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Analyses for Flavonoid Aglycones in Fresh and Preserved *Hibiscus* Flowers*

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In the past, perennial and temperate-zone tree-form *Hibiscus* species and hybrids have been relegated to the status of little-known garden plants, at least in the United States. However, their potential as a new source of edible flowers and natural food colorants (all within the anthocyanin class of edible flower pigments) elevates them to the status as—possibly the newest—of the New Crops.

The 17 indigenous native *Hibiscus* species of North America range from USDA Zones 4–10 and, in the wild, are confined to wetland areas. In cultivation, the plants are installed in permanent plantations, using zero-runoff protocols under passive flooding, or drip irrigation protocols. Other hardy perennial and tree-form species from Asia, including *H. mutabilis*, *H. syriacus*, and the Pan-Pacific species *H. hamabo* and *H. tileaceous* are suitable for USDA Zones 8–10. Potential products from these cultivars include fresh food (primarily edible flowers for the restaurant trade) and natural food colorants, as well as edible seed meals and seed proteins (for nutraceutical applications), seed oils, lubricants, and fiber, mucilages and complex polysaccharides from fruiting organs, roots, canes, and/or branches.

In this paper, the flowers of selected native North American, non-native Asiatic and pan-Pacific species and North American hybrid cultivars were selected for preliminary analysis of pigments. The 29 species and hybrid Malvaceae utilized for the chemical characterizations of the pigments in the fresh flowers are listed in Table 1. These include *Hibiscus* species native to the continental US: *Hibiscus aculeatus* (light yellow with small red eye); *H. coccineus* (solid orange/red); *H. laevis* (cream to blush with red eye); *H. martianus* (solid red); *H. moscheutos* (white to cream or blush with red eye); *H. striatus lambertianus* (light purple with small light-red eye); and several hybrids of three of these native species [including BOSTx®HHHybrids: 'Governor Ann' (mulitple types); 'Nathan's Star'; 'Pink Hybrids' (mixed types); 'Purple Hybrids'; 'Razberri Rhapsody'; and 'Razberri Ruffles'] (Fig. 1). Representative photos of BOSTx®HHHybrids cultivars are available for viewing at BOSTx.com.

In addition, some non-*Hibiscus* genera of Malvaceae that are native to the US were also analyzed, including *Kosteletzkya virginica* (pink with yellow eye), *Malvaviscus arboreus drummondii* (solid orange), *Pavonia lasiopetalus* (solid pink), and *Sida spinosa* (solid yellow). Non-native Malvaceae used in the analyses included *Abelmoschus moschatus* (two forms, orange and red, tropical Asia), *H. calyphyllus* (yellow, Madagscar), *H. mutabilis* (peach, China), *H. paramutabilis* (red, China), *H. rosa-sinensis* (orange, pan-Pacific), *H. syriacus* (blue, China), and *M. arboreus mexicana* (orange, Central Americas). Our methods and the results of the analyses are detailed below.

METHODOLOGY

Preparation of Standards

Various suppliers provided 18 flavonoid aglycone standards with purity levels of 95%–99%. Catechin and five epicatechin-type compounds along with luteolin, naringenin, hesperetin and kaempferol were purchased from Sigma Chemical Co. (St. Louis, Missouri). Myricetin and apigenin were obtained from Aldrich Chemical Co. (Milwaukee, Wisconson) while delphinidin was obtained from Spectrum Chemical Co. (New Brunswick, New Jersey). The remaining aglycones—including cyanidin, petunidin, pelargonidin, peonidin, malvidin, and quercetin—were purchased from Sarsyntex (Merignac, France).

Initially, solutions containing 2–5 aglycones were used to obtain retention time and spectral information for the identification of the flavonoid aglycones present in the *Hibiscus* flower extracts. These solutions were prepared by dissolving standard compound in methanol (1–2 mg/mL of each aglycone). After removing an aliquot for HPLC analysis, the standard solution was mixed with 5 mL of Reagent A (Reagent A is a 4:1 mix

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of 62.5% aqueous methanol with 0.5 g/L *tert*-butylhydroquinone and 6 N HCl) and refluxed for 2 hr. This reacted solution was also analyzed by HPLC.

Two series of standard solutions (each series containing different aglycones found in the *Hibiscus* flowers) were analyzed by HPLC to determine the quantity of compound versus chromatograph peak area information. The most concentrated solution in each series was precisely prepared from four solid standards. Specifically, 300–500 µg of each of the four aglycones was weighed to the nearest 0.1 µg. The amounts were combined, then dissolved and diluted to 5.00 mL with Reagent A. Next, the concentrate was diluted 1.00 mL to 10.00 mL and 0.100 mL to 10.00 mL with Reagent A to produce two other solutions for the series. The standard solutions were analyzed by HPLC using 2, 10, and 20 µL injections.

HPLC Analysis

The LC system employed is a Hewlett-Packard (HP) 1050 Series modular system equipped with an 1100 Series diode array detector and a HP Kayak computer with HP HPLC 3D ChemStation software for instrument operation and data analysis. The LC method uses a HP Zorbax Eclipse XDB-C18 column (250 × 4.6 mm) preceded by a guard column of similar stationary phase. The columns are maintained at room temperature. The mobile phase is run at 1.00 mL/min and consists of a gradient of water, methanol (MeOH) and

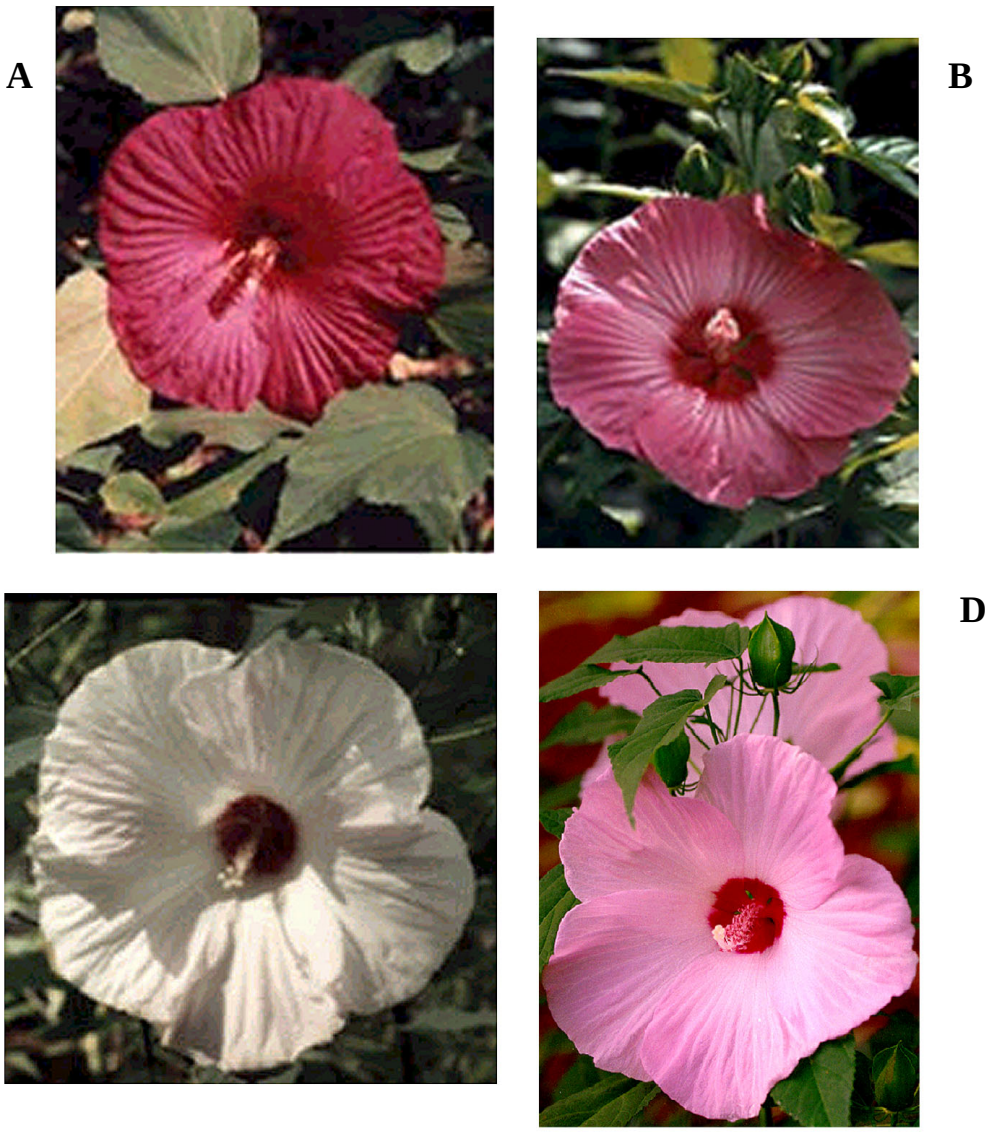

Fig. 1. BOSTx®HHHybrids: (A) ‘Razberri Ruffles’, (B) ‘Michelle’s Passion’, (C) ‘Jacki’s K.O.’, and (D) ‘Grace Coolidge’.

acetonitrile (ACN), each containing 0.05% trifluoroacetic acid. The gradient is as follows: 0–5 min at 90% H_2O, 6% MeOH, and 4% ACN; 5–30 min at 85%, 9%, and 6%; 30–60 min at 71%, 17.4%, and 11.6%; 60–61 min at 0%, 85%, and 15%; 61–66 min at 90%, 6%, and 4%. For the anthocyanidins, the chromatogram signal is monitored at 520 nm (20 nm bandwidth) with reference to 570 nm (60 nm bandwidth) while, for all other flavonoid aglycones, the chromatogram signal is monitored at 210 nm (20 nm bandwidth) with reference to 550 nm (100 nm bandwidth). In addition to the chromatogram signals, spectra are collected over 190–600 nm. Spectra of peaks within the flower extract chromatograms were compared to those of standards for identification purposes.

Extraction of Fresh Flower Petals

Fresh blossoms of 29 species and hybrids of *Hibiscus* were examined over a course of 4 days (Table 1). The flowers from eight or fewer entries were harvested in the morning after blossoms were fully open, placed in clear domed trays and transported under air conditioning (as would be done for a fresh delivery to potential restaurant clients) to the laboratory, then stored at 2ºC in a laboratory cold room until extraction was performed. Generally, four sets of two extractions were run concurrently for each day of freshly harvested samples, so that eight flower varieties could be examined per day while limiting the interval between harvest and extraction to 10 hr.

The extraction procedure was as follows: For small to very small flowers, 6–8 whole flowers were selected for extraction. For medium to large sized flowers, 4–6 petals from a single flower were used, depending upon the size of the petals. Flower diameters range from as small as 3 cm (*Pavonia* spp.) to as large as 30 cm (*Hibiscus* hybrid cultivars).

Whole flowers or individual petals (depending upon blossom size) were cut into 2–5 mm by 5–10 mm pieces. Then, 1.0–1.2 g of fresh tissue were weighed to the nearest 0.1 mg into a 50 mL round-bottom flask. The amount of tissue used for the air-dried and freeze-dried samples was ± 0.1 g, in order to attain pigment concentrations comparable to fresh blossoms and compensate for the water weight lost in the dehydrated samples. Blossom dry weights are routinely about 10% of fresh weight for both hot-air dried and freeze dried samples. Levels of petal hydration in frozen samples (which were bagged in plastic) were equivalent to fresh samples. The tissue was covered with 25 mL of Reagent A and refluxed for 2 hr. After cooling to room temperature, the reaction mixture was sonicated for 5 min, transferred to a 100 mL volumetric flask and diluted to the mark with methanol. The dilute sample was well mixed and a 4 mL portion was centrifuged. An aliquot of the clear supernatant was transferred to HPLC vial for analysis. HPLC analysis was carried out using a 20 µL injection and was accomplished within 2.5 hr of extract preparation.

RESULTS

Chromatography of Standards

The retention times for the 18 flavonoid aglycones initially investigated compare well with those of Merken and Beecher (2000). Of the 18 listed therein, eight aglycones were found in the *Hibiscus* flower extracts, including delphinidin, cyanidin, petunidin, myricetin, pelargonidin, malvidin, quercetin, and kaempferol. Calibration of these eight aglycones yielded straight lines with coefficients of 0.998 or greater. All intercepts were close to zero. In the standards, the limits of detection (LODs) ranged from 38 ng for kaempferol to 149 ng for delphinidin. For the anthocyanidins, these LODs determine the minimum of compound detectable in the flower extracts. For myricetin, quercetin and kaempferol, however, the minimum of compound detectable in the extracts is limited by background noise that results in LODs of 450–520 ng. For a 20 µL injection and 100 mL of extract, the LODs convert to minimum detectable concentrations in flowers ranging between 0.32 mg/g for cyanidin and 2.6 mg/g for kaempferol.

Analysis of Hibiscus Flowers

The flavonoid aglycones identified in the fresh flower petals of the 29 species and hybrids are presented in Table 1. The two most commonly found flavonoid aglycones were the flavonol *quercetin* and the anthocyanin *cyanidin* (Fig. 2). Quercetin levels in the flowers ranged from 2 to 192 mg/g with the highest levels found

in those flowers with white or cream colored petals. These included *H. moscheutos* 'White', *H. laevis* 'Slight Blush', and BOSTx®HHHybrids 'Light Pinks'. No quercetin was detectable in the two *H. syriacus* cultivars (Table 1) or in the two *Malvaviscus arboreus* varieties. Cyanidin was identified in all of the flowers, with concentration ranging from 1 to 77 mg/g. The highest concentrations of cyanidin were found in the very dark magenta-red flowers from BOSTx® 'Razberri Rhapsody' and, surprisingly, the lighter more orange-red flowers of *H. martianus*. The lowest levels of cyanidin were found in cultivars with pink, white, yellow, or purple flowers.

In addition to quercetin and cyanidin, several other aglycones were detected in the flowers, but only in specific genomes. Delphinidin, petunidin, and malvidin (all anthocyanidins) were found at levels of 1–5 mg/g in the flowers of the two cultivars of *H. syriacus* (an Asiatric species of *Hibiscus*), 'The Blues', and 'Purple Red' (Table 1). These results are similar to those of Kim and Fujieda (1991), although they also reported the presence of pelargonidin (flower reds) and peonidin, compounds in many *H. syriacus* cultivars which were not detected in the *H. syriacus* cultivars examined in this study, because only blue-flowered cultivars (rather than the more common red or pink of this species) are produced at Hibiscus Hill Plantation, Waller county, Texas. Pelargonidin was also identified in the dark red-orange flowers of *Malvaviscus arboreus drummondi* and *M. arboreus mexicana*, both of which are New World species. In these cultivars, pelargonidin levels were ca. 10 mg per g flower tissue.

Kaempferol was found at high levels (41–145 mg/g) in four species, *Kosteletzkya virginica*, *H. striatus lambertianus*, *M. arboreus drummondi* and *M. arboreus mexicana*, all of which are New World natives. It was also tentatively found at very low concentrations (3–8 mg/g) in the Asiatic species *H. mutabilis*, *H. paramutabilis* and the two *H. syriacus* cultivars. The presence of kaempferol in *H. mutabilis* f. *versicolor* has been reported by Ishikura (Ishikura 1973, 1982). Kaempferol has also been reported in *H. moscheutos* (Ohmoto et al. 1988) (a New World native) and *H. rosa-sinensis* (Subramanian and Nair 1972) (a pan-Pacific tropical species), although it was not found in those two *Hibiscus* cultivars in our study.

One last aglycone was identified, i.e. myricetin. This compound was found in *H. aculeatus*, the only *Hibiscus* species in section Furcaria that is native to the New World. This species is related to a number of African and Australian annual and biennial species in section Furcaria, including kenaf (*H. cannabinus*), roselle (*H. sabdariffa*), *H. radiatus*, red shield hibiscus (*H. acetosella*), and numerous perennial Australian genomes (e.g., *H. splendens*, *H. heterophyllus*). The fresh flowers of *H. aculeatus* are yellow with a small reddish eye and were found to contain about 16 mg/g of myricetin. Small amounts of myricetin were also found in *H. calyphyllus* and BOSTx® 'Pink Hybrids' (Table 1). Three quercitin glycosides were identified in BOSTx® 'Razberri Ruffles', as well as at least four unknown cyanadin and quercetin glycosides.

VITAMIN CONTENT

Preliminary analyses were performed on a handful of hibiscus flower samples for vitamin A and E presursors, specifically, the carotenes and tocopherols. The analysis procedure used was an adaptation of that of Kurilich et al. (1999). To extract the vitamin presursors, flower petals were cut into little pieces and refluxed for 30 min in ethanol containing the anti-oxidant butylhydroquinonone. Potassium hydroxide then was added to the mixture and refluxing was continued for 30 min more. The reacted sample was filtered and then the filtrate rotoevaporated under vacuum to a reduced volume. After the addition of water, the sample was extracted with hexane:toluene (10:8). The hexane:toluene layer was collected and rotoevaporated under reduced vacuum to a dry residue. The residue was then reconstituted in tetrahydrofuran and the extract analyzed by high performance liquid chromatography (HPLC).

HPLC was performed using a Hewlett-Packard (HP) 1050LC equipped with an 1100 Diode Array Detector module and a computerized ChemStation operating system. The HPLC method for the separation of carotenes and tocopherols employs a Phenomenex Prodigy ODS-2 (5 µm, 4.6 × 250 mm) column maintained at room temperature and an isocratic mobile phase of acetonitrile:methanol:tetrahydrofuran (52:40:8) run at 2.0 ml/min. The runtime is 25 min with tocopherols appearing at 5–6 min and carotenes eluting at 14–15 min. The chromatogram signal is monitored at 290 nm for tocopherols and 450 nm for carotenes (both referenced to 650 nm). Spectra are collected over 210–750 nm.

Table 1. Flavonoid aglycones in 29 *Hibiscus* species and hybrids, fresh flowers.

Species Cultivar name	Relative size and appearance of flower	Concentration (mg/g fresh tissue)								Total pigments (mg/g fresh flower)
		Quercitin	Kaempferol	Myricetin	Delphinidin	Cyananidin	Petunidin	Pelargonidin	Malvidin	
Abelmoschus moschatus	Very small; light red (fresh only)	6				19				25
Abelmoschus moschatus	Small; light orange pink	2				14				16
Hibiscus aculeatus	Medium; yellow with red eye	127		16		3				146
Hibiscus calyphyllus	Medium; yellow with dark red eye	9			1	5				15
Hibiscus coccineus	Large; orange red star	16				31				47
Hibiscus grandiflorus	506	74			10				590	
Hibiscus laevis ‘Slight Bush’	Medium; white w/ slight blush, pink eye	160				7				167
Hibiscus martianus	Very small; red with red-yellow eye	23				72				95
Hibiscus moscheutos ‘White’	Large; cream with red eye	192				5				197
Hibiscus mutabilis ‘Single Pink’	Large; pink	80	8			5				93
Hibiscus paramutabilis	Large; dark pink	91	4			20				115
Hibiscus rosa-sinensis	Medium; light red, whitish eye, long red style (fresh only)	7				36				43
Hibiscus striatus lambertianus	Medium; purple	17	145			1				163
Hibiscus syriacus ‘Purple Red’	Small; purple-red		3		3	1	3		5	15
Hibiscus syriacus ‘The Blues’	Medium; blue		6		1	1	1		1	10
Hibiscus syriacus alba		96			7				103	
Kosteletzkya virginica	Medium; light purple/pink, sm. yellow eye	16	119			4				139

Malvaviscus arboreus drummondii	Very small; orange, tubular		41		2	10		53
Malvaviscus arboreus mexicana	Small; orange, tubular		42		3	11		56
Pavonia hastata	Medium; striped light-dark pink	8			28			36
Pavonia lasiopetalus	Medium-small; pink	4			3			7
Sida spinosa	Very small; golden yellow	6			5			11
BOSTx® ‘Delilah’	764	49		146		959		
BOSTx® ‘Governor Ann’ (type)	Large; orange red	46			38			84
BOSTx® ‘Lady Bird’		1186			158			1344
BOSTx® ‘Marty’s Star’		613			253			866
BOSTx® ‘Orange Star’		387			102			489
BOSTx® ‘Purple Backs’		1379	96		69			154
BOSTx® ‘Quatro Rojo’		547			183			730
BOSTx® ‘Razberri Rhapsody’	Large; very dark pink	125			77			202
BOSTx® ‘Razberri Ruffles’	Medium; deep raspberry red	111			44			155
BOSTx® ‘Rosalyn Carter’		1632			44			1676
BOSTx® ‘Turk’s Cap’		27	674		11			712
BOSTx® ‘Yoda’		1032			276			1308
BOSTx® Hybrid light pinks (lg)	Large; white with dark pink eye	170			4			174
BOSTx® Purple hybrids (mixed)	Large; purple-pink	90			22			112
BOSTx®HHHybrid ‘Nathan’s Star’	Large; dark pinky-red	6			39			45
BOSTx®HHHybrid Pink - dark red eye	Large; pink with dark pink eye	119			8			127
BOSTx®HHHybrid Pink - med. red eye	Large; pink with medium pink eye	81			7			88
BOSTx®Pink hybrids (mixed)	Very large; pink w/dark pink to cherry red eye	77			13			90
Pink Abutilon		239	174		8			421

In the preliminary analyses, α- and γ-tocopherol were found in the flower petals of all eight of the hibiscus varieties analyzed. These varieties include *Hibiscus grandiflorus* and the Bostx®hybirds of 'Razberri Rhapsody', 'Governor Ann', 'Rosalyn Carter', 'Razberri Ruffles', 'Lady Bird', 'Quatro Rojo', and 'Marty's Star'. From the rough quantitation, we can say that there is at least 20–80 ppm of α-tocopherol and 2–15 ppm of γ-tocopherol in the flowers petals. There is a possibility that α- and β-carotene are also present but additional analyses are needed to positively identify these precursors. We are currently in the process of optimizing the extraction procedure for accurate quantitative evaluation of the tocopherol and carotene content of the hibiscus flowers. We are also performing analyses to confirm or refute the identification of the carotenes. When the hibiscus plants bloom this year and more samples can be harvested, the eight hibiscus analyzed and additional varieties will be analyzed for their vitamin precursor content using the soon-to-be new more accurate analysis procedure.

VALUE-ADDED PRODUCTS

Several potential FDA regulatory barriers may have to be overcome in order to introduce the use of value-added *Hibiscus* products to the market place. In the case of three North American native endemic Malvaceae species (*Hibiscus moscheutos, H. aculeatus* and *Kosteletskya virginiana*), there is some documentation of ethnobotanical usage (Plants for a Future 2002; Dr. Duke's Phytochemical and Ethnobtanical Data-

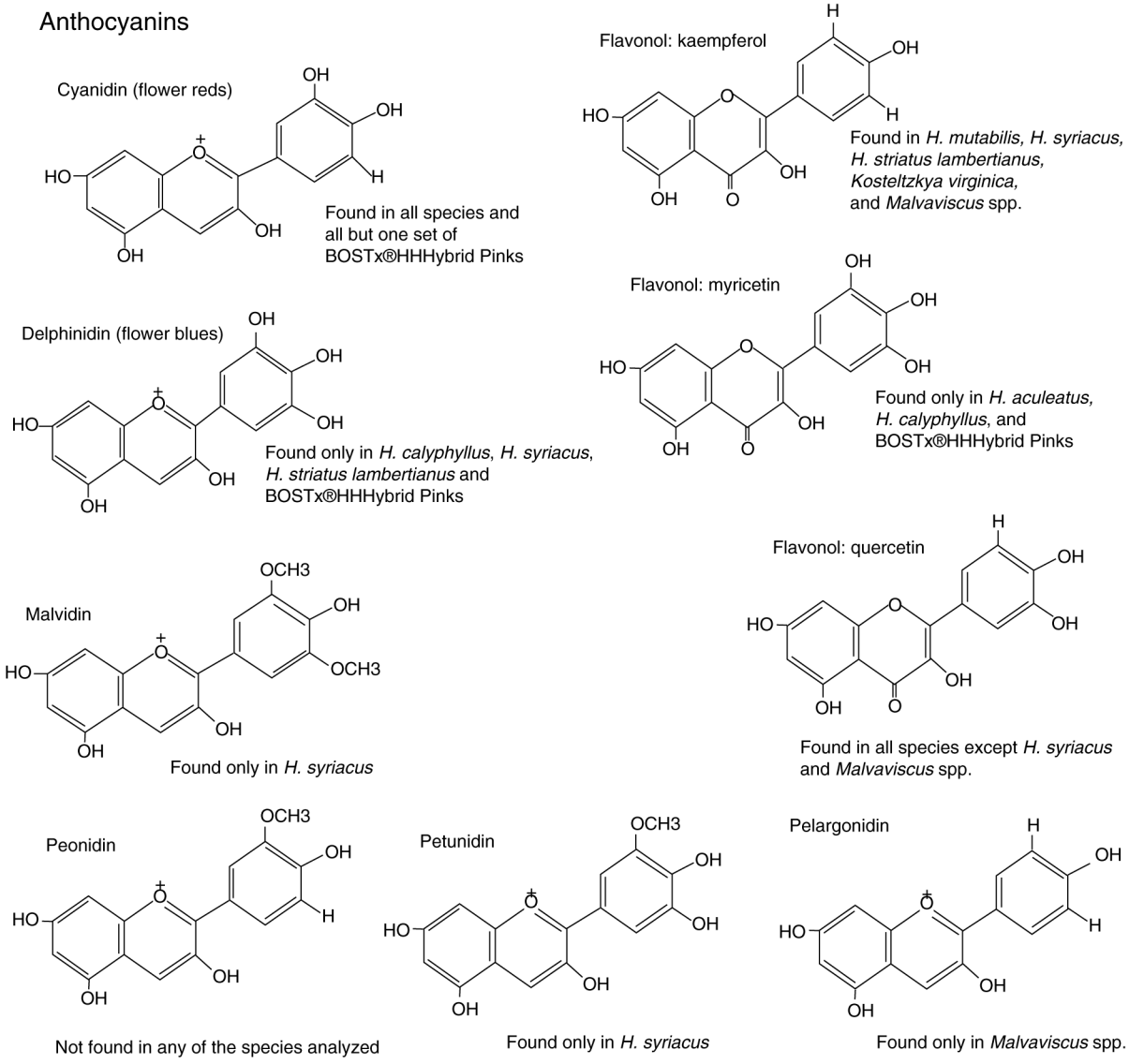

Fig. 2. General chemical structure of plant flavonoids found in BOSTx®HHHybrids and selections, 2001.

bases 2002). For all the other native American endemic species of *Hibiscus*, however, there is little or no phytochemical data. The Old World *Hibiscus* species (and many other genera within the Old World Malvaceae), however, are well documented. The lack of data for North American Malvaceae genomes is one of the driving forces behind the chemical investigations we have pursued in recent years, many of which are documented herein. Without such data, we cannot move ahead with development of our formulations of *Hibiscus* value-added food products and skin-care formulations.

In addition to edible pigments, all of the North American endemic species also have good yields of industrial fibers, as do all of the non-endemic species and varieties listed in Table 1. In addition, the roots and flowers of many of these species have been used to make root and flower teas, and have shown activity against tumors, viruses, and cancers. All species are also very mucilagenous and many (if not all) have been used for centuries in many countries—including the US—as demulcents, emollients, nervines, and stomachics, as well as in the treatment of dysentery, lung, and urinary ailments. In the US, *Kosteletskya* has been used as a potherb (leaves) and flowers are eaten uncooked. Roots have been used as vegetables (cooked) and can be ground, made into a paste and roasted to make "marshmallows;" teas may be made from the flowers, and any part of the plant can be boiled and used as an egg-white substitute for making meringues (the root, flower and/or stem sugars and mucilages are extracted and concentrated by boiling in water).

Other species that we currently grow (or have grown) have similar efficacies (Plants for a Future 2002; Dr. Duke's Phytochemical and Ethnobtanical Databases 2002). These include *H. mutabilis* and *H. paramutabilis*, *H. rosa-sinensis*, and *H. syriacus*. We have also grown several annual and/or biennial species (such as *H. acetosella*, *H. cannabinis*, and *H. sabdariffa*), but are concentrating on perennial and tree-type Malvacea because of the much greater potential for sustainable agriculture with these long-lived species.

The results of previous investigations of nutritional analyses of dehydrated petals (analyses performed by Siliker Laboratories, Dallas, Texas) indicate substantial yields of dietary proteins, sugars, fiber, and unsaturated fats, as well as most of the major dietary vitamins. These could be recovered from floral harvests on a commercial basis. Applications may include use of whole fresh, dried (dehydrated or freeze-dried), or frozen petals for use in nutraceutical products and specialty gourmet foods, or as a base for extraction of natural food colorants for applications in foods and beverages. Other applications for fresh and processed blossoms and their extractions may include formulation of cellulosic and mucilagenous dietary products, transdermal carriers for non-alimentary drug delivery, and skin-care formulations.

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