereira, & Esteuinho, 2008), by the substituting the absorbance values into the equation:

$$[\mathrm{GAE}]_{\mathrm{mg/ml}} = (714.36 \times \mathrm{abs@760\ nm} - 45.234); \quad R^2 = 0.988$$

The total polyphenolic content, expressed in mg gallic acid equivalents per gram of pollen (Table 4) was calculated by considering that 200 μl were used for the samples and standards and the pollen concentration was 20 mg of pollen residue per ml of MeOH.

2.7. Determination of flavones and flavonol equivalents

A modification of a method reported by Kosalec, Bakmaz, Pepeljnjak, and Vladimir-Knežević (2004) was employed. Exactly 250 μl of the methanolic pollen extract (20 mg/ml) were combined with MeOH (750 μl), water (1.4 ml) and 1 M sodium acetate (50 μl). The sample solution was treated with 0.784 M aluminum chloride (50 μl) and the blank treated with DI water 50 μl. Exactly 200 μl was transferred to each well of a microplare and the absorbance was recorded at 415 nm. Blank absorbance values were subtracted from the samples. Quercetin was used as a standard and the results expressed as mg quercetin per gram of pollen (QE) (Table 4) by substituting the absorbance values, volume of MeOH required for 20 mg/ml and the mass of the pollen into the following equation:

$$[\mathrm{QE}]_{\mathrm{mg/g}} = (383.56 \times \mathrm{abs@415\ nm} + 8.6726) \times V_{\mathrm{ml}}(\mathrm{extract})/\mathrm{mass_g}(\mathrm{pollen}); \quad R^2 = 0.9983$$

2.8. Determination of flavonones equivalents

Exactly 40 μl of the pollen extract (20 mg of residue per ml) was combined with 80 μl of a 50.5 mM 2,4-dinitrophenyl- hydrazine solution (100 mg 2,4-dinitrophenylhydrazine dissolved in 10 ml MeOH with 200 μl H_2SO_4 (conc.)) in a 5 ml screw cap test tube. The contents were heated for 50 min at 50 °C on a water bath, then allowed to cool to room temperature. Exactly 280 μl of a 10% KOH (w/v) in MeOH was added. As previously reported by Kosalec et al. (2004), to generate the calibration curve, methanolic standards of naringenin at (0, 0.25, 0.50, 0.75, 1.0, 2.0, 5.0 and 10.0 mg/ml) were added instead of the sample volumes. The values are reported (Table 4) as naringenin equivalents (NE) by the following equation. This method was also reported by Kosalec et al. (2004)

$$[\mathrm{NE}]_{\mathrm{mg/ml}} = (13.77 \times \mathrm{abs@486\ nm} - 0.7554) \times V_{\mathrm{ml}}(\mathrm{extract})/\mathrm{mass_g}(\mathrm{pollen})$$

2.9. Determination of phenolic compounds in pollen extracts by solid phase micro-extraction followed by gas chromatography–mass spectrometry

The gas chromatography–mass spectrometry (GC–MS) procedure is a modification of a method reported by Daher and Gülacar (2008). Freeze dried pollen extracts were prepared in methanol at 15 mg/ml. To a 1.8 ml GC vial with a silicone septa 300 μl of the pollen extract was mixed with 1500 μl of a NaCl buffer (prepared by dissolving 4 g of NaCl in 40 ml water (18 mΩ and adding 800 μl glacial acetic acid). A polyacrylate fiber (PA) (cat # 57304) (Supelco, Bellefonte, PA), which was previously conditioned according to the manufacturers instructions, was supported with a manual holder and was immersed into the solution for exactly 60 min. The fiber was immediately placed into the injection port of the Varian 3800 series GC apparatus with a Varian (Palo Alto, Ca) 2200 Series Saturn ion trap mass detector and allowed to desorb for 30 min. The injection port was set at 300 °C and the column over was set at 40 °C for 1 min hold, then at 3 °C/min. The total run time was 87.67 min. The helium flow-rate was 1 ml/min. The column was a Varian Factor Four (30 m × 0.25 mm ID). The Varian MS version 6.9 software was used to process the chromatograms using the ions: 316, 302, 298, 286, 270, 269, 153, 131, 105, 77 and 51. The compounds were identified from spectral comparisons with NIST (2005 version, Gaithersburg, MD) and Wiley (2008 version, Hoboken, NJ) spectral libraries. A standard curve, using naringenin as a standard, was used to determine the concentration of the polyphenolics. Final values are reported in milligrams per gram of crude pollen.

$$[\mathrm{Naringenin}]_{\mathrm{mg/ml}} = (\mathrm{Area}) \times 6\mathrm{E} - 7; \quad R^2 = 0.9924$$

2.10. Statistics

For the solvent extracts, DPPH and FRAP results were compared among the groups using the ANOVA technique with XLStat 2007.6 (Addinsoft®). When ANOVA indicated differences among the means, a Tukey (HSD) analysis of the differences was used for all comparisons. All data are reported as mean ± standard error.

3. Results and discussion

In this study, eight solvents were employed to compare the antioxidant activity, to determine the most effective extraction solvent(s) (Fig. 1). The most effective extraction solvents were determined by comparing the DPPH and FRAP values. Non-polar solvent extracts, for example petroleum ether and chloroform, showed little activity in our assays, so we reported only the solvents that provided high antioxidant activity by the DPPH and FRAP methods

Table 4
Total polyphenolics phenolics of pollen extracts expressed as gallic acid equivalents (GAE) per mg/g pollen. Flavones and flavonols are expressed as quercetin equivalents (QE) per mg/g pollen and flavonones are expressed as naringenin equivalents (NE) per mg/g pollen.

		Pollen Type					
		Mesquite (mg/g)	Yucca (mg/g)	Palm (mg/g)	Terpentine bush (mg/g)	Mimosa (mg/g)	Chenopod (mg/g)
GAE	Polyphenolics	29.38 ± 0.22	19.48 ± 0.53	15.91 ± 0.05	29.12 ± 0.02	34.85 ± 0.08	26.84 ± 0.02
QE	Flavonols and flavones	3.54 ± 0.05	3.78 ± 0.11	2.66 ± 0.07	3.42 ± 0.18	5.48 ± 0.09	4.25 ± 0.14
NE	Flavonones	23.02 ± 2.05	23.57 ± 2.13	19.94 ± 2.42	27.48 ± 3.32	22.57 ± 1.67	19.94 ± 1.84

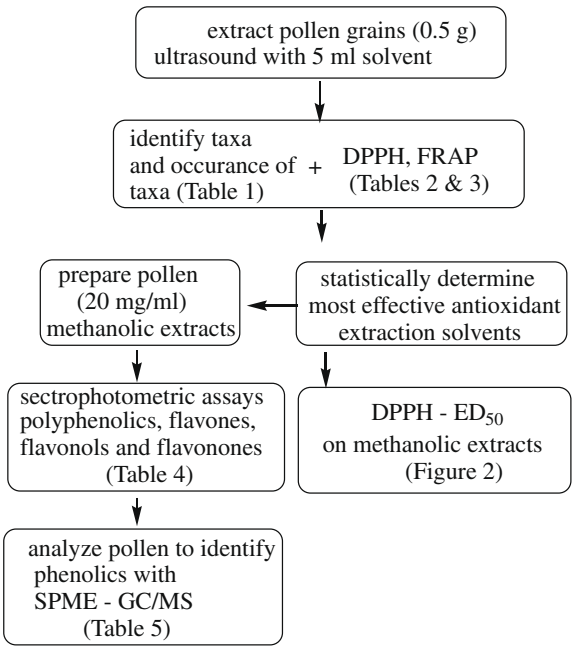

Fig. 1. Flow chart of experimental steps taken to determine the most effective extraction solvents and quantitative analysis to determine the phenolic components of the pollen extracts.

(Tables 2 and 3). These finding are analogous to recent reports on the effects of solvents and solvent combinations. For instance, Miraliakbari and Shahidi (2008) reported that a chloroform/methanol combination was superior for extracting antioxidants from raw nuts, compared to hexane.

The literature indicates that many of the compounds of interest in pollen grains are within the outer wall of pollen which consists of cellulose, pectin and a substance called callose, which is composed of B-1,3 polyglucan (Stanley & Linskens, 1974). For solvent extraction, we first investigated extracting the pollen with a Waring® blender, with ethanol as a solvent, but little radical scavenging ability activity was observed with the DPPH assay. We then investigated heating the samples in a heating block at 60–80 °C and vortexing intermittently. However, the ultrasound method produced higher antioxidant activities; therefore, we reported this method only. For taxa identification (Table 1), the residue from the methanolic extracts of the six samples were: acetylized, stained and mounted on microscope slides. Comparisons were made at magnification 460× with a pollen library from taxa identified from the Sonoran Desert. The samples are named according to the highest percentage taxa in the representative sample (Table 1). Therefore, the botanical origin of the pollen and percentage of taxa are reported. Mimosa plant foliage, which has been investigated as a renewable energy crop, has been reported to have high levels of polyphenolics and is reported to have the highest antioxidant activity compared to other renewable energy crops such as: *sericea*, kudzu, arunzu, switchgrass, velvet bean and caster (Lau, Carrier, Howard, & Lay, 2004). The Palm pollen sample was the only sample to be of one taxa and is 100% pure and is *washingtonia*, which is otherwise known as the fan palm.

The pollen samples were different in terms of the morphology and color of the pellets and the radical inhibition activity was greater with the pollen samples having more pigment. For instance, the beige colored Palm pollen (Table 1) was the least active of all pollens tested with the 2,2-diphenyl-1-picryhydrazyl (DPPH) (Table 2) or the ferric reducing-antioxidant power (FRAP) assay (Table 3). The most active was Mimosa pollen closely followed by the Chenopod pollen and the Mesquite pollen both of which exhibited yellow and orange pellets (Table 1). The FRAP assay, which occurs in a 0.3 M NaOAc/HOAc at pH 3.6, produced similar results as the DPPH, except that the FRAP assay predicts the Mesquite pollen to be more active than the Chenopod using both methanol (MeOH) and dimethylformamide (DMF) as extraction solvents. The results in Tables 2 and 3 show that MeOH and DMF were the most effective extraction solvents, with the latter being slightly more effective. The ED_{50} values, which are expressed as the concentration of pollen in mg per ml to quench 50% of the DPPH radicals, reveal the same order of pollen activity for MeOH (Fig. 2). Using MeOH, the Mimosa and Chenopod samples required the lowest concentration of all pollen samples tested to quench 50% of the DPPH radicals, while the Palm pollen, which is was found the least active, requires the highest concentration. MeOH and ethanol are reported to extract a wide range of compound types, including flavonoids and catechins that are components of pollens (Leja et al., 2007). It should be mentioned that acidified aqueous MeOH solvents are also effective antioxidant extraction solvents, but the DPPH method is compromised, as H^+ is a well known UV–Vis shift reagent (Markham, 1982).

The extracts also contained relatively high levels of polyphenolics, which are expressed as gallic acid equivalents per gram of pollen (Table 4). Methanolic extracts in a similar report were found to contain high levels of UV-B absorbing compounds in *Vica Faba*, *Betula pendula* and *Helleborus foetidus* (Rozema et al., 2001). A recent report on bee pollen samples from New Zealand, which is considered the highest UV region in the world, and in Portugal reveals: flavonoid glycosides, flavonoid aglycons and caffeic acid derivatives. The amounts of total phenolics in the ethanolic extract of the taxa *Eucalyptu globulus Labill* was reported at 32.59 mg/g gallic acid equivalents (GAE) of pollen (Leja et al., 2007). This is very near the values for some of the total polyphenolics for the most potent Sonoran Desert pollen extracts, as expressed in terms of GAE of pollen (Table 4). Polyphenolics are excellent scavengers of radicals and the number of hydroxyl groups on the phenyl ring seems to enhance the antioxidant capacity of a polyphenolic molecule (Wettasinghe & Shahidi, 2000).

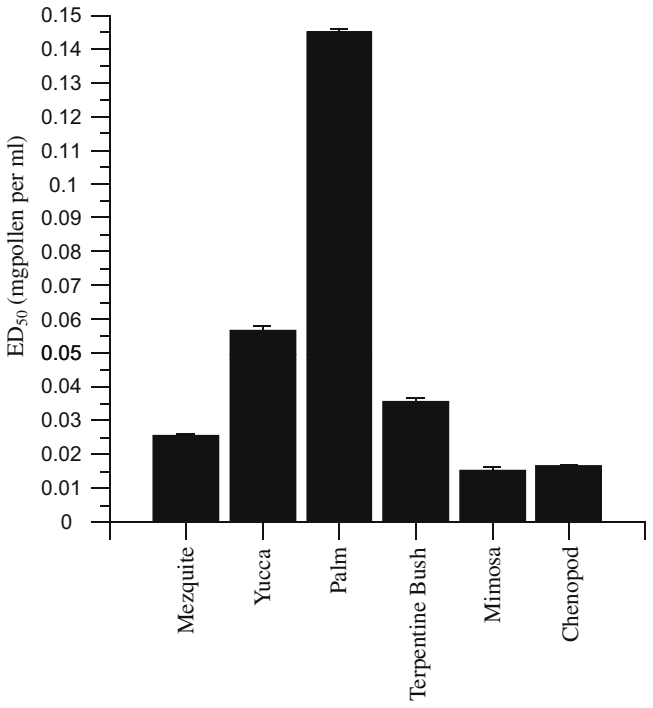

Fig. 2. Comparison of the ED_{50} values in mg of pollen per ml methanol (lower values indicate more powerful antioxidant capacity).

Table 5
Compounds identified by SPME coupled with GC–MS and quantified in terms of mg/g of crude pollen according to a standard curve of naringenin.

Pollen	Phenolic compounds identified in pollen by SPME – GC/MS
Mesquite	Naringenin (16.5 ± 3.57); 4′,5-dihydroxy-7-methoxyflavanone (0.38 ± 0.035); 7,8,2′,4′-tetrahydroxy isoflavone (0.34 ± 0.01); benzene acetic acid, α-oxo, methyl ester (0.21 ± 0.02); anthraquinone derivative (0.0956 ± 0.003); 5-methoxy-7-methyl-1,2-naphthoquinone (0.066 ± 0.009); 7-hydroxy-1-indanone (0.062 ± 0.009); 1-p-tolyl-anthraquinone (0.047 ± 0.008)
Yucca	Naringenin (0.58 ± 0.077); 2-methyl-5-hydroxybenzofuran (0.092 ± 0.004); 4′,5-dihydroxy-7-methoxyflavanone (0.09 ± 0.0038); anthraquinone derivative (0.062 ± 0.008); 5-methoxy-7-methyl-1,2-naphthoquinone (0.041 ± 0.009); 1,2,3,4-tetrahydro-2-(2-hydroxy-3-phenoxypropyl)-6,7-dimethoxyisoquinoline (0.034 ± 0.013); 1-)2-methoxy phenyl)-9,10-anthracenedione (0.014 ± 0.001)
Palm	2,6-Dihydroxy-6-methylbenzaldehyde (0.38 ± 0.07); 2-formyloxy-1-phenylethanone (0.20 ± 0.026); 4′,5-dihydroxy-7-methoxyflavanone (0.058 ± 0.0086); anthraquinone derivative (0.085 ± 0.01); 5-methoxy-7-methyl-1,2-naphthoquinone (0.070 ± 0.008); 7-hydroxy-1-indanone (0.038 ± 0.007); naringenin (0.0013 ± 0.0002); methyl benzoate (0.010 ± 0.001)
Terpentine Bush	Naringenin (2.99 ± 0.38); benzene acetic acid, α-oxo, methyl ester (0.35 ± 0.11);4′,5-dihydroxy-7-methoxyflavanone (0.26 ± 0.023); 5-methoxy-7-methyl-1,2 naphthoquinone (0.093 ± 0.0098); 1,1-diphenyl-9-methyldeca-3,5-dien-1,9-diol-8-one (0.027 ± 0.0033); 1-p-tolyl-anthraquinone (0.022 ± 0.0016)
Mimosa	Naringenin (20.86 ± 1.84); 4′,5-dihydroxy-7-methoxyflavanone (0.45 ± 0.033); benzene acetic acid, α-oxo, methyl ester (0.51 ± 0.012); 5-hydroxy-7-methoxy-2-methyl-3-phenyl-4-chromene (0.075 ± 0.0014); 1-p-tolyl-anthraquinone (0.029 ± 0.0015)
Chenopod	Naringenin (6.62 ± 0.324); α-oxo, methyl ester (4.27 ± 0.14); 4′,5-dihydroxy-7-methoxyflavanone (0.34 ± 0.022); 5-methoxy-7-methyl-1,2-naphthoquinone (0.19 ± 0.013); 1-(3-methoxy phenyl)-anthraquinone (0.104 ± 0.02); 5-hydroxy-7-metoxy-2-(4-methoxyphenyl)-4H-1-benzopyran-4-one (0.11 ± 0.0.031); anthraquinone derivative (0.078 ± 0.014); 7-methoxy-6-(3-methyl-2-butenyl)-2H-1-benzopyran-2-one (0.027 ± 0.0011)

Since the MeOH extracts showed high antioxidant activity, and it is facile to evaporate MeOH at low temperatures as opposed to DMF, the methanolic extracts were used for the quantitative determination of the total flavonoids, flavones, flavonols and flavonones (Table 4). The Mimosa pollen had the highest level of polyphenolics at 34.85 ± 0.08 mg/g GAE, with Mesquite pollen have the second highest amount. Palm pollen contained the lowest level of polyphenolic compounds with a value of 15.91 ± 0.05 mg/g GAE. The Palm pollen, correspondingly, had the lowest observed radical quenching ability, while the Mimosa pollen had the highest (Tables 2 and 3). Also, the amounts of flavonols and flavones, were determined in terms of milligrams of quercetin equivalents (QE) per gram of pollen. The Mimosa pollen sample had the highest amount 5.48 ± 0.09 mg/g QE versus the other pollen samples, while Palm pollen had the lowest at 2.66 ± 0.07 mg/g QE.

The amounts of flavonones are expressed as milligrams of naringenin equivalents (NE) per gram of pollen. The Terpentine bush pollen, had the highest levels of NE at 27.48 ± 3.32 mg/g and the Yucca pollen had the second highest amounts of NE at 23.57 ± 2.13. This is interesting as the Yucca pollen had relatively low antioxidant activity (Tables 2 and 3). The data between the antioxidant activity and the total phenolics correlated well in this study for the observed activity. For instance, when the DPPH% activity (Table 2) is plotted verses the total polyphenolics (Table 4) for the pollen samples in the order of increasing activity: Palm, Yucca, Terpentine Bush, Mesquite, Chenopod and Mimosa for the MeOH, the R^2 coefficient is 0.8502. When the FRAP values (Table 3), for the methanolic pollen extracts are plotted in the order of increasing activity: Palm, Yucca, Terpentine Bush, Chenopod, Mesquite and Mimosa are plotted against the total polyphenolics, the R^2 value is 0.873. The DMF extracts for the FRAP assay revealed a correlation of 0.9221. The FRAP and DPPH assays are in close agreement with the exception that the DPPH assay predicts the Chenopod to be more active than the Mesquite pollen, while the former predicts the Mesquite pollen to be more active. The FRAP and DPPH assays, predict the Mimosa pollen to be the most active in terms of radical quenching ability. The ED_{50} values, which are generated from DPPH assay results, suggest that the Mimosa pollen has slightly more radical quenching capacity than the Chenopod pollen (Fig. 2) for the MeOH extracts. We also tested the extracts for anthocyanins using cyanidin hydrochloride as standard using an AOAC differential pH method (2005); no anthocyanins were detected in all samples tested.

The analysis of the pollen samples for active phenolic constituents with GC–MS was conducted by first adsorbing the phenolic components with a 85 μM polyacrylate SPME fiber. The PA fiber was chosen because it was found to be superior for adsorbing molecules bearing phenolic moiety compared to the other commercially available fibers (LeBlanc et al., 2008). This observation has been reported by Daher and Gülacar (2008). The chromatography peak for naringenin, which was identified by the NIST 2005 spectral library and confirmed by retention time, was the dominant peak for the Mimosa, Mesquite and Chenopod pollen, with the former containing the most naringenin of any pollen sample we tested. Naringenin, which is a component of grapefruit juice, is a reported antioxidant and is active in terms of slowing the replication of the Hepatitis C virus in *in vitro* studies (Nahmias et al., 2008). In addition to naringenin, 4′,5-dihydroxy-7-methoxyflavanone was found in all of the pollen samples but mostly in the Mimosa, Mesquite and Chenopod and Terpentine. The latter mentioned flavanone, which is reported to be a component of *Larrea tridentate*, which is a taxa component of the Mesquite and Mimosa pollen samples at 28.3% and 10.8%, respectively (Table 1), has been found active against human breast cancer cells (Lambert et al., 2005). In addition, 2,6-dihydroxy-6-methylbenzaldehyde and 2-formyloxy-1-phenylethanone were only detected in Palm pollen (Table 5) and carnegine, an alkaloid of the cactus Carnegiea gigantica first discovered by Bruhn and Lundström (1976), was detected in the Mimosa pollen. In addition, the methyl ester of linoleic acid was detected in the Chinopod pollen.

Palm pollen, which is the only beige color pollen with sparse amounts of any pellets with any pigments, has the lowest antioxidant activity (Tables 2 and 3) of any of the pollen samples and the lowest amounts of polyphenolics (Table 4), flavonols and flavone equivalents (Table 4) and naringenin and 4′,5-dihydroxy-7-methoxyflavanone (Table 5) of any of the pollen samples. The Palm pollen was the last pollen collected in November, when the UV intensity is much lower. Therefore, this type pollen would not require the extent of DNA protection as a plant that pollinates in the summer months. It is interesting that in a study where caged bees were fed pollen from the Sonoran Desert for survival studies, that Mesquite and Saguaro pollen, proved superior to Palm for bee longevity (Schmidt, Theones, & Levin, 1987).

In summary we showed that the most effective solvents that we investigated for extracting antioxidants from pollen are MeOH and DMF. The Mimosa pollen sample, which was collected from the July to August period had the highest antioxidant activity of the tested pollen samples in either the DPPH or FRAP assays (Tables 2 and 3) and it also had the highest polyphenolics (Table 4) and the highest levels of naringenin (Table 5). By the DPPH assay results, Chenopod pollen exhibited the second highest activity, clo-

sely followed by Mesquite pollen, which was collected during the Spring. Palm pollen had the lowest antioxidant activity. The FRAP assay results suggest the same order of activity except that the Mesquite pollen was more active in terms of its antioxidant activity than the Chenopod. Our data supports the hypothesis that pollen collected during the more UV-intense period of early through late summer, has more antioxidant activity than pollen, such as palm, collected in the fall season. Future studies will be directed at determining whether phenolic compounds protect plant pollens against UV radiation and perhaps offer some protection to bees in immune responses against pathogens.

Acknowledgement

We wish to express our appreciation to Mr. Fred Terry and for collecting the pollen samples and for his suggestions in terms of proposing the botanical origin of the pollen, based on the time collected.

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