SUNFLOWERS AND HONEYBEES: A STUDY OF THE MUTUALISTIC RELATIONSHIP FROM A BIOCHEMICAL AND MORPHO-ANATOMICAL PERSPECTIVE

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DEDICATION

For my husband, Thomas Wojtaszek, and my daughter, Alisa Wojtaszek, my gratitude for your endless supply of encouragement, faith and support cannot be expressed in words.
For my mom, Linda Arms, for always believing in me and for never giving up.

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ABSTRACT

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SUNFLOWERS AND HONEYBEES: A STUDY OF THE MUTUALISTIC RELATIONSHIP FROM A BIOCHEMICAL AND MORPHO-ANATOMICAL PERSPECTIVE

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The mutualistic relationship between *Helianthus annuus* (*Asteraceae*) and *Apis mellifera* is reflected in their co-evolutionary adaptations. The corolla morphology and pigmentation of sunflowers help form a target pattern under UV, recognizable by bees. While collecting rewards, bees cross-pollinate the disk florets. Morpho-anatomical co-adaptations of the sunflower and honeybee were studied with LM, SEM, and CLSM. This study reports for the first time the presence of one to three rows of transitional papillae on stigma, which may function in protection of the receptive stigma from self-pollination. A model of the cross-pollination of sunflower inflorescence by honeybees is presented. The chemical characterization of flavonoid pigments in disk florets, known to contribute to the target pattern of the inflorescence, accomplished with chromatographic and MS techniques, revealed the presence of luteolin and pelargonidin pigments. This is the first report on the presence of luteolin and pelargonidin in sunflower disk florets. Results of this study will contribute to the metabolomics of the phenylpropanoid pathway in *H. annuus* in addition to enhancing understanding of mutualism and biosemiotic relationships between flowering plants and pollinators.

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LIST OF ABBREVIATIONS

Abbreviation

BRF: basal ray floret

CAD: collisionally active dissociation

CE: collision energy

CEM: channel electron multiplier

CID: collision induced dissociation

CLSM: confocal laser scanning microscope

CXE: collision cell exit potential

DF: deflector.

DF: disk floret extract

DRF: distal ray floret

EP: entrance potential

ESI-MSMS: electrospray ionization tandem mass spectrometry

FP: focusing potential

FW: fresh weight

H. annuus: Helianthus annuus

HPLC: high performance liquid chromatography

LM: light microscope

$[M+H]^+$: positive ion mode

$[M-H]^-$: negative ion mode

m/z: mass to charge ratio

MODE: ion mode\

MS: full mass spectrometry

MW: molecular weight

t_R: retention time

SEM: scanning electron microscope

SM: stereomicroscope

SPE: solid phase extraction

TLC: thin layer chromatography

CHAPTER I

INTRODUCTION

Sunflower, *Helianthus annuus*, *Asteraceae*, has evolved morpho-anatomical and biochemical adaptations to maximize seed production success primarily by means of cross pollination by insects, most notably honeybees (*Apis mellifera*, Hymenoptera: Apidae). A sunflower plant has a specialized heterogamous inflorescence called capitulum or head (Fig.1). The outer whorl of the capitulum is made of zygomorphic yellow ray florets, which are sterile. The center of the capitulum is occupied by actinomorphic tubulate or disk florets, which are fertile, typically burgundy in color, and arranged in arcs radiating from the center of the inflorescence into distinct left and right turning spiral rows (Fambrini *et al*., 2003).

The ray ligulate florets are non-reproductive and have a vestigial ovary. Each zygomorphic ray floret is composed of three to five elongated petals which are fused to form a ribbon-like structure ending with a short corolla tube positioned above a vestigial ovary with or without a vestigial stigma (Berti *et al*., 2005).

Each disk floret is has a five-lobed tubular corolla and an inferior ovary. The five anthers are fused forming an anther column inside the corolla tube. The filaments of the stamen are not fused and are attached to the base of the tubular corolla. The style, which ends in a bi-lobed stigma, is located inside the anther tube. Surrounding the base of the style is the nectary (Samanta *et al*., 2011).

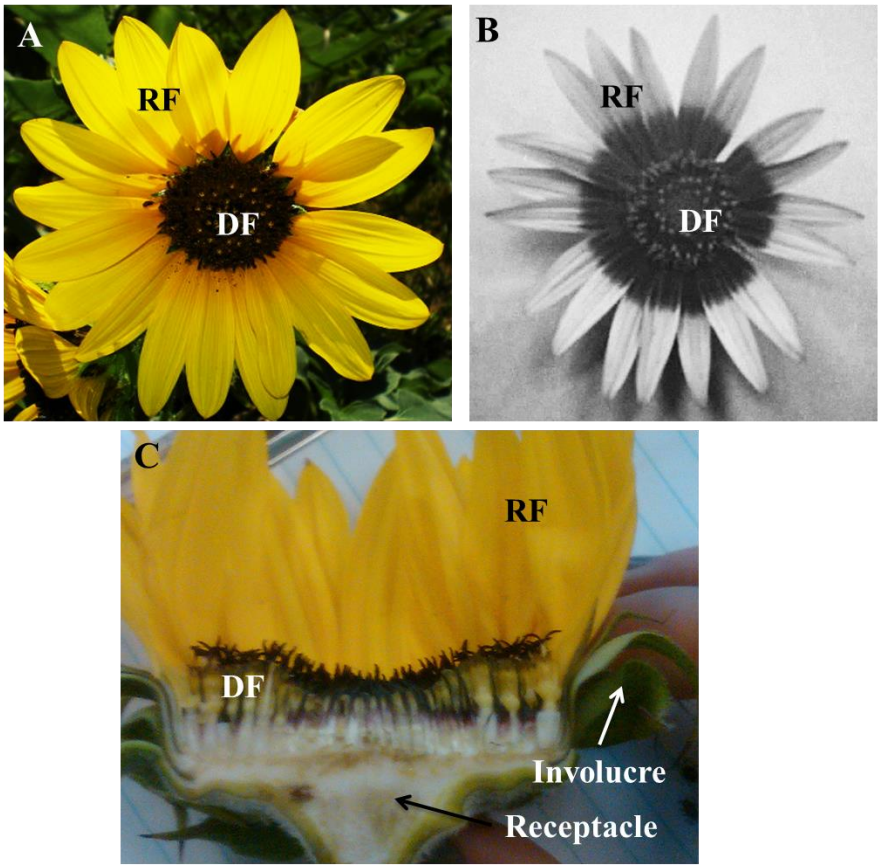

Fig. 1. Sunflower (*H. annuus*, *Asteraceae*) inflorescence in visible light and under UV radiation. A) Sunflower inflorescence photographed in visible light showing the yellow ray florets (RF) and brown disk florets (DF). B) Sunflower inflorescence photographed under UV showing the target pattern. C) Longitudinal section through the sunflower capitulum showing the disposition of ray (RF) and disk (DF) florets on the receptacle (from trachtenNS201, www.flickr.com/photos/53604369@N06/with/5039822010/).

Inflorescence shape, floral pigmentation and pollinator vision evolved as a biosemiotic relationship (Chittka and Menzel, 1992). Previous studies showed that honeybees recognize the target pattern, displayed by the radial symmetry of sunflower inflorescences, which is visible only in the UV spectrum (Chittka and Kevan, 2005, Briscoe and Chittka, 2001; Dyer, 1996) (Fig. 1B). Honeybees land on the distal ray florets, and the basal ray florets ‘guide’ them toward the disk florets in the center of

inflorescence to collect their rewards (McCrea and Levy, 1983, Horridge, 2000) and during this process they cross pollinate the disk florets. Honeybees are rewarded with offerings of nectar and pollen, which they consume as sources of protein, lipids, vitamins, minerals, carbohydrates, and water (Herbert and Shimanuki, 1978).

During the last decades, a wealth of information has been collected on how honeybees visualize and cognitively process color information (Chittka and Walker, 2006). Research has shown that honeybees visualize their environment mainly in the wavelengths of the UV spectrum (Briscoe and Chittka, 2001). Honeybees have three types of color receptors located in their compound eyes, each of which absorb different wavelengths of light, specifically blue, green and ultraviolet light (Chittka and Menzel, 1992). These three color receptors have maximum sensitivities near 340 nm (UV receptors), 440 nm (blue receptors), and 530 nm (green receptors) (Briscoe and Chittka, 2001). Honeybees use their green photoreceptors for detection of flower fields from a distance and the UV photoreceptors for distinguishing individual flowers up close (Guirfa and Lehrer, 2001).

The radial symmetry of the sunflower inflorescence and the UV-absorbing and reflecting floral pigments are important adaptations that contribute to the target pattern of the capitulum (Dyer 1996). There are three groups of floral pigments in the plant world, namely flavonoids, carotenoids, and betalains (Grotwold, 2006). Sunflower inflorescence pigments are flavonoids and carotenoids only (Schlangen *et al*., 2009).

Flavonoids are phenolic secondary metabolites produced via the phenylpropanoid pathway (Fig. 3). Through a series of complex reactions starting with phenylalanine,

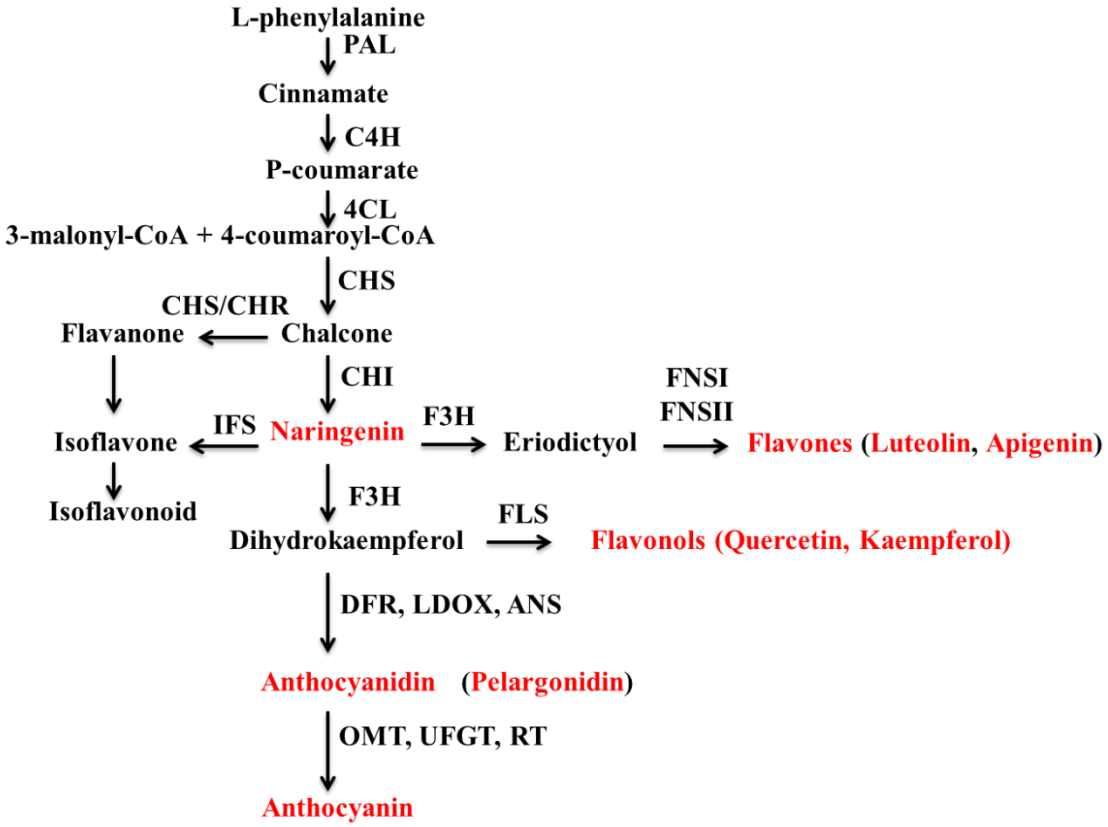

Fig. 3. Diagram of the phenylpropanoid biosynthesis pathway. Enzymes are as follows: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate; CHS, chalcone synthase; CHI, chalcone isomerase; CHR, chalcone reductase; F3H, flavonone 3-hydroxylase; FLS, flavonol synthase; FNSI /FNSII, flavone synthase; DFR, dihydroflavonol-4-reductase; LDOX, leucoanthocyanidin reductase; ANS, anthocyanidin synthase; OMT, O-methyltransferase; UFGT, UDP-flavonoid glucosyltransferase; RT, rhamnosyl transferase (Adapted from Winkel-Shirley, 2001). Compounds in red have previously been shown to contribute to the target pattern in plants (Harborne, 1994; Schlangen *et al.*, 2009**)**.

different types of flavonoid compounds are synthesized along the pathway. These secondary compounds are classified into six major chemical subgroups: chalcones, flavones, flavonols, flavandiols, anthocyanins and condensed tannins (or proanthocyanidins) (Winkel-Shirley, 2001).

Flavonoids and other polyphenols in *Asteraceae* species have been previously shown to create the contrasting light and dark areas of the target pattern seen in the sunflower inflorescences. More specifically, anthocyanins, flavones and flavonols are known to contribute to the target pattern of sunflowers (Harborne, 1994; Winkel-Shirley, 2001; Schlangen *et al.*, 2009).

In the ray florets, flavonols and flavones are the pigments contributing to the dark bases under UV (Harborne and Smith, 1978: McCrea and Levy, 1983). Flavones are one of the largest subgroups of flavonoids present in almost all vascular plant tissues ranging in color from pale yellow in the visible spectrum to blue in the UV spectrum.

Anthocyanidins, water-soluble pigments in plants, are the aglycones of modified anthocyanins (Stintzing and Carle, 2004). These compounds contribute to most of the blue, purple and red colors of fruits, flowers, and vegetables and are the major pigments responsible for the visible brown-burgundy color of the disk florets in addition to the UV absorption in the disk florets (McCrea and Levy, 1983; Samanta *et al*., 2011). Anthocyanins synthesized in the *H. annuus* achene hulls have been studied as a possible source of antioxidants other human consumer applications such as fabric dyes and food colorants (Vaccari *et al*., 1982; Mazza *et al*., 2004).

Carotenoids are isoprenoids and are essential components of photosynthetic organisms where they play an important role in the protection of chlorophyll against photoinhibition (Demmig-Adams and Adams, 1996; Hirschberg, 2001). In plants, carotenoids are synthesized in the plastids from pyruvate and glyceraldehyde-3-phosphate (Hirschberg, 2001). Carotenoids are classified as carotenes and xanthophylls

and are responsible for most of the visible yellow pigmentation of plants in the *Asteraceae* family (Valadon and Mummery, 1971).

Betalains are water-soluble nitrogen-containing conjugates of betalamic acid derived from the aminoacid tyrosine (Strack *et al*., 2002). Betalains are characteristic pigments of most families of the *Caryophyllales* and in some genera of *Basidomycetes.* They contribute to the attraction of pollinators and replace anthocyanins in the flowers of *Caryophyllales* (Strack *et al*., 2002). Anthocyanins and betalains appear to be mutually exclusive and have never been found together in the same plant (Stintzing and Carle, 2004).

Sunflower flavonoid biochemistry has been studied in leaves, seeds and honey for many years (Rieseberg *et al*., 1987; Weisz *et al*., 2009). Flavonoids previously identified in leaves, seeds and honey are: chalcone (flavonoid), pelargonidin (anthocyanidin), apigenin (anthocyanin), luteolin (flavones), kaempferol (flavonol), cyanidin (anthocyanidin) and malvidin (anthocyanidin) (Bohm and Stuessy, 2001; Vaccari *et al*., 1981). Specific *Helianthus* ray floret pigments that have been identified include quercetin and chalcone (Harborne, 1978; Schilling *et al.*, 1987). Previous studies of *H. annuus* established the presence of luteolin in foliage and pollen (Reiseberg *et al*., 1987; Yao *et al*., 2004), but thus far no published articles on the presence of luteolin in the floral tissues related to the target pattern recognized by bees could be found in the literature.

The goal of this study was to identify flavonoid pigments in the ray and disk florets which contribute to the formation of the target pattern of the inflorescence as part of the complex adaptations of the sunflower for cross-pollination by bees. The objectives

of this project were to characterize co-evolutionary mutualistic adaptations of sunflower and honeybees and to isolate and chemically characterize one flavonoid pigment in the ray or disk florets that contributes to the target pattern of the sunflower inflorescence.

CHAPTER II

MATERIALS AND METHODS

Plant Materials and Chemicals

Fresh sunflower (*Helianthus annuus, Asteraceae*) inflorescences were collected from spontaneous populations in the North Texas area. HPLC grade methanol, hydrochloric acid, chloroform, formic acid, acetone, glacial acetic acid, and acetonitrile were obtained from Fisher Scientific (Somerville, NJ). Flavonoid standards: apigenin, chalcone, kaempferol, luteolin, naringenin, pelargonidin chloride and quercetin, and glycerin for confocal microscopy were purchased from Sigma-Aldrich (St. Louis, MO).

Pigment Extraction

The extraction of flavonoid pigments was carried out following the method described by Lewis et al. (1998) with some modifications. Disk and ray florets from sunflower inflorescences were manually separated. The distal and basal areas of the ray florets were separated from each other based on the target pattern seen under UV radiation (Fig. 1B in Chapter I). The basal area of the ray florets of the sunflowers is dark under UV radiation and the distal area is light in color. Fresh weights (FW mg) were determined for the disk florets and the ray floret fractions, after which the plant material was submerged in liquid nitrogen, homogenized to a powder using a mortar and pestle and stored at -80°C.

Dry frozen floret powders were extracted at room temperature in 90% methanol (10 ml g^{-1} FW) for 72 h. At the end of the extraction time, samples were centrifuged at 1200 x g for 10 min. The supernatants were decanted and stored at -4°C. The pellets were re-extracted with 50% methanol (5 mL/g^{-1} FW) for 24 h. The 50% methanol extracts were centrifuged at 1200 x g for 10 min, and the supernatants from both 90% and 50% methanol extractions were combined. The floret extractions were treated with chloroform (1:1) to remove carotenoids and other low polarity molecules. The upper aqueous layer was decanted and stored at -4°C in amber glass vials for further use. A flow chart outlining the methodology for fractionation and identification of pigments is presented in Figure 1.

Chromatography

Solid Phase Extraction and Acid Hydrolysis

Solid Phase Extraction (SPE) and acid hydrolysis were carried out by employing a modified method of Lewis et al. (1998). Thermo Scientific HyperSep C18 solid phase extraction 15 mL columns (Fisher Scientific, Somerville, NJ) were activated with 5 mL methanol followed by 5 mL water. Each floral extract (4 mL) was loaded on the column and the SM fraction was obtained and discarded. Fractions F20 and F80 were eluted with 20% and 80% methanol, respectively.

For acid hydrolysis, 2 N HCl was added to the F80 fractions (1 mL HCL/9 mL F80 fraction), which were then placed in a 95°C water bath for 1 h. The SPE-hydrolyzed F80 fractions were dried under nitrogen gas and re-suspended in 100% methanol for further chromatography studies.

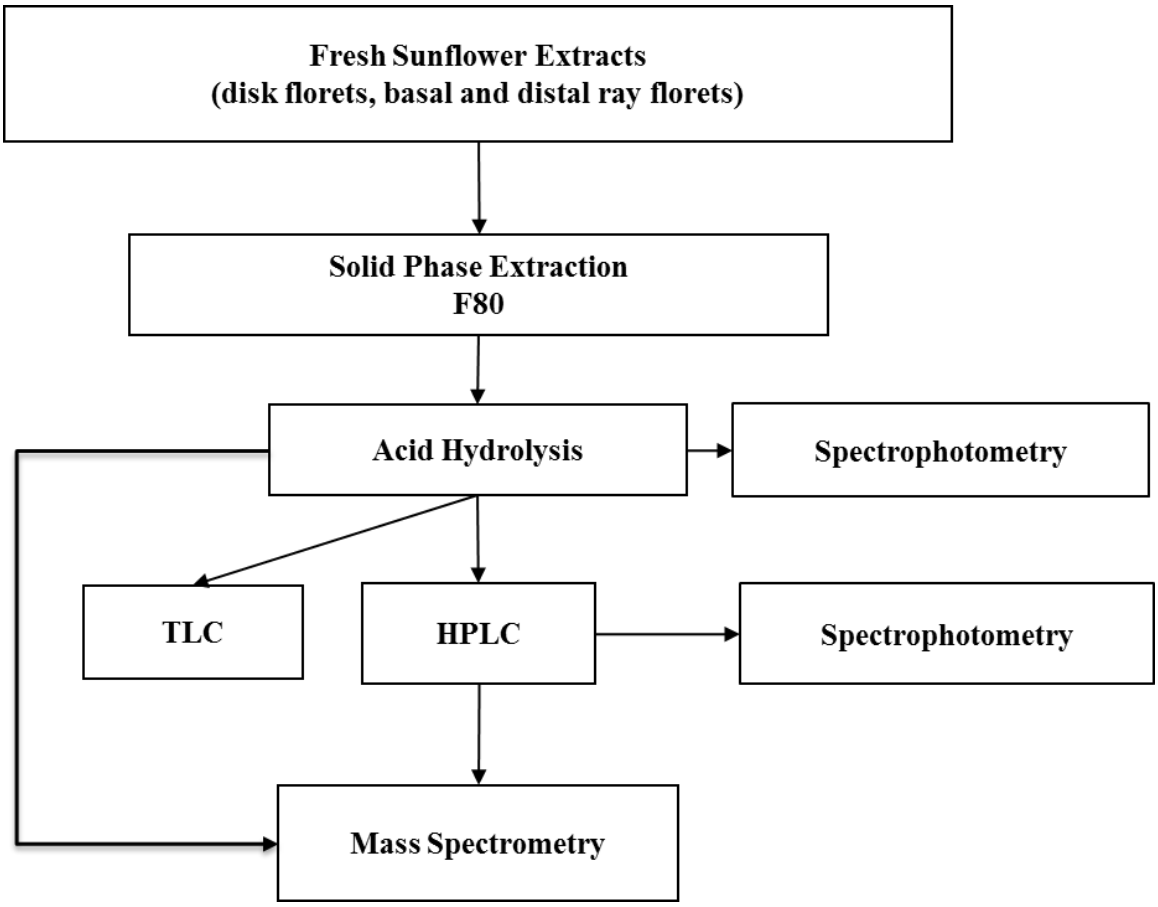

Fig. 1. Flow chart outlining the methodology for fractionation and identification of sunflower floral pigments.

Thin Layer Chromatography (TLC)

Whatman silica gel TLC plates (20 x 20 cm wide, 500 µm thick) with fluorescent indicator (Fisher Scientific, Somerville, NJ) were spotted with 10 µL (1mg/mL methanol) of the flavonoid standards apigenin, chalcone, kaempferol, luteolin, naringenin, pelargonidin, and quercetin, and 25 µL of the distal and basal ray floret SPE-hydrolyzed F80 fraction and disk floret SPE-hydrolyzed F80 fraction. The TLC plates were

developed with 9:2:1 chloroform:acetone:formic acid (Lewis *et al.,* 1998). When the solvent front reached 1 cm from the upper edge of the TLC plates, the plates were removed from the developing chamber, dried completely, and photographed on a UV-bed.

High Performance Liquid Chromatography (HPLC)

A combined and modified method by Jung *et al.* (2009) and Weisz *et al.* (2009) was used for HPLC analyses with a Gilson 322 Pump at room temperature. A Thermo Scientific Hypersil Gold aQ (5 µm 250 x 4.6mm) RP-C8 HPLC column (Fisher Scientific, Somerville, NJ) was used. Absorbance was measured with a Gilson 153 UV/VIS detector at 520nm and 254nm The mobile phase consisted of 2% acetic acid in ultrapure water (solvent A) and 0.5% acetic acid in ultrapure water and acetonitrile (50:50, solvent B). A linear gradient was used beginning with 75% A: 25% B to 0% A: 100% B over 60 min at a flow rate of 0.5 ml min^{-1}. Flavonoid standards (1 mg/mL) were dissolved in 100% methanol and used as controls. Fractions were collected every 10 minutes from the SPE-hydrolyzed F80 disk floret samples. Each 10 minute sample (a total of 6 fractions) was re-injected to visualize the peaks that were present in each of these fractions. The fractions that were collected between 50-60 minutes showed peaks consistent with the retention times of some flavonoid standards. These fractions were collected twelve times, pooled, concentrated under N_2 gas, and used for mass spectrometry studies. HPLC peaks were captured and analyzed using Gilson Inc. software Trilution LC version 2.1.

Mass Spectrometry

Mass spectrometry (MS) was carried out on an AB Sciex model API 3000 triple quadropole electrospray tandem mass spectrometer (Framingham, MA). Flavonoid standards, SPE-hydrolyzed F80 disk floret samples, and the HPLC 50-60 minute fractions were injected at a rate of 10 uL/min. Curtain gas interface and nebulizer gas were both set to 8, and the ion spray voltage was set to 4200 in positive mode $[M+H]^+$ and -4200 in negative mode $[M-H]^-$. The channel electron multiplier (CEM) was set to 2100 V for all samples. The entrance potential (EP) was set to 10/-10, while the focusing potential (FP) was set to 200/-200 and the declustering potential (DP) was set to 30/-30 for all samples for both positive/negative modes, respectively.

For electrospray ionization tandem mass spectrometric scans (ESI-MSMS), the collisionally active dissociation (CAD) gas was set to 4 for all samples. The ion gauge pressure for full mass spectrometry (MS) was 0.8 x $10e^5$ Torr, and for ESI-MSMS was 3.6 x $10e^5$ Torr. Negative and positive ion mass spectra of the standards, and the spectra of the SPE-hydrolyzed F80 disk floret samples and the 50-60 minute HPLC fractions of the SPE-hydrolyzed F80 disk floret samples were scanned in the 50-400 *m/z* range to obtain parent and daughter fragment ions. Sample specific conditions applied for mass spectrometry can be seen in Table 1.

Table 1. MS/MSMS conditions for the flavonoid standards, the SPE-hydrolyzed F80 disk floret samples and the 50-60 minute HPLC fractions. MW: molecular weight, MODE: ion mode; CE: collision energy; CXE: collision cell exit potential, and DF: deflector.

SAMPLE	MW	MODE	CE	CXP	DF
Quercetin MS	302	neg	NA	NA	200
Pelargonidin MS	306	pos	NA	NA	-400
Naringenin MS	272	neg	NA	NA	200
Luteolin MS	286	neg	NA	NA	200
Kaempferol MS	286	neg	NA	NA	200
Apigenin MS	270	neg	NA	NA	200
Disk Floret MS		neg	NA	NA	200
Disk Floret MS		pos	NA	NA	-400
Quercetin MSMS_301_CE30	302	neg	-30	-15	200
Pelargonidin MSMS_271_CE30	306	pos	30	15	-400
Pelargonidin MSMS_271_CE40	306	pos	40	15	-400
Naringenin MSMS_271_CE30	272	neg	-30	-15	200
Luteolin MSMS_285_CE40	286	neg	-40	-15	200
Kaempferol MSMS_285_CE30	286	neg	-30	-15	200
Apigenin MSMS_269_CE30	270	neg	-30	-15	200
Disk Floret HPLC fraction MSMS _285_CE30		neg	-30	-15	200
Disk Floret HPLC fraction MSMS_285_CE40		neg	-40	-15	200
Disk Floret HPLC fraction MSMS_305_CE15		neg	-15	-15	200
Disk Floret HPLC fraction MSMS_306_CE10		pos	10	15	-400

The flavonoid standards were prepared as 0.5mg/500 µL solutions of water:methanol (50:50). For each standard, 1 µL of the above solution was combined with 10 µL acetic acid, diluted with 495 µL water and 450 µL methanol. Samples of the SPE-hydrolyzed F80 disk floret extract and the HPLC 50-60 minute fractions were also prepared for MS/ESI-MSMS as mentioned above. Each standard, the SPE-hydrolyzed F80 disk floret samples, and the HPLC 50-60 minute fractions were scanned in MS mode to visualize parent ion mass-to-charge ratios. ESI-MSMS mode also was employed with these same samples to produce the daughter fragments. Validation of fragment mass-to-

charge ratio was corroborated using the phytochemical spectral database ReSpect for Phytochemicals (Sawada et al., 2012).

Spectrophotometry

Spectrophotometry of flavonoid standards in methanol, SPE-hydrolyzed F80 disk floret samples in methanol, and HPLC 50-60 minute fractions in methanol were performed using a Shimadzu UVmin-1240 UV-Vis Spectrophotometer as shown in Figure 1. A blank of 100% methanol was used. Samples were scanned between 200nm to 700nm.

Microscopy

Ray and disk florets were dissected and sectioned for visualizing with the following microscopes: Hitachi TM-1000 SEM, Nikon A1 Confocal System (CLSM), and Olympus BH-2 LM equipped with a Nikon DXM200 camera.

Whole and dissected florets were mounted on aluminum stubs (Ted Pella, Inc., Redding, CA) for observations with the TM-1000 SEM. Same types of specimens and cross sections of florets were mounted in glycerin on glass slides for CLSM. Samples were viewed under the following wavelengths: DAPI, 400 nm; FITC, 588 nm; TexasRed, 561 nm; and Cy5, 626 nm. Cross sections of ray florets were mounted in water on glass slides for light microscopy (LM). Whole ray florets were visualized with an Olympus JM stereoscope (SM) as well.

Honeybees were dissected after being kept in a -20°C freezer for 10 min. Heads and legs were mounted on carbon stubs (Ted Pella, Inc., Redding, CA) and were viewed with the TM-1000 SEM.

Photography

Photographs of the sunflower inflorescences were taken using a Nikon D50 SLR utilizing the Nikkor 50-220 mm lens and an 18-55 mm lens on a Targus TG-t60p tripod. Ultraviolet photographs were taken with a B+W UV-pass filter (model #430) in a reflector box equipped with twelve FEIT electric BPESL/13watt/120vac mercury vapor black lights.

CHAPTER III

A MICROSCOPIC REVIEW OF THE SUNFLOWER AND HONEYBEE MUTUALISTIC RELATIONSHIP

A manuscript for submission to

International Journal of AgriScience

ABSTRACT

The mutualistic relationships between plants and pollinators have been extensively studied. However, few studies on the sunflower-honeybee relationship are present in the scientific literature. The aim of this study was to present a complete description of the co-evolutionary adaptations of the wild sunflower (*Helianthus annuus*, *Asteraceae*) and honeybee (*Apis mellifera*, Hymenoptera: Apidae) reflective of their mutualism. A model for cross-pollination of sunflower florets by honeybees is presented. The floral pigments and the arrangement of florets in the sunflower inflorescence contribute to the formation of a target pattern, recognizable by bees in the UV spectrum. Conical epidermal cells on the abaxial epidermis of the sunflower ray florets reflect UV radiation, thus forming a landing site for bees, which are then guided to the disk florets in center of the inflorescence by the UV-absorbing basal area of the ray florets. The concentric pattern of disk floret maturation and offering of nectar and pollen rewards are key adaptation for cross-pollination. Nectar and pollen are offered in a timely manner that facilitates deposition of pollen on receptive stigmas of disk florets. Honeybees move through rows

of pollen-receptive stigmas of pistillate florets, thus depositing the pollen acquired during previous visits to other inflorescences, before reaching the pollen and nectar of the staminate florets towards the center of the inflorescence. This review based on a comprehensive literature research and own microscopic investigations presents a complete description of the morpho-anatomical adaptations of the sunflower-honeybee mutualistic relationship with possible practical applications in pollination biology and crop production.

Keywords: *Apis mellifera,* biosemiotic relationship, co-evolution, disk florets, *Helianthus annuus,* microscopy, mutualism, sunflower capitulum, ray florets

INTRODUCTION

The common sunflower (*Helianthus annuus* L., *Asteraceae*) is a native plant species of North America, southern Canada and Mexico (USDA Plants Database). Originally domesticated by the Native Americans 3,000 years ago, the sunflower is an important crop today. It was selected for increased seed size as a source for cooking oil (Yarnell 1978). Sunflower seeds, leaves, roots and flowers have been used as a source of medicine by Native Americans (Moerman 1986). Pigments extracted from the sunflowers were used as dyes for clothing and other household items (Heiser 1976). Parts of the sunflower plants are used as a food source for humans today (seeds for oil and confection markets). Wildlife, such as game birds, songbirds, chipmunks, mice, gophers and squirrels eat the sunflowers seeds. Antelope, deer and moose also graze on the plants. The sunflower stalks are used for food and fodder for livestock and poultry (Heiser 1976).

During its evolution, *H. annuus* have developed morpho-anatomical and biochemical adaptations to maximize pollination success and seed set, mainly through cross pollination by insects, especially bees. Wild populations of common sunflower are self-incompatible, thus enforcing outcrossing, whereas modern cultivars, inbreds, and hybrids are self-compatible and partially-to-strongly self-pollinated, although benefit from outcrossing as well (Heiser et al. 1969; Fick 1978). Cross pollination increases the seed set in wild and cultivated sunflower inflorescences (Free 1964) and ensures genetic variability, and therefore the hybrid vigor (heterosis) with important consequences for agricultural yields (Kaya 2005).

The honeybee, *Apis mellifera* L. (Hymenoptera: Apidae), is managed mostly for honey production and less for pollination services (Morse and Calderone 2000; Aizen and Harder 2009) although it is the single most important crop pollinator (McGregor 1976). Non-*Apis* bees, also known as wild bees, are also valuable for cross pollination of many wild as well as crop species (Losey and Vaughan 2006). Honeybees, the major pollinators of sunflowers, are rewarded with offerings of nectar and pollen, which they utilize as sources of protein, lipids, vitamins, minerals, carbohydrates, and water (Herbert and Shimanuki 1978). Specialized pollinators, such as honeybees and some other insects, are particularly beneficial to their partner plant species, as they are more likely to effectively transfer pollen to conspecific plants than generalist pollinators. This process called flower constancy prevents the loss of pollen during intraspecific flights and prevents other pollinators from clogging stigmas with pollen of other flower species (Chittka et al. 1999).

Inflorescence shape, floral pigmentation and pollinator vision evolved as a biosemiotic relationship (Chittka and Menzel 1992). The shape and floral pigments are meaningful signals for insect pollinators. It has been shown that honeybees recognize the target pattern displayed by sunflower inflorescences (Figure1 A and B; Chittka and Kevan 2005), since they visualize their environment in the wavelengths of the UV spectrum (Briscoe and Chittka 2001). The radial symmetry of the inflorescence and the UV-absorbing and reflecting floral pigments are important adaptations that contribute to the target pattern of the capitulum (Dyer 1996). The specific localization of different classes of carotenoids and flavonoids in inflorescence tissues contributes to the target pattern of the *Asteraceae*. Flavonols and flavones are believed to be the pigments responsible for UV absorption in the dark basal parts of the sunflower ray florets (Harborne and Smith 1978, McCrea and Levy 1983) and carotenoids play a role in creating the light portion of the distal ray floret. Anthocyanins, a class of flavonoids which are 3-glucosides of anthocyanidins, are the main pigments responsible for UV absorption in the dark disk florets (McCrea and Levy 1983, Samanta et al. 2011). Honeybees land on the ray florets, which 'guide' them toward the disk florets in the center of inflorescence to collect their rewards (McCrea and Levy 1983, Horridge 2000) and during this process they cross pollinate the disk florets. In North America and Europe, supplies of domestic honeybees have declined, in part because of problems caused by parasitic mites and pesticide misuse (Allen-Wardell *et al*. 1998, Holden 2006). Several documented examples show that reductions in bee abundance can cause reduced crop yields (Wilcock and Neiland 2002).

The goal of this microscopic review was to illustrate the co-evolutionary adaptations of sunflower and honeybee as a result of their mutualistic relationship, especially since no complete descriptions of these adaptations are available in the scientific literature. This microscopic study focuses mainly on the reproductive developmental stages of the sunflower florets leading to the offering of pollen and nectar rewards for honeybee pollinators and the honeybee adaptations for interpreting the *H. annuus* signals, collecting, and transporting rewards, thus contributing to cross pollination. The authors hope that the results of this study enhance our knowledge of the mutualistic relationship between sunflowers and honeybees with possible practical applications in pollination biology.

MATERIALS AND METHODS

Sunflower inflorescences and honeybees were collected from spontaneous populations in North Texas area. Glycerin for confocal microscopy was purchased from Sigma-Aldrich (St. Louis, MO) and aluminum and carbon stubs for SEM were from Ted Pella, Inc. (Redding, CA).

Ray and disk florets were dissected and sectioned for visualizing with the following microscopes: Hitachi TM-1000 SEM, Nikon A1 Confocal System (CLSM), and Light Microscope Olympus BH-2 LM equipped with a Nikon DXM200 camera. Whole and dissected florets were mounted on aluminum stubs for observations with the TM-1000 SEM. Same types of specimens and cross sections of florets were mounted in glycerin on glass slides for CLSM. Samples were viewed under the following wavelengths: DAPI, 400 nm; FITC, 588 nm; TexasRed, 561 nm; and Cy5, 626 nm. Cross sections of ray

florets were mounted in water on glass slides for light (LM). Whole ray florets were visualized with an Olympus JM stereoscope (SM) as well.

Honeybees were dissected after being kept in a -20°C freezer for 10 min. Heads and legs were mounted on carbon stubs and placed in the SEM for microscopic study.

Photographs of the sunflower inflorescences were taken using a Nikon D50 SLR utilizing the Nikkor 50-220 mm lens and an 18-55 mm lens on a Targus TG-t60p tripod. Ultraviolet photographs were taken with a B+W UV-pass filter (model #430) in a reflector box equipped with twelve FEIT electric BPESL/13watt/120vac mercury vapor black lights (Figure 1 A and B).

RESULTS AND DISCUSSION

Floral Biology – Adaptations for Cross Pollination

A sunflower plant has a specialized heterogamous inflorescence called capitulum or head. The outer whorl of the capitulum is made of zygomorphic yellow ray florets, which are sterile. The center of the capitulum is occupied by actinomorphic tubulate or disk florets, which are fertile, typically burgundy in color, and arranged in arcs radiating from the center of the inflorescence into distinct left and right turning spiral rows (Fambrini et al. 2003).

The ray ligulate florets are non-reproductive and make up the yellow circle at the edge of the head. Each zygomorphic ray floret is composed of three to five elongated petals which are fused to form a ribbon-like structure ending with a short corolla tube positioned above a vestigial ovary with or without a vestigial stigma (Figure 2 A and C; Berti et al. 2005). The adaxial epidermis of the ray florets contains all conical cells

(Figure 2 B, D, and E), and the abaxial epidermis is made up of flat cells. The reflective cones with typical patterns of striations of the adaxial epidermis cells contribute to the reflectance of light in all directions. Figures 1 G and H illustrate the fluorescing conical epidermal cells in the light portion of the target pattern of the sunflower inflorescence seen by honeybees. The abaxial epidermal cells are not conical in shape, and appear to have no fluorescent properties (Figure 1 G).

While ray florets are homogeneously yellow in visible light, each floret corolla has a UV-reflecting distal tip and a UV-absorbing base (Schlangen et al. 2009) due to different types of carotenoids and flavonoid pigments localized in the cytoplasm and vacuoles of epidermal cells (Figure 1 H; Samanta et al. 2011). As viewed by bees, the distal parts of the ray florets make up the light portion of the target pattern, and the basal parts of the ray florets contribute to the dark portion of target pattern of the sunflower inflorescence (Figure 1 B). The UV-reflecting distal parts of the ray floret provide honeybees with cues for long-distance recognition of the inflorescence. In close proximity, they act as landing sites. Once the bee lands on the ray floret, the UV-absorbing basal parts act as a 'honey-guide' toward the nectar and pollen within the disk florets (Schlangen et al. 2009). Also, the conical shape of the adaxial epidermal cells allows bees a better grip of the corolla surfaces aiding in efficient foraging and transfer of pollen (Whitney et al. 2009).

The disk florets are located in the center of the sunflower capitulum. Each disk floret is made up of a five-lobed tubular corolla and an inferior ovary. The five anthers are fused forming an anther column inside the corolla tube. The filaments of the stamen are not fused and are attached to the base of the tubular corolla. The style, which ends in a bi-

lobed stigma, is located inside the anther tube (Figure 3 A; Samanta et al. 2011). Surrounding the base of the style is the nectary (Figure 3 G and H). Fertile disk florets develop in nine different stages according to Schneiter and Miller (1981), but the present study focuses on the three reproductive developmental stages: a) immature, b) staminate, and c) pistillate stages (Figure 1 C; Sammataro et al. 1985).

The immature developmental stage is characterized by an unopened corolla. The abaxial layer of immature corolla is populated with dense non-glandular and glandular trichomes (Figure 1 F). The glandular trichomes are short, globular, multicellular structures that secrete sequiterpene lactones, which protect the florets against pest attacks. These trichomes may also secrete aromatics, which attract pollinators (Gopfert et al. 2005). The non-glandular trichomes are long, multicellular structures with pointed tips, also contributing to herbivore deterrence (Gershenzon and Mabry 1984).

In the staminate stage, the fused anther column extends through the apex of the open corolla. At this developmental stage, the crown of the corolla tube shows five tips. The abaxial side of the corolla tips is yellow, but as the corolla fully matures, the tips reflex exposing the burgundy adaxial epidermis with conical cells. The upper anther tube edge above the corolla tube also has five tips. The abaxial side of an anther concave tip has mostly glandular trichomes and few non-glandular trichomes (Figure 1 D and Figure 3 D, E and F), which secrete attractants for pollinators, as well as deterrents for herbivores (Gershenzon and Mabry 1984, Gopfert et al. 2005). The pollen grains are released from the fused anthers, accumulate inside the anther column, and are visible as stars between the five anther tips of the disk florets (Figure 5), providing visible cues as semiotic

signals for pollinators. Honeybees interpret these signals that nectar and pollen are available for harvest (Wojtaszek et al. 2008).

Pollen and nectar are produced only during the staminate stage of disk floret development. The sunflower nectary is located at the base of the corolla tube on top of the ovary (Figure 3 G, H, and I). Sunflower nectary size and shape varies among the wild and cultivated varieties of *H. annuus* (Neff and Simpson 1990). The nectaries studied here were ring-shaped organs, triangular in cross section, surrounding the base of the style. Prominent stomata were randomly spaced among the epidermal cells of the walls and especially on the ridge of the nectary (Figure 3 H and I). Nectary wall height varies from 200-360 µm, the internal diameter from 470-800 µm, and the width at the base between 70-100 µm. Nectar production is constant during the staminate phase (Neff and Simpson 1990) and is presumably secreted mainly from the specialized stomata on the ridge of the nectary, but this process has not been thoroughly investigated (Sammataro et al. 1985). Mean nectar volumes in the sunflower staminate disk florets range from 0.02-0.32 µl with a nectar solute concentration of 26-70% with up to 569 µg nectar-sugar (Wist and Davis 2006).

In the pistillate stage of disk floret development, the anther column retracts inside the corolla tube, the style and stigma elongate upward emerging from inside the anther column, and stigma opens its two lobes (Figure 1 C and E and Figure 3 A and B). Initially, the stigmatic lobes extend upwards tightly pressed together and upon maturation, each lobe curls downward into a coiled conformation. The adaxial side of the mature stigma is covered with short conical papillae of similar height, which form the

receptive stigmal area. The abaxial stigmal side is layered with elongated non-receptive papillae of varied length, some with pointed tips and others with rounded tips (Figure 1 E). One to three files of obvious 'transitional papillae' are found between the short papillae area and the elongated papillae area of the stigma (Figure 3 C). A review of the literature does not reveal information on this transitional layer of stigma papillae in sunflowers or other *Asteraceae* species. The transitional papillae along with the elongated papillae most likely function in protecting the receptive stigmatic areas during emergence from the anther column. The elongated papillae on the abaxial side of the stigma lobes function to brush own pollen and break the star-shape compaction of pollen as the style-stigma formation extends through the anther column (Figure 3 B). It seems that the short papillae are receptive to pollen during the staminate floral stage while inside the anther column full of pollen (Hiscock et al. 2002). Fully extended at maturity, the stigmal lobes reflex and expose the receptive papillae for cross pollination, thus reducing the possibility of clogging the stigma with own pollen (Neff and Simpson 1990, Hiscock et al. 2002).

Bee Adaptations for Collecting and Transporting Nectar and Pollen

Worker honeybees are adapted for collecting pollen and nectar for nutritional and building activities in the hive. This microscopic study of the honeybee illustrates the morphological structures involved in locating, harvesting, and transporting pollen and nectar. Bees locate flowers with their compound eyes. The bee compound eye (Figure 4 D) is a collection of hexagonal shaped simple eyes with each hexagonal omatidium being able to process visual information independently of each other. Each omatidium receives and processes either UV, blue, or green light waves. The balance of different color

receptors is believed to be an evolutionary adaptation with which the compound eye achieves high visual performance (Menzel and Blakers 1976). Honeybee compound eyes visualize the sunflower target pattern and identify it as a source of pollen and nectar. Honeybees also have three simple eyes called ocelli located on top of the head in a triangular pattern with one ocellus centered with the middle point between the antennae (Figure 4 G). The function of the ocelli is not well understood, but it is believed that they help honeybees orient themselves within their environment using the location of the sun in the sky at any point during the day (Michael and Chittka 2013).

On the flower, ready to harvest, honeybees accurately ascertain the quality and quantity of available nectar by means of thousands of sensory cells on their antennae and mouthpieces (Figure 4 A; Rogers and Vallortigara 2008). The bee mouth adapted mainly for sucking nectar. It is structurally complex, made up of two mandibles and a proboscis. The worker bee mandibles are very small compared to those of other insects and are used for chewing and molding wax, chewing wood and other objects, clean other bees, and bite intruders in the hive. The proboscis is a multi-component structure, which functions in nectar sucking, food exchange with other bees (trophallaxis) and water extraction from nectar (Figure 4 B and C; Winston 1991). The proboscis consists of two maxillae and two labial palps surrounding a protrusible tongue or glossa that ends with labellum (de Brito Sanchez 2011). When the proboscis is not being used, it is folded into a space beneath the head (Winston 1991).

Bee bodies are covered with simple and branched hairs (Figure 4 E). The hairs found on the compound eye (Figure 4 D) perceive airflow and help the honeybee orient

themselves in windy conditions (Thorp 1979). The occurrence of densely packed long branching hairs is specific to bees (Figure 4 J, K, and L; Thorp 1979) and enhances pollen acquisition by providing a large surface area for pollen attachment and transport between flowers and to the hives. The forelegs and feet have specialized rakes, combs and brushes for collecting the pollen from the body hairs and packaging it for transport (Figure 4 F and I). The brushed pollen is mixed with nectar to facilitate compaction. With a series of scraping and rubbing movements in combination with push and pull strokes of the rakes and combs, the pollen is moved into the baskets located on the hind legs. The pollen basket or corbicula is a concavity on the outer upper femur of each hind leg in which a single pin hair in the middle and a series of long hairs on the edge of the basket hold the packed pollen in place for transport (Figure 4 H; Thorp 1979).

Cross-pollination of Sunflower by Honeybees

Honeybees are the main pollinators of sunflowers (Free 1964). Their mutualistic relationship consists of sunflower cross pollination by bees and floret rewards of nectar and pollen for bees. The co-evolution of honeybee pollinators and flowering plants is believed to have begun in the Cretaceous period. Based on the bee-amber fossil samples, it is speculated that bees could have arisen during the middle Cretaceous period when flowering plants were widespread or earlier than flowering plants and consumed gymnosperm pollen, or at the same time, or very soon after the rise of flowering plants (Baker and Chmielewski 2003).

Bees select flowers for pollen and nectar harvest based on many criteria. Most important of this criteria are the floral visual signals interpreted by bees. The common

sunflower synthesizes flavonoids and carotenoids, which are deposited in a manner that creates the target pattern that is recognizable and preferred by bees. The bee interprets this signal that the flowers have nectar and pollen readily available for harvest. The contrasting feature between light and dark areas on the sunflower inflorescence is what draws bees in for closer investigation.

The sunflower head is comprised of 50-400 disk florets which mature in a concentric manner beginning with the floret rows at the outer edge of the capitulum and moving inward where each floret row matures within 1-2 days of each other (Minckley et al. 1994). Usually, the honeybee lands on the distal portion of the ray floret, which is viewed as the white rings of the target pattern of the sunflower inflorescence. The dark basal part of the ray floret guides the bee towards the disk florets. As the honeybee passes through the outer disk floret rows on their way to collect rewards from the staminate florets, foreign pollen on the bee bodies is deposited on the receptive stigmas of the pistillate disk florets assuring cross pollination (Figure 5; Knox et al. 1976). Honeybees are attracted to the staminate florets by the display of the pollen star which signals production of nectar in these florets (Figure 5). Thus, the specifically timed development of reproductive disk florets increases the likelihood of cross pollination and is a key component of the co-evolutionary nature of the relationship between sunflower and honeybee.

Honeybees are more likely to harvest from the sunflowers when there are larger number of disk florets actively producing pollen and nectar at the same time. With multiple nectar and pollen producing disk florets at one time and for an extended period, sunflowers are an excellent source of nutrition for the honeybee pollinator. Nectar

production is constant during the staminate phase, and honeybee visits and the time spent on foraging increase as nectar accumulates in the disk floret corolla (Neff and Simpson 1990). Thus the possibility of cross pollination and fertilization opportunities increases as honeybee visits and as time spent foraging increase.

The length of the disk floret corolla can vary among varieties of sunflowers, but the relatively short length of the corolla corresponds to the short length of the honeybee proboscis (Herrera 1989). Honeybees have an average proboscis length of 5.1 mm and the average corolla length of wild sunflowers ranges from 4.0-6.5 mm (Heiser 1947). There is evidence to suggest that pollinators with short proboscis lengths prefer flowers with short corolla tubes (Inouye 1980). The correlation between corolla tube length in staminate disk florets, where nectar is produced, and the short length of the honeybee proboscis, is an important characteristic of the co-evolution of these two species.

CONCLUSIONS AND REMARKS

This microscopic study of the common sunflower and the honeybee illustrates the co-evolution of these two organisms in establishing a successful mutualistic relationship that serve both plant and insect, as well as the human society. This review enhances our understanding of the importance of flower-pollinator mutualism for crop production, especially of the sunflower cross pollination process by honeybees. Pollination is essential for the production of many crops, and therefore, pollinator management and use should become a key component of methodologies for enhancing crop production, especially since some areas in the world are suffering a pollination crisis (Garibaldi et al. 2011). Enhancing honeybee pollination efficiency is economically important for hybrid

sunflower seed production since this crop plant is frequently under pollinated (Greenleaf and Kremen 2006). An increase in the per-visit pollination efficiency of honeybee individuals may be immensely valuable for global food production in the future.

Although this study focused on the Apis species, it is known that the presence of wild bees enhances the pollination efficiency of honeybees in sunflower (Greenleaf and Kremen 2006). Sunflowers that are visited by bees native to the area in which they grow are more likely to have increased seed set than sunflowers visited by non-native bees (Parker 1981). When domestic honeybees pollinate in conjunction with wild bees, pollination efficiency can increase 5-fold. The abundance and diversity of wild bee communities increase crop pollination and therefore the crop yield (Greenleaf and Kremen 2006). Given the current issue of colony collapse disorder (CCD) in the domestic bee-keeping industry, it seems imperative to support and conserve wild bee populations surrounding commercial crop fields. Current research indicates that not only commercial bees are impacted by the use of pesticides, currently believed to be one of the main contributors to CCD, but that the widespread use of these chemicals and habitat fragmentation by the agricultural practices are also harming the wild bee population (Krupke et al. 2012). Applications of the knowledge gained from this study could be important for pollination biology and crop improvement, as well as for conservation programs aimed at species recovery and maintenance of ecological services, such as pollination.

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FIGURE LEGENDS

Fig1. Sunflower (*H. annuus, Asteraceae*) inflorescence and floret morpho-anatomy. A) Sunflower inflorescence photographed in visible light showing the yellow ray florets and brown disk florets, some of which exhibit pollen and pollinated stigmas. B) Sunflower inflorescence photographed under UV showing the target pattern, with UV reflectance restricted to the distal portion of the ray florets and pollen; dr, distal ray florets; br, basal ray florets; d, disk florets. C) The three reproductive stages of disk floret development under study: a) immature, b) staminate, and c) pistillate; ac, anther column; c, corolla; ov, ovary; pp, pappus; st, stigma. D) CLSM with DAPI laser (blue, 402 nm) of an anther tip with glandular trichomes (gt), and non-glandular trichomes (t). E) CLSM with DAPI (blue, 400nm)/FITC (green, 588nm)/TexasRed (red, 561nm)/Cy5 (pink, 626nm) lasers of a disk floret stigma lobe with pollen (p), and short (sp) and elongated papillae (ep). F) CLSM with DAPI (blue, 400nm)/FITC (green, 588nm)/TexasRed (red, 561nm)/Cy5 (pink, 626nm) lasers of the disk corolla outer layer with pollen (p), glandular trichomes (gt), non-glandular trichomes (t), and adaxial epidermis with conical epidermal cells (ce). G) CLSM with DAPI (blue, 400nm)/FITC (green, 588nm)/TexasRed (red, 561nm)/Cy5 (pink, 626nm) of the ray floret cross section showing conical epidermal cells reflective cone (ce), less fluorescent abaxial non-conical epidermal cells (ae) and vascular bundles (vb). H) CLSM with DAPI (blue, 400nm)/FITC (green, 588nm)/TexasRed (red, 561nm)/Cy5 (pink, 626nm) lasers of the ray floret abaxial epidermal cells with flavonoids in the cytoplasm (cy, green) and vacuole (v, blue). Scale bars for confocal images 1 D and F-H are 100 µm. Scale bar for confocal image 1 E is 50 µm.

Fig2. Sunflower (*H. annuus, Asteraceae*) ray floret morpho-anatomy. A) Photograph of the distal portion of the ray floret corolla (c). B) LM of the ray floret cross section showing the adaxial conical epidermal cells (ce), the abaxial flat epidermal cells (ae), and a vascular bundle (vb). C) Photograph of the basal portion of the ray floret corolla (c) and the vestigial ovary (ov). D) SEM image of the adaxial epidermal layer showing conical epidermal cells. E) Detail of the conical epidermal cells in D showing the reflective cones.

Fig3. Sunflower (*H. annuus, Asteraceae*) disk floret morpho-anatomy visualized with SEM. A) Pistillate stage disk floret with corolla (tc), anther tips (a), and stigma (s). B) Late pistillate stage bi-lobed stigma with elongated papillae (ep) on the abaxial side, and short papillae (sp) on the adaxial side. Notice the own pollen attached to the elongated papillae. C) Details of a stigma lobe in B showing three different types of papillae: elongated (ep), transitional (tp), and short (sp). Only the short papillae are receptive. D) Tips of the fused anther tube in the staminate stage. The abaxial side of anthers shows clefts populated with glandular (gt) and non-glandular (t) trichomes. E) Detail of anther tip cleft showing glandular (gt) and non-glandular (t) trichomes. F) Detail of a glandular trichome (gt) on the abaxial side of the anther cleft. G) Disk floret nectary (n) surrounding the style (st); c, corolla. H) Disk floret nectary in the shape of a ring after the style removal. I) Nectary rim with specialized stomates (representative stoma is circled).

Fig4. Honeybee (*A. mellifera,* Hymenoptera: Apidae*)* morpho-anatomical adaptations for pollen and nectar harvesting, visualized with SEM. A) Photograph of a representative honeybee. Notice the hairs on the body and the pollen basket with pollen on the outer femur of the hind leg. B) Honeybee proboscis with maxillae (m), two labial palp (lp), glossa (g), and labellum (lb). C) Detail of glossa (g) and labellum (lb). D) Head with compound eyes (c), antennae (a), and one of three simple eyes (s). E) Honeybee compound eye showing the individual hexagonally shaped ommatidia. F) Simple eye or ocellus (o) surrounded by many branched hairs. G) Antenna comb (c) located on each of the honeybee's front forelegs. H) Pollen basket (pb) on the outer upper femur of each hind leg. Pollen grains mixed with nectar are pinned into place with a specialized pinning hair (not seen) and kept in place with a row of curved hairs (h). I) Representative honeybee foot showing the empodia (em), the rake (r) utilized for cleaning pollen and debris from the body, and a claw (cl). J) Representative bee leg covered with hairs. K) Branched hairs located at the joint between the coxa and the leg. L) Details of hairs on legs.

Fig5. Bee cross pollinating pathway on a wild *H. annuus (Asteraceae)* inflorescence. The disk florets flower in concentric circles. The three stages of disk floret reproductive development are shown between the white lines. From the outer ray floret ring towards the center of capitulum: (p) late pistillate and early pistillate disk florets, s) staminate disk florets with pollen star formations, and i) immature buds of disk florets. The arrow indicates a bee pathway from the landing site on the ray floret to the staminate disk floret to harvest pollen and nectar rewards. In passing though the pistillate disk floret circle, the bee cross pollinate these flowers by wiping the pollen collected from a previously visited plant against the receptive stigma lobes. Once in the staminate circle, the bee body hairs collect new pollen grains, which are transported to a new plant.

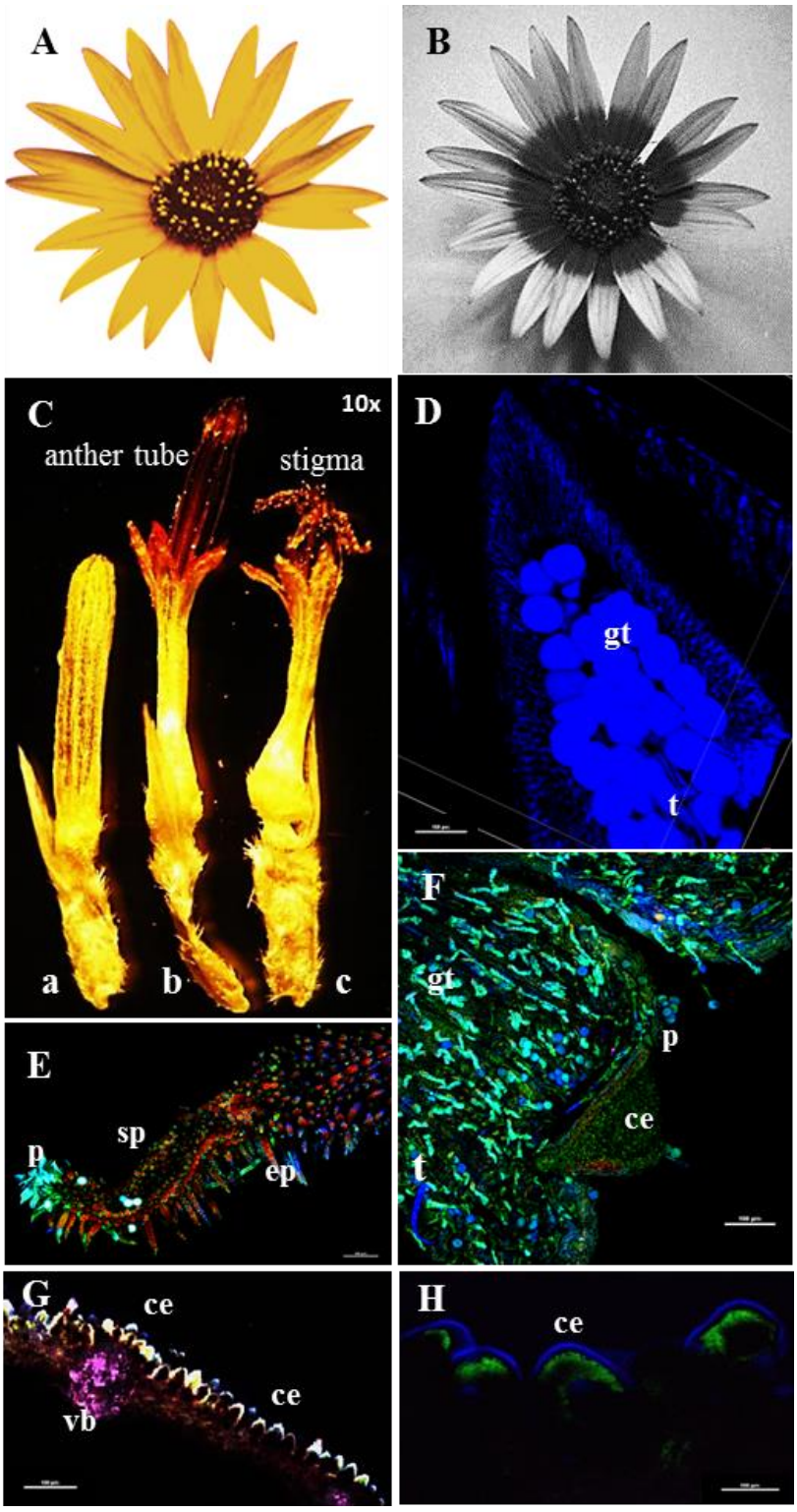

Fig1. Sunflower (*H. annuus, Asteraceae*) inflorescence and floret morpho-anatomy.

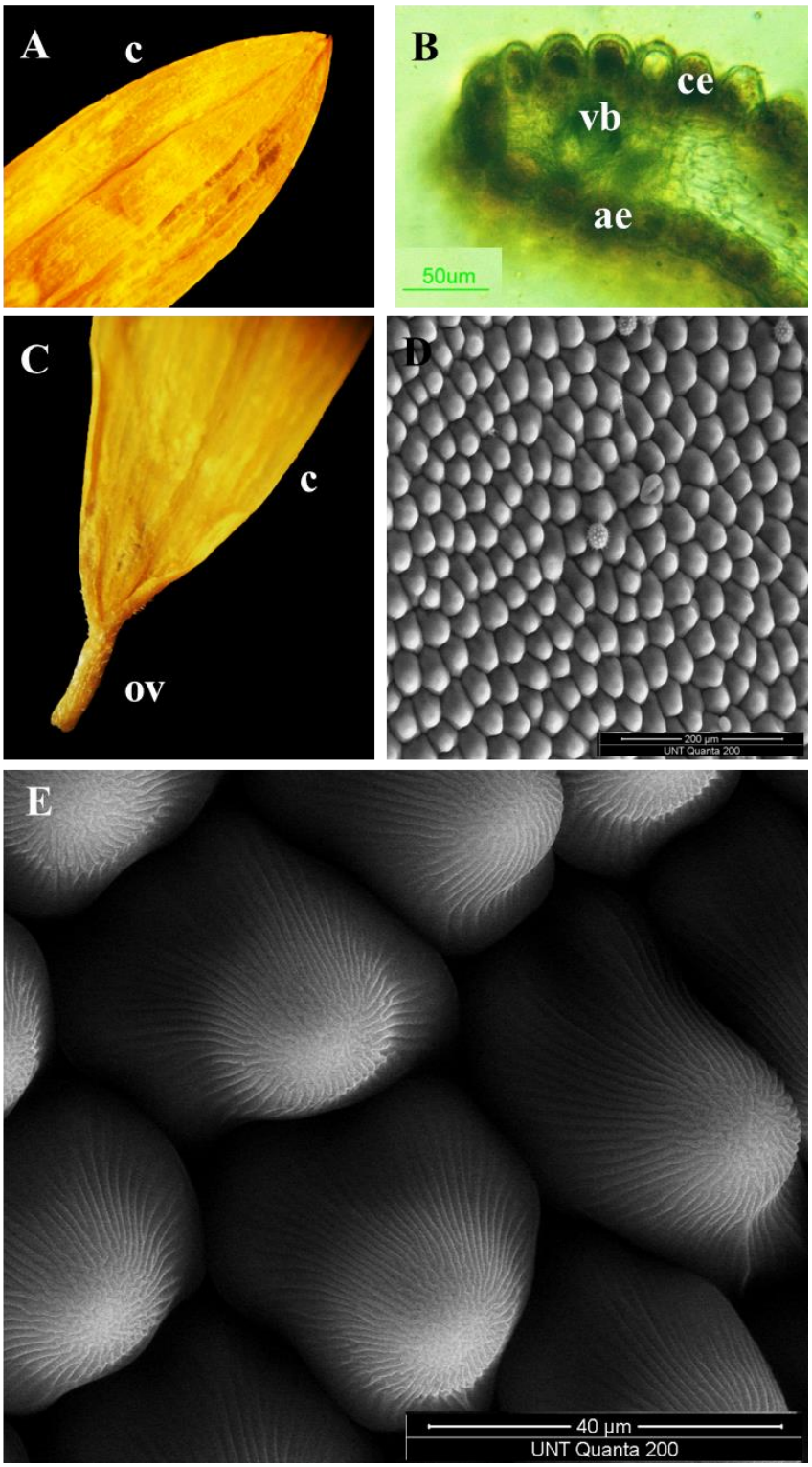

Fig2. Sunflower (*H. annuus, Asteraceae*) ray floret morpho-anatomy.

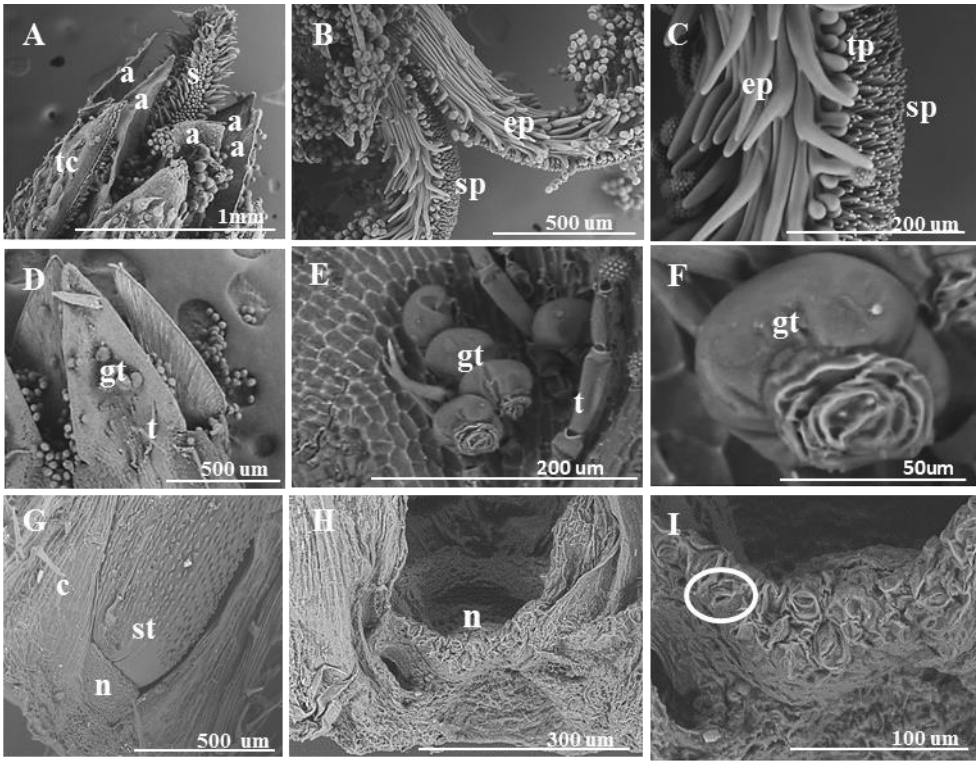

Fig3. Sunflower (*H. annuus, Asteraceae*) disk floret morpho-anatomy visualized with SEM.

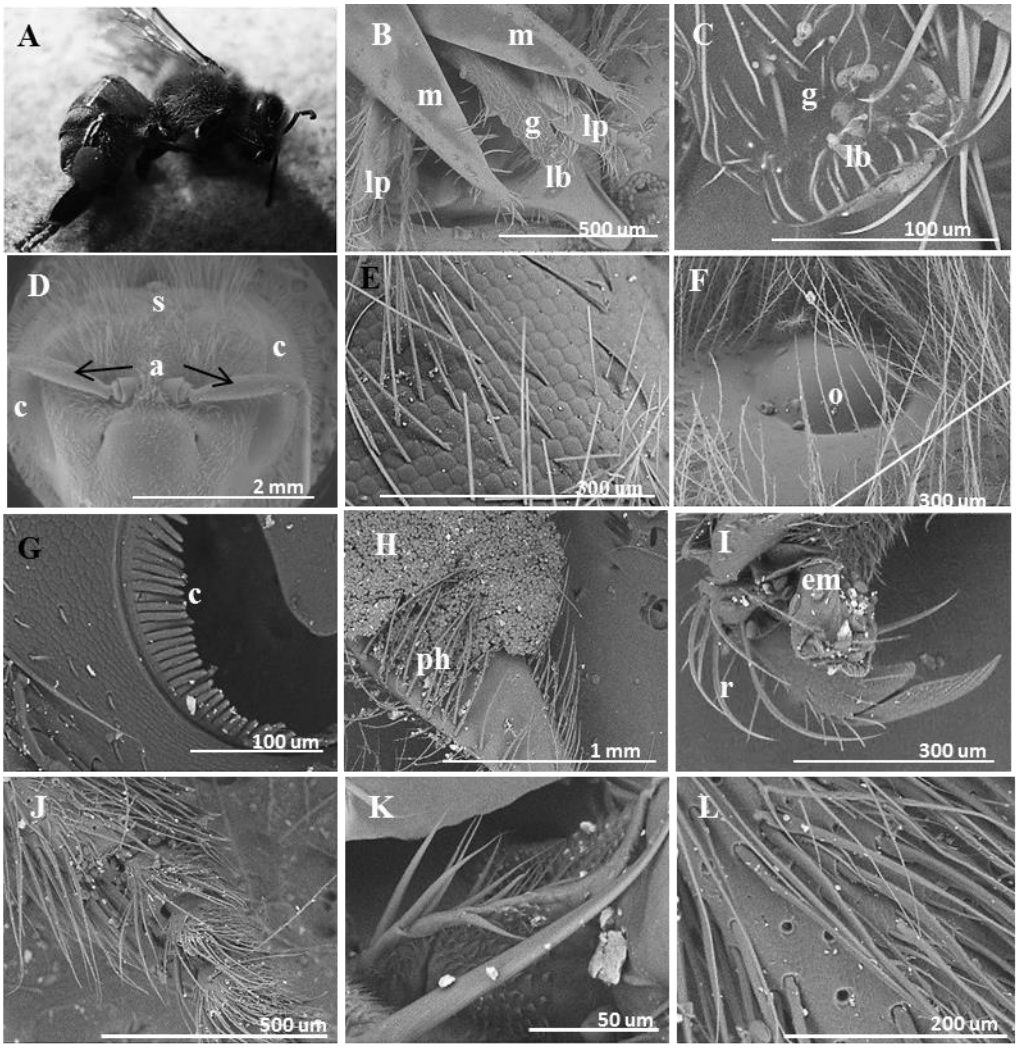

Fig4. Honeybees (*Apis mellifera*) morpho-anatomical adaptations for pollen and nectar harvesting, visualized with SEM.

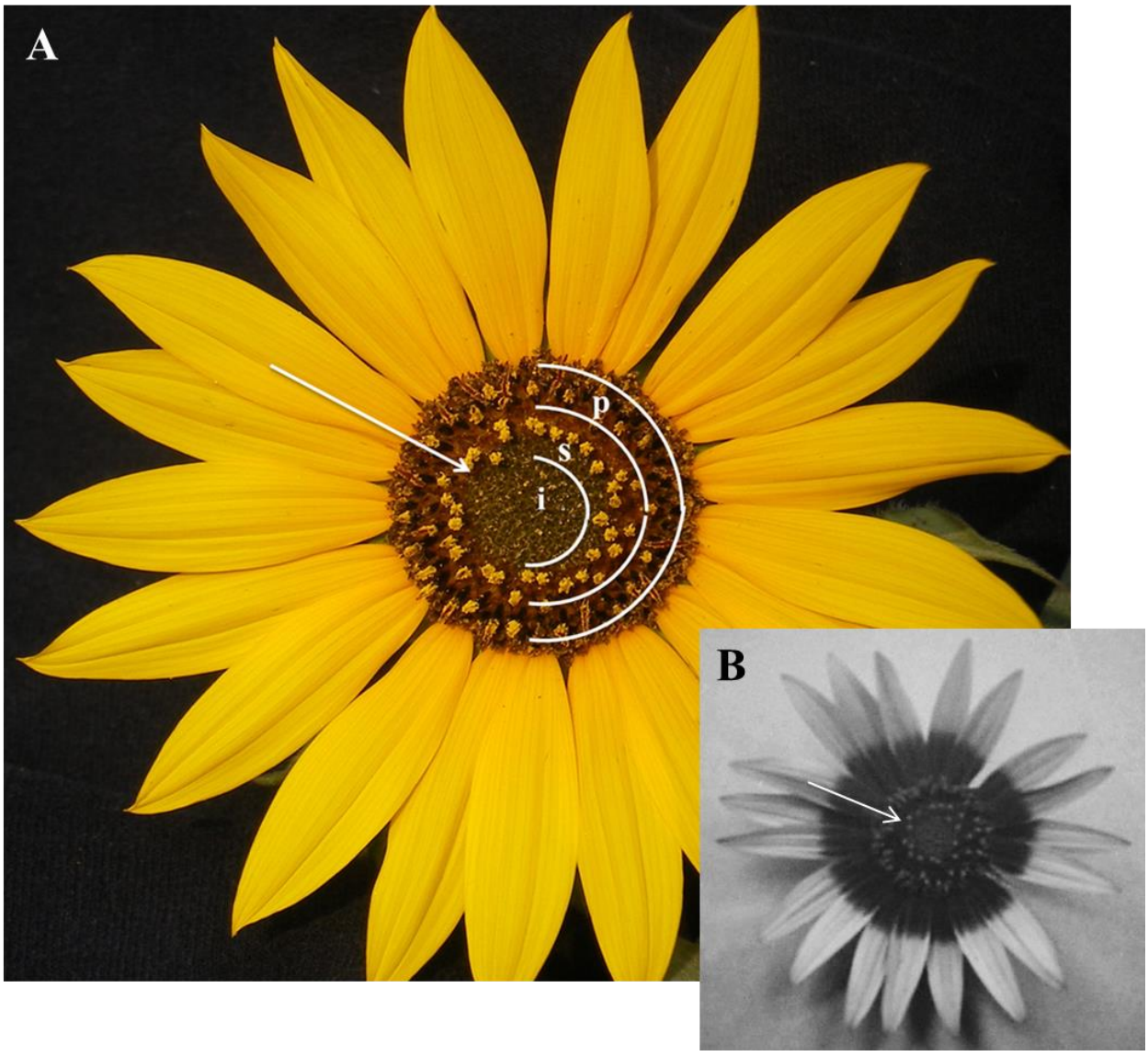

Fig5. Bee cross-pollinating pathway on a *H. annuus, Asteraceae* inflorescence shown in A) visible light, and (B) under UV radiation.

CHAPTER IV

IDENTIFICATION OF FLAVONOID PIGMENTS IN SUNFLOWER DISK FLORETS

Flavonoid pigments in the SPE-hydrolyzed F80 disk floret extract that may contribute to the target pattern of *H. annuus*, were separated by HPLC. The chemical structures of the flavonoid standards apigenin, kaempferol, luteolin, naringenin, pelargonidin chloride, quercetin are presented in Fig. 1. The above standards were selected based on previous studies on sunflower pigments which showed their presence in leaves, pollen, seeds and honey (Vaccari *et al*., 1982; Rieseberg *et al*., 1987; Bohm and Stuessy, 2001; Yao *et al*. 2004; Mazza *et al*., 2004; Weisz *et al*. 2009**).** The anthocyanidin standard pelargonidin chloride was specifically chosen based on floral studies showing that other *Asteraceae* species contain this pigment in their inflorescences (Harborne, 1978; Harborne, 1994; Schlangen *et al*., 2009).

The retention times (t_R) of the flavonoid standards based on the HPLC analyses are presented in Table 1. Most flavonoid standards showed multiple peaks and shoulders, as a possible sign of chemical degradation. The greatest peak area for each standard was considered the corresponding t_R for that standard (Fig. 2). The HPLC trace of the standard apigenin (**1**) shows two large peaks with t_R = 43.2 and 44.2 minutes, respectively. Kaempferol (**2**) has one peak with t_R = 44.8 minutes indicating that this compound has not degraded. Luteolin (**3**) HPLC trace presents two peaks with t_R = 39.0

and 39.2 minutes, respectively, and an additional low peak at t_R 40.3. Naringenin (**4**) shows one peak with t_R = 43.2 minutes and two low peaks with t_R = 43.2 and 43.8 minutes, respectively. Pelargonidin chloride (**5**) has a peak at t_R = 35.9 minutes with one shoulder at t_R = 37.2 and an additional peak at t_R = 40.0 minutes. Quercetin (**6**) shows two very close peaks with t_R = 48.3 and 48.5 minutes, respectively, and one shoulder with t_R = 49.9 minutes (Fig. 2).

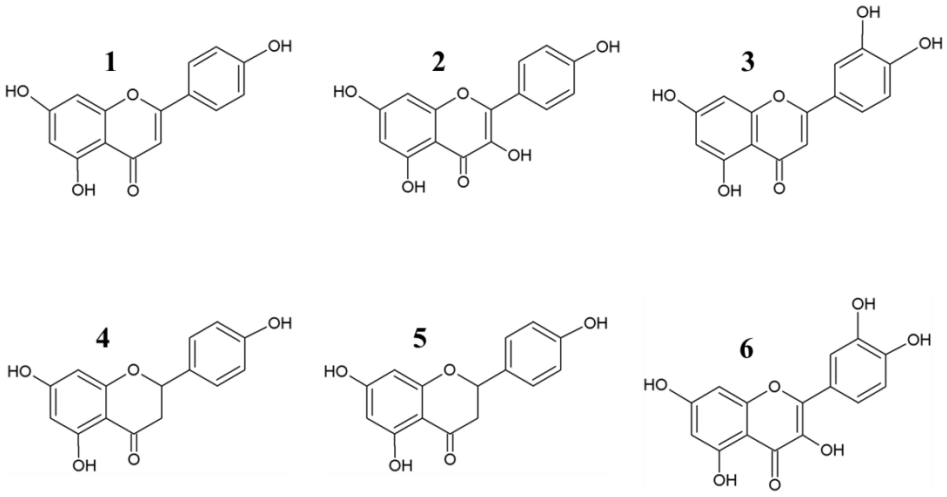

Fig. 1. Chemical structures of flavonoid pigment standards. (1) apigenin (2) kaempferol (3) luteolin (4) naringenin (5) pelargonidin (6) quercetin.

The presence of multiple peaks and shoulders may be also indicative of impurities in the samples, or of a possible incompatibility with the mobile phase, known to induce fronting or tailing peaks (Strasser and Varadi, 2000). However, the mobile phase used in this study is comparable to those employed in flavonoid HPLC studies based on literature (Hughes *et al*., 2001; Volpi and Bergonzini; 2006, Weisz *et al*., 2009). It is known that anthocyanins are sensitive to light and heat, and thus degrade easily. It is possible that pelargonidin degradation in this study occurred due to exposure to mostly light (Chiste *et*

al., 2010).

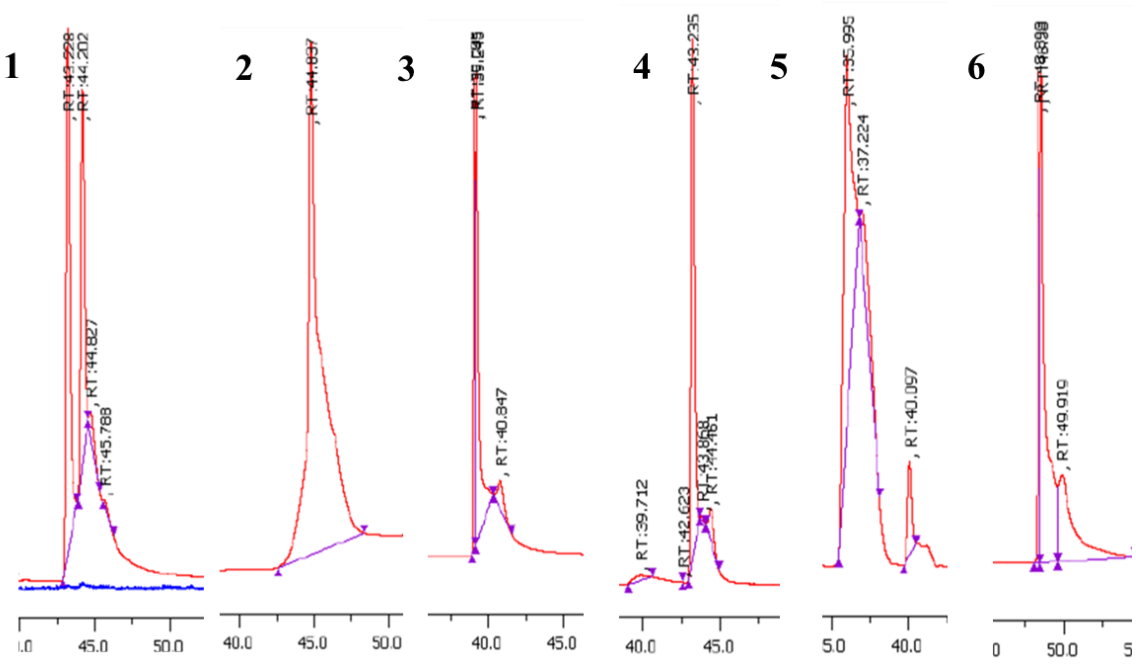

Fig. 2. HPLC chromatograms of flavonoid pigment standards. (1) apigenin, (2) kaempferol, (3) luteolin, (4) naringenin, (5) pelargonidin chloride, (6) quercetin.

Table 1. HPLC retention times (minutes) of flavonoid standards.

HPLC Retention Times (t_R) of Flavonoid Standards	
Flavonoid Standards	t_R (minutes)
(1) Apigenin	43.2
(2) Kaempferol	44.8
(3) Luteolin	39.0
(4) Naringenin	43.2
(5) Pelargonidin Cl_2	35.9
(6) Quercetin	48.3

* t_R values correspond to the highest peak area for each standard.

HPLC of the SPE-hydrolyzed F80 disk floret extract was performed in triplicate and a representative trace is shown in Fig. 3. The HPLC trace displays peaks detected at 254 nm and reveals that the SPE-hydrolyzed F80 disk floret extract is a complex mixture of polar and non-polar compounds. The peaks with the greatest peak area have t_R = 3.1, 6.2, 29.5, 37.8 and 50.6 minutes, indicating that these compounds are present in the disk florets at a higher concentration than the other compounds. Two peaks were of interest since their retention time values (37.8 and 36.5 minutes) were close to those of the flavonoid standards luteolin [(**3**), t_R = 39] and pelargonidin chloride [(**5**), t_R = 36], respectively.

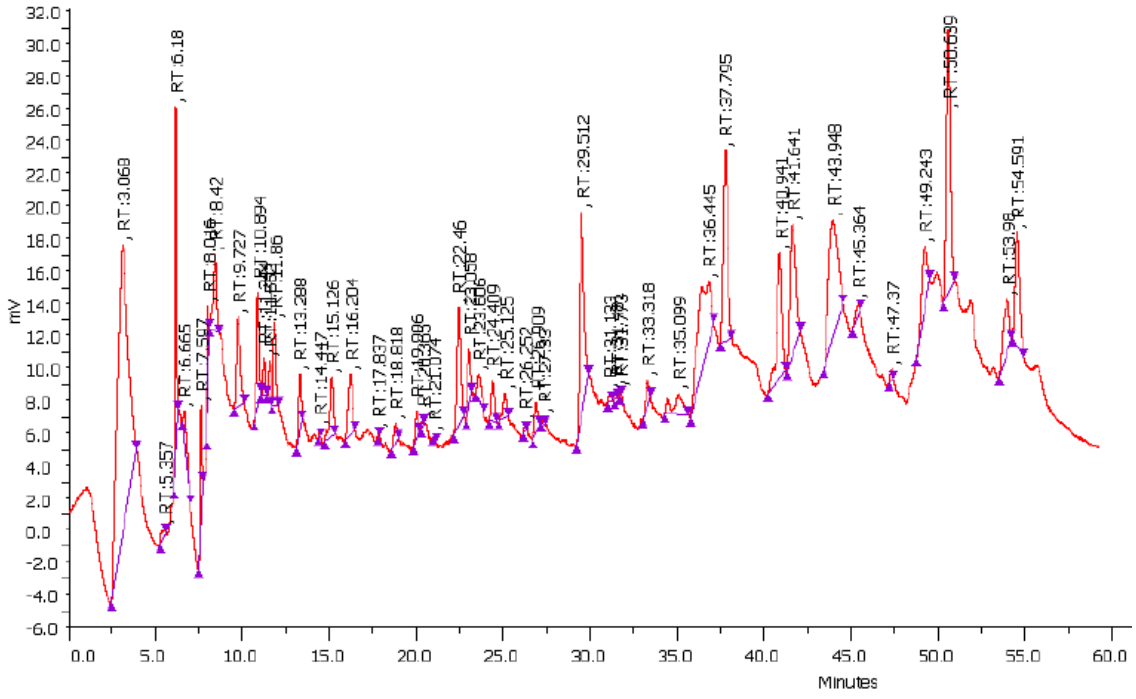

Fig. 3. Representative HPLC chromatogram of SPE-hydrolyzed disk floret extract. The mobile phase consisted of 2% acetic acid in ultrapure water (solvent A) and 0.5% acetic acid in ultrapure water and acetonitrile (50:50, solvent B). The UV detector was set at 254 nm.

To isolate the SPE-hydrolyzed F80 disk floret extract peaks whose t_Rs were comparable to the flavonoid standards luteolin and pelargonidin chloride, HPLC fractions were collected every ten minutes, re-injected, and evaluated for peaks which may correspond to the above flavonoid standards. Since the SPE-hydrolyzed F80 disk floret extract is complex and concentrated, same peaks are seen in successive fractions, which indicate that these compounds are being carried over from one fraction to the next. Fractions collected between 35 and 49 minutes, did not show visible peaks that corresponded to those of standards. However, the fraction collected between 50-60 minutes, produced two peaks with t_Rs of 36.0 and 37.4 min, similar to those of the standards (Fig.4).

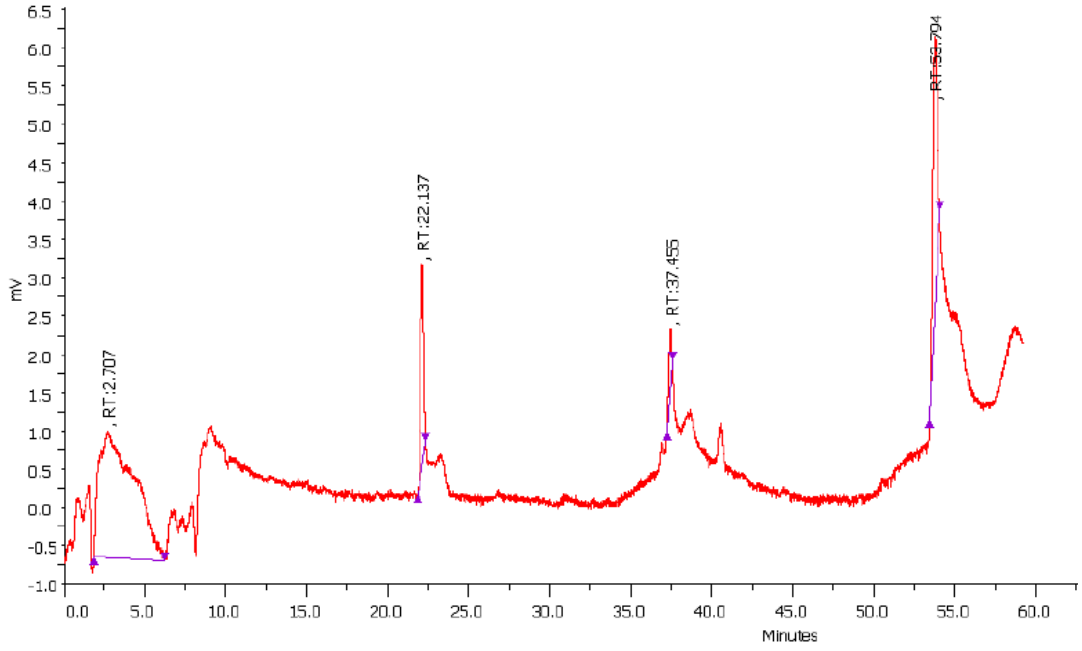

Fig. 4. Representative chromatogram of the 50-60 minute HPLC fractions of the SPE-hydrolyzed disk floret extract. The mobile phase consisted of 2% acetic acid in ultrapure water (solvent A) and 0.5% acetic acid in ultrapure water and acetonitrile (50:50, solvent B). The UV detector was set at 254 nm.

Flavonoid separation by HPLC continues to be the most commonly used scientific method for flavonoid research and has been utilized to study sunflower flavonoids for many years (Vaccari *et al*., 1981, Rieseberg *et al*., 1987, Bohm and Stuessy, 2001, Yao *et al*., 2004, Weisz *et al*., 2009). Historically, identification of plant flavonoids was accomplished by NMR with supportive analysis using UV spectrophotometry, SPE, TLC and HPLC (Mabry *et al.,* 1970, Markham *et al.,* 1978; Harborne, 1978; Harborne, 1994). Since the early 1990's, flavonoids in a wide variety of flowering plants have largely been characterized by means of mass spectrometry coupled with liquid chromatograph (LC-MSMS) (Yong, 1993; Cuyckens and Clayes, 2004; Weisz *et al.,* 2009; Aldini *et al.,* 2011; Chen *et al.,* 2012). As technology advances, flavonoid extraction and identification protocols are refined, and databases of phytochemicals are developed and updated on a regular basis with reliable standardized data.

Studies on flavonoid of *H. annuus* have been ongoing for many years utilizing techniques such as spectrophotometry, TLC, HPLC, gas chromatography, mass spectrometry, phenolic content assays and NMR spectroscopy, as exemplified in the following examples. Apigenin, chalcone, quercetin, kaempferol, luteolin, nepetin, hidpidulin, and nevadensin, have been identified in the sunflower leaves (Schilling and Mabry, 1981; Schilling, 1983; Rieseberg *et al.,* 1987; Schilling *et al.,* 1987; Macias *et al.,* 1997; Yue *et al.,* 2008). Floral flavonoid pigments in a variety of *Helianthus* species have been identified as quercetin (and derivatives), chalcone, kaempferol, myricetin, cyanidin, delphinidin, and pelargonidin (in red sunflowers) (Sando, 1925; Harborne, 1978; Schilling and Spooner, 1988). More specifically, ray floret flavonoid pigment studies

have identified quercetin, apigenin, luteolin, coreopsin, and marein (Schilling and Mabry, 1981; Schilling, 1983; Schilling *et al.,* 1987; Schilling and Spooner, 1988). A recent study stated that the anthocyanins pelargonidin, cyanidin, and taxfolin, in addition to flavones, flavanones, and flavonols might be present in ornamental sunflowers but no definitive chemical identification was provided (Zhang *et al.,* 2011). Sunflower hulls also have been investigated as a source of flavonoids. The hulls were found to be a rich source of anthocyanins (Vaccari *et al.,* 1981; Gao and Mazza, 1996; Weisz *et al.,* 2009). Sunflower oils have been investigated for their flavonoid content as a source of antioxidants and have revealed the presence of luteolin, kaempferol, myricetin, and quercetin (Roedig-Penman and Gordon, 1998; Skerget *et al.,* 2005). Sunflower oil is produced by compressing the achenes, hulls and seeds, under high pressure to express the oil (Weisz *et al.,* 2009). Therefore, the flavonoid content of sunflower oils could be tracked back to the hulls. Studies on the honey also have identified the presence of quercetin, myricetin, tricetin and luteolin (Yao *et al.,* 2004; Yao *et al.,* 2005; Marghitas *et al.,* 2009). In the case of sunflower, the honey is produced by bees from the nectar and pollen collected from the disk florets. It seems that the flavonoids present in honey originate in the collected pollen.

In order to identify the compounds corresponding to the t_Rs of luteolin and pelargonidin standards, the SPE-hydrolyzed F80 disk floret HPLC 50-60 fraction was analyzed by mass spectrometry. To visualize the parent ion of the flavonoid standards, full mass spectrometry (MS) scans was completed and used to compare them with the

MS of the SPE-hydrolyzed disk floret extract (Fig. 5) and the HPLC 50-60 min fractions (Fig. 6).

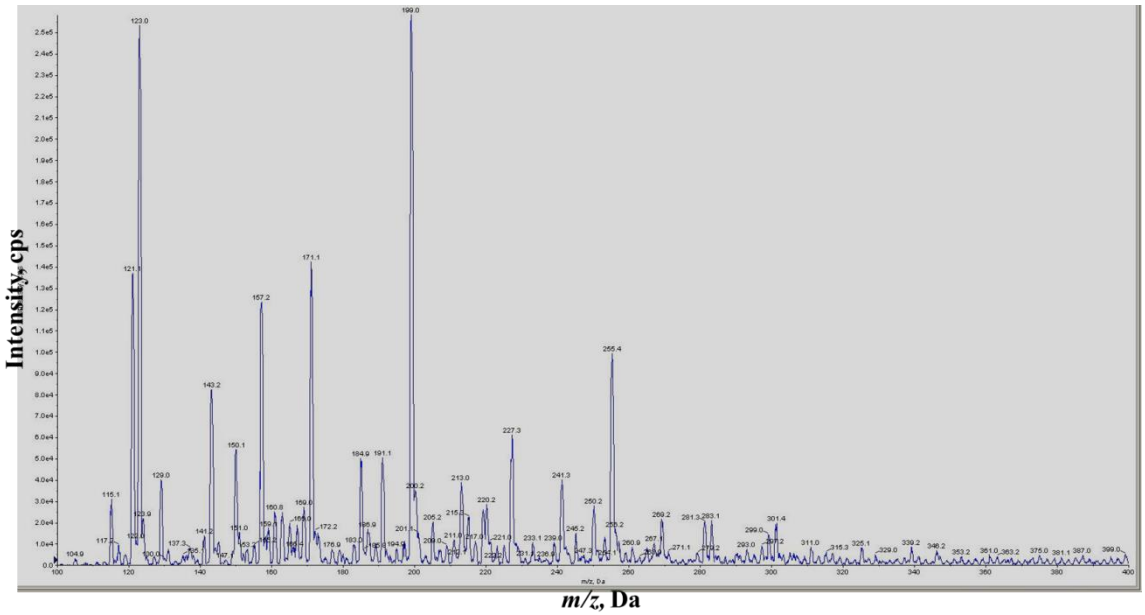

Fig. 5. MS of SPE-hydrolyzed disk floret extract in negative ion mode $[M-H]^-$.

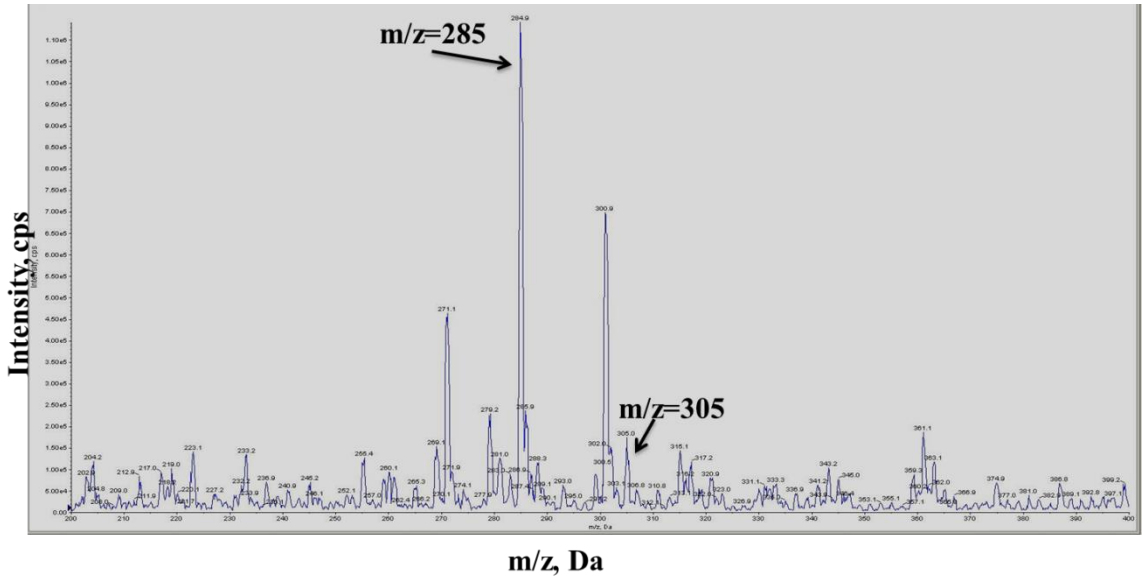

Fig. 6. MS of the disk floret HPLC 50-60 minute fractions in negative ion mode $[M-H]^-$. Parent ion *m/z* of 285 and 305 are present.

To determine fragmentation pattern of the daughter ions of the flavonoid standards, electrospray tandem mass spectrometry (ESI-MSMS) was employed. The spectrograms of the flavonoid standards were compared to the spectrograms of the SPE-hydrolyzed disk floret extract and the HPLC 50-60 min fractions. MS and ESI-MSMS scans were performed in positive $[M+H]^+$ and negative ion mode $[M-H]^-$.

The flavonoid standards luteolin (**4**) and pelargonidin chloride (**5**) *m/z* traces produced daughter ions, under specified collision induced dissociation (CID), consistent with the information found in ReSpect (Sawada *et al.,* 2012). ESI-MSMS with CID 271 of pelargonidin chloride (**5**) was traced in positive mode $[M+H]^+$ and produced one fragment with *m/z* = 271 (Fig. 7). ESI-MSMS with CID 285 of luteolin (**3**) was traced in negative mode $[M-H]^-$ and produced fragments with *m/z* = 284, 283, 151, 133, and 107 (Fig. 8). The fragmentation patterns produced by the flavonoid standards used in this study were consistent with the flavonoid fragmentation patterns found in the ReSpect phytochemical database (Sawada *et al.,* 2012).

The disk floret HPLC 50-60 min fractions were analyzed in positive $[M+H]^+$ and negative ion mode $[M-H]^-$. It was noted that in MS mode, the parent ions with *m/z* 285 and 305 were present (Fig. 6). Due to these parent ions being present in MS mode, ESI-MSMS in negative ion mode $[M-H]^-$ with CID 285 and collision energy (CE) of 40 V was carried out to investigate the presence of luteolin (**3**) in the disk floret HPLC 50-60 minute fractions (Fig. 9). The ESI-MSMS fragmentation pattern was consistent with the fragmentation pattern of the standard luteolin (**3**), and thus the presence of luteolin (**3**) in the disk floret HPLC 50-60 minute fractions was definitively confirmed.

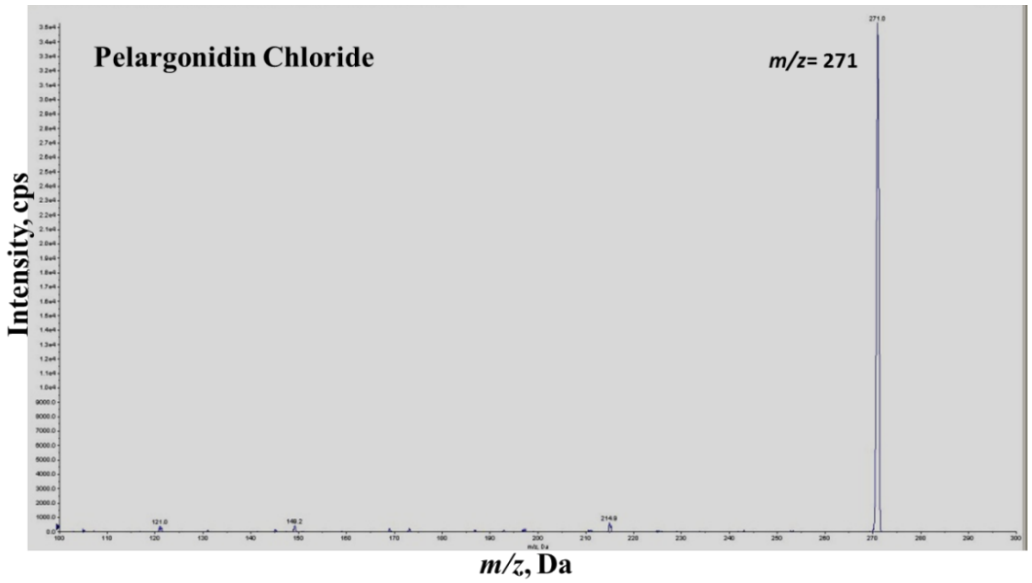

Fig. 7. ESI-MSMS of flavonoid standard pelargonidin chloride. Pelargonidin chloride was detected by tracing the *m/z* 306 ion $[M-H]^-$ with CID 271 and CE30.

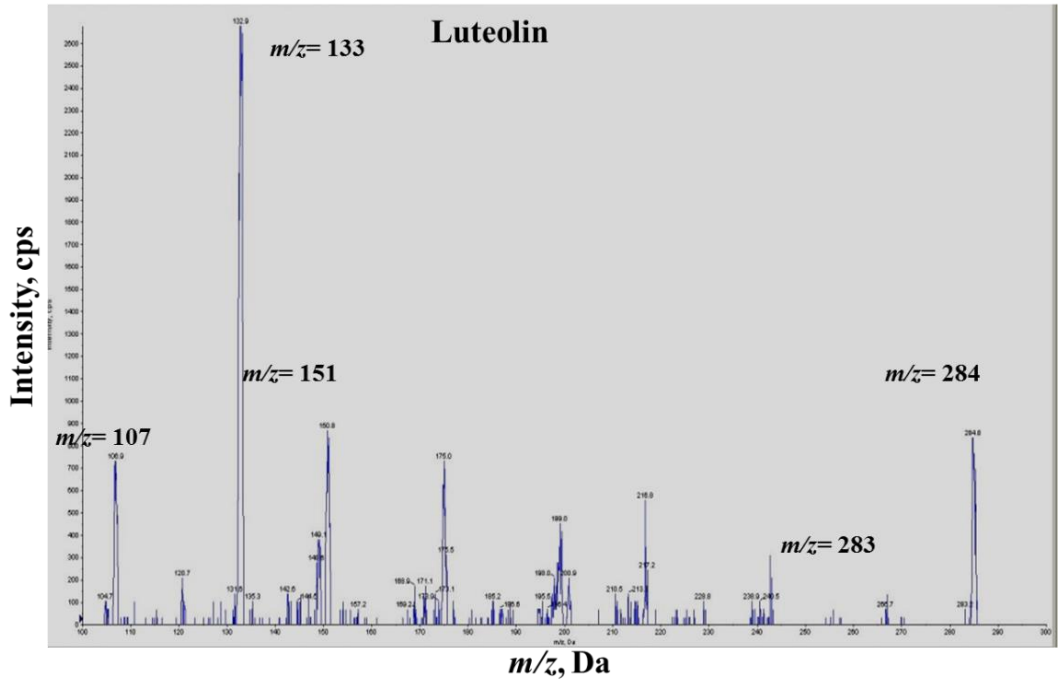

Fig. 8. ESI-MSMS of flavonoid standard luteolin. Luteolin was detected by tracing the *m/z* 286 ion $[M-H]^-$ with CID 285 and CE40.

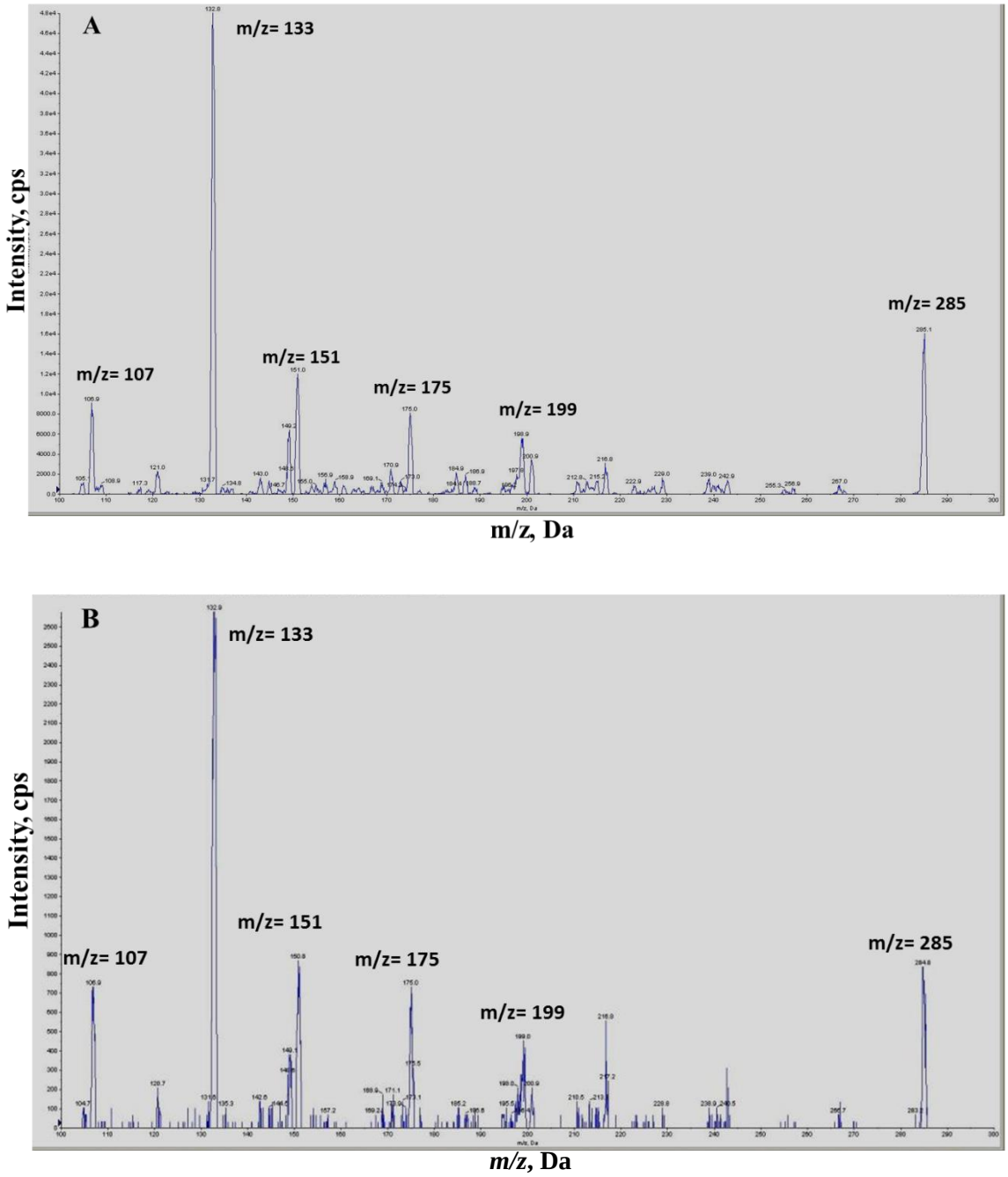

Fig. 9. ESI-MSMS of disk floret HPLC 50-60 minute fractions (A) as compared to that of luteolin (B).

MS in positive mode $[M+H]^+$ did not provide a parent ion *m/z* that compared to the pelargonidin (**5**) samples, but in negative mode a parent ion with a *m/z* ratio of 305 was noted (Fig. 6), therefore ESI-MSMS in negative ion mode $[M-H]^-$ with CID 305 and CE of 40 V was carried out to investigate the presence of pelargonidin (**5**) in the disk floret HPLC 50-60 minute fractions (Fig. 10). A fragment with *m/z* 269 was produced, which can be identified as the fragmentation pattern similar to that found in the positive mode for pelargonidin chloride (**5**), which produced a fragment with *m/z* 271. The ESI-MSMS fragmentation pattern was consistent with the fragmentation pattern of the standard pelargonidin (**5**), and thus definitively confirms the presence of pelargonidin (**5**) in the disk floret HPLC 50-60 minute fraction sample.

To gain supportive evidence for the presence of flavonoids in the SPE-hydrolyzed disk and ray floret extract samples, thin layer chromatography (TLC) was utilized. A representative TLC analysis (Fig. 11) shows comparable relative mobility between the SPE-hydrolyzed disk floret extract (DF) and the flavonoid standards apigenin, kaempferol, luteolin, naringenin, pelargonidin and quercetin. The SPE-hydrolyzed distal ray floret (DRL) extract spots show similar relative mobility with the standards apigenin, kaempferol and pelargonidin. The SPE-hydrolyzed basal ray floret (BRL) extract spots show similar relative mobility with standards apigenin and kaempferol. There was no correlation of relative mobility with the standard chalcone.

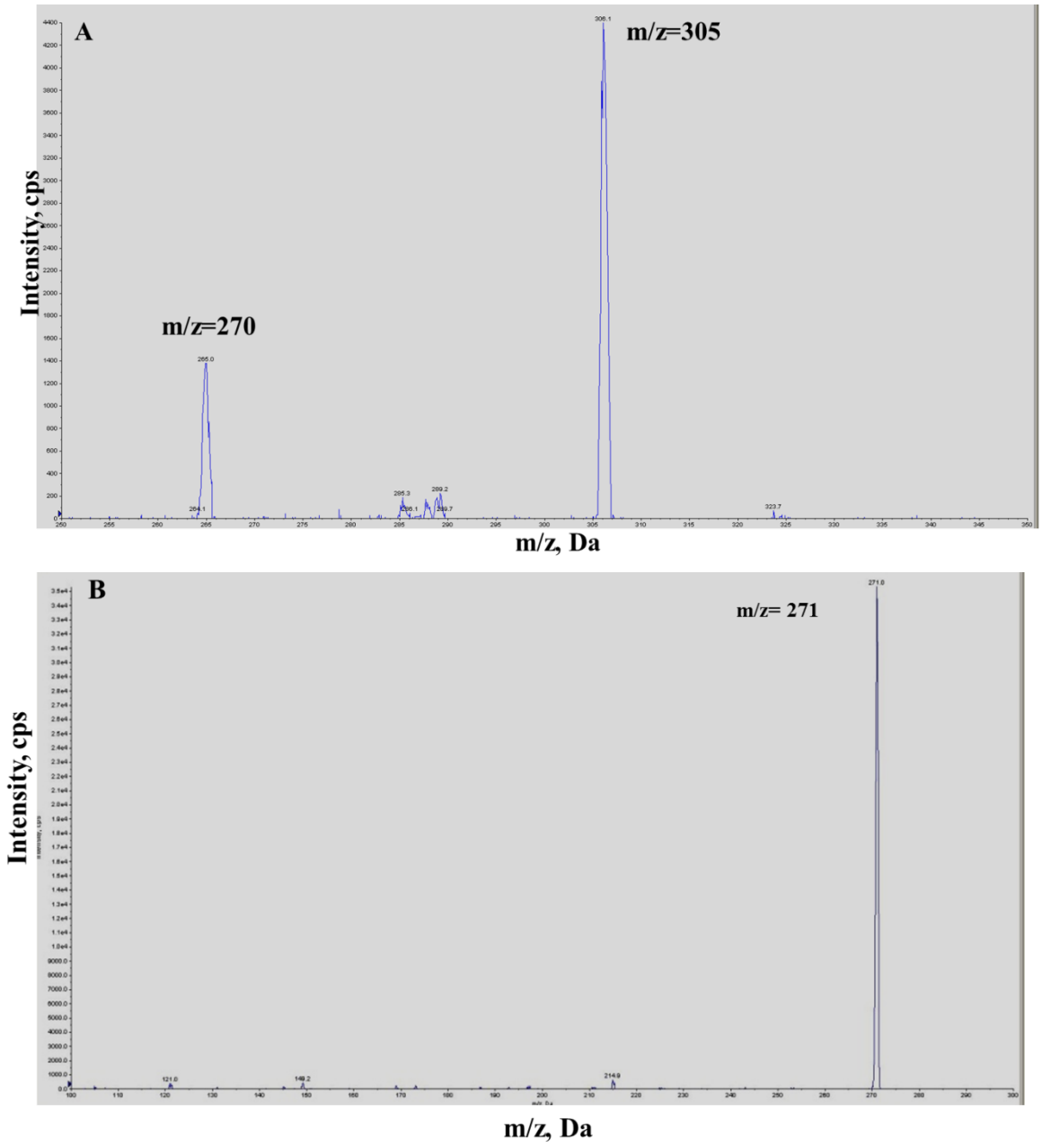

Fig. 10. ESI-MSMS of disk floret HPLC 50-60 minute fractions (A) compared with that of pelargonidin (B).

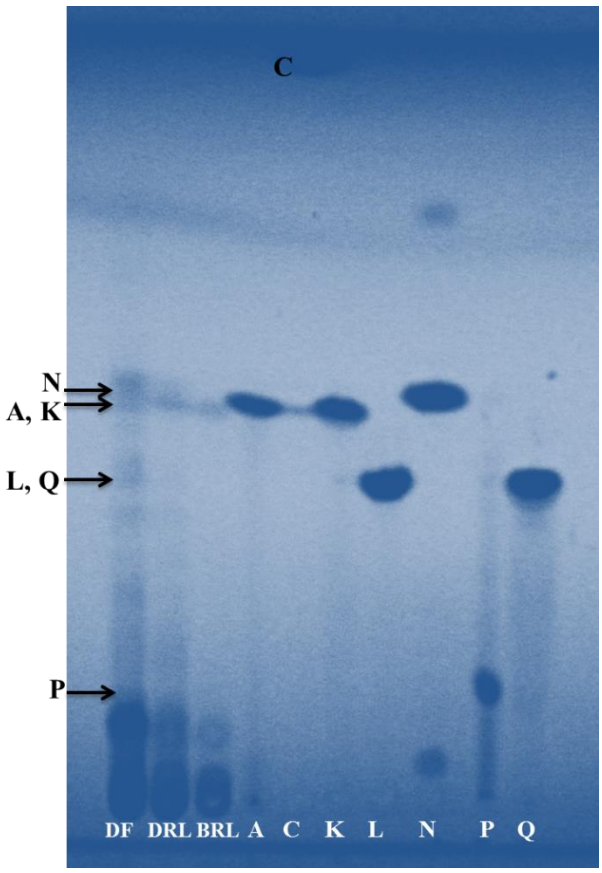

Fig. 11. Representative Thin Layer Chromatography on silica plate with UV indicator. Solvent system was chloroform:acetone:formic acid (9:2:1). DF, disk floret extract; DRL, distal ray floret extract; BRL, basal ray floret extract; A, apigenin (flavone); C, chalcone (flavonoid); K, kaempferol (flavonol); L, luteolin (flavone); N, naringenin (flavonoid); P, pelargonidin Cl_2 (anthocyanidin); and Q, quercetin (flavonol).

Spectrophotometry of the flavonoid standards and the of SPE-hydrolyzed disk floret samples was completed under a UV-visible range (200 – 700 nm) and utilized as supportive evidence for the identification of flavonoids in the disk floret samples (Fig. 12). The spectrographs show complex traces, indicative of many compounds in the SPE-hydrolyzed disk floret and the HPLC 50-60 minute fraction samples. Spectrograph traces of both samples (Fig. 12 A and B) show two peaks, 210 nm and 290 nm, in common.

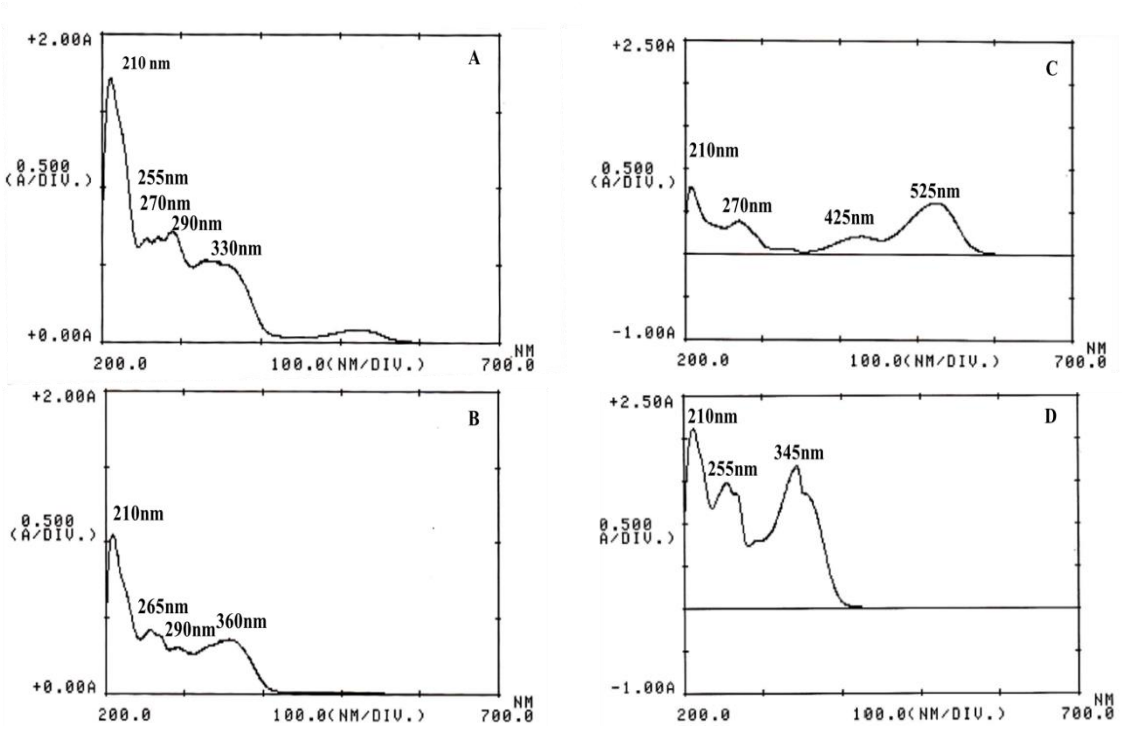

Fig. 12. Spectrophotometry of SPE-hydrolyzed disk floret samples and flavonoid standards. A) SPE-hydrolyzed disk floret samples. B) SPE-hydrolyzed disk floret HPLC 50-60 min fractions. C) Pelargonidin chloride and D) Luteolin.

Similarities between these two samples are expected since the HPLC 50-60 minute fraction originated from the SPE-hydrolyzed disk floret sample. However, the SPE-hydrolyzed disk floret sample, a more complex mixture of compounds, has two major peaks at 255 nm and 270 nm that were not individualized in the HPLC 50-60 fraction spectrograph, although they may be present at lower concentrations. The HPLC 50-60 minute fraction spectrograph has two peaks at 265 nm and 290 nm that are not marked on the SPE-hydrolyzed disk floret sample spectrograph. Since the HPLC 50-60 minute fraction samples were collected according to the retention times of luteolin and

pelargonidin, it is possible that the 265 nm and 290 nm peaks represent other compounds which were concentrated in this sample.

According to the literature, flavone and flavonol spectrographs should have two major peaks between 240 – 280 nm, and 300 – 380 nm, respectively (Mabry *et al.,* 1970). Anthocyanins spectrograph should show a specific peak approximately at 520 nm (Lee *et al.,* 2005). The peaks in the spectrographs of the SPE-hydrolyzed disk floret (255 nm, 270 nm and 330 nm) and the HPLC 50-60 minute fraction samples (260 nm and 360 nm) that are consistent with the range of flavonols. Due to the complexity of flavonoid biosynthesis, it is likely that the disk floret samples contain not only flavonols and anthocyanins but also flavones, flavanones, dihydroflavanols, isoflavones, proanthocyanidins and even chalcones, stilbenes, and coumarins (Winkel-Shirley, 2001). Since a flavonoid-specific extraction was employed for the disk floret sample, it is possible that the spectrograph peaks that do not correspond to those of the flavonol luteolin and anthocyanidin pelargonidin standards represent other types of flavonoids.

Anthocyanidin UV absorption is impacted by the pH of the extraction solution since anthocyanidins undergo structural changes at a pH range between 1 and 4.5 (Lee *et al.,* 2005). The aglycone pelargonidin is notoriously unstable *ex vivo* due to the presence of a flavylium cation at its core. The counterion of the flavylium ion is typically chloride, which aids in maintaining the electric neutrality of the compounds (Garcia-Viguera and Bridle, 1999; Freitas *et al.,* 2011). Anthocyanidin aglycones are purchased bound to the chloride ion in order to preserve chemical and structural stability. The pelargonidin chloride standard used for this study was prepared in methanol and its pH was recorded

as 3.0. The SPE-hydrolyzed disk floret sample and HPLC 50-60 minute fraction were also acidic, with pHs of 1.5 and 1.8, respectively. The acidic pHs were due to acid hydrolysis with HCl employed in the fractionation methodology and to acetic acid used in the HPLC solvent system. It is possible that the 525 nm specific peak of pelargonidin is not seen in neither the SPE-hydrolyzed disk floret sample nor the HPLC 50-60 minute fraction due to structural changes caused by acidic pH.

In order to visualize the UV absorption of the standards and the sunflower extracts used in this study, TLC paper spotting methodology was employed (Fig. 13). Photographs of the paper TLCs were taken under UV radiation with a normal lens, as well as with a UV-pass lens to visualize the absorptive and reflective properties of the flavonoid pigments. Flavonols and flavones are believed to be the pigments responsible for UV absorption in the dark basal parts of the sunflower ray florets, and carotenoids play a role in creating the light portion of the distal ray floret (Harborne and Smith 1978; McCrea and Levy, 1983). Anthocyanins are the main pigments responsible for UV absorption in the disk florets, which make the dark portion of the target pattern of the sunflower inflorescence (McCrea and Levy 1983; Samanta *et al*., 2011).

The SPE-acid hydrolyzed disk floret spots (Acid hydrolysis T, Fig. 13 A and B) are darker than the upper (UL) and lower (LL) ray floret extracts, indicative of its flavonoid content rich in UV absorptive flavones, flavonols and anthocyanins. This is consistent with the dark basal ray and disk floret portion of the target pattern of the inflorescence (Fig. 13 D). Since a flavonoid specific extraction method was used to obtain the floret samples, the SPE-acid hydrolyzed disk floret spots show the absorptive

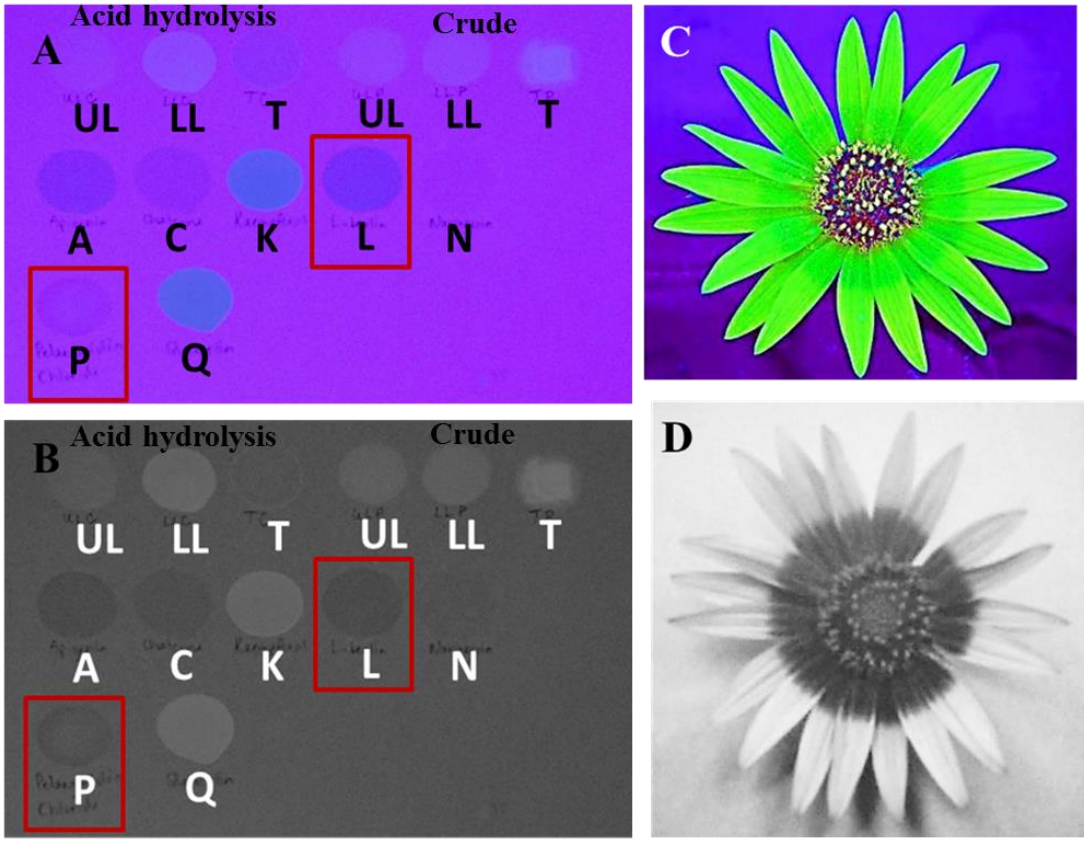

Fig. 13. Representative paper TLC of flavonoid standards and sunflower extract spots under UV radiation and corresponding sunflower inflorescence photos. A) Paper TLC of pigment spots under UV photographed with a normal lens. B) Paper TLC of pigment spots under UV photographed with a UV-pass lens. C) Photograph of a sunflower inflorescence under UV. D) Photograph of the sunflower inflorescence in C) under UV using a UV-pass lens. UL, upper ligulate (distal ray floret) extract; LL, lower ligulate (basal ray) floret extract; T, tubulate (disk) floret extract; A, apigenin; C, chalcone; K, kaempferol; L, luteolin; N, naringenin; P, pelargonidin chloride; Q, quercetin; 'Acid hydrolysis' represents crude extract samples that were flavonoid specifically extracted followed by SPE and acid hydrolysis; 'Crude' represents the initial methanol extraction of floral samples with no further processing.

and reflective properties of the flavonoid pigments only. The crude extracts contain carotenoids in addition to flavonoid pigments. Therefore, the TLC spots of Crude UL,

LL, and T (Fig. 13 A and B) show more UV reflection than absorption due to presence of carotenoids, which are known to contribute to the light portion of the target pattern as seen in Fig. 13 C and D (McCrea and Levy, 1983).

The flavonoid standards in Fig. 13 A and B show spots that are both UV reflective and absorptive. Apigenin (A), chalcone (C), luteolin (L), naringenin (N), and pelargonidin chloride (P) are darker and therefore more UV absorptive than kaempferol (K) and quercetin (Q) which appear to be lighter and UV reflective (Fig. 13 A and B). In *Rudbeckia hirta*, another *Asteraceae* species, quercetagetin (a 6-hydroxyquercetin flavonol) has been found in the basal portion of the ray florets and shown to contribute to the UV absorption in that area of the inflorescence (Schlangen *et al.*, 2009). It is noted, that the methoxyl group in position 6 of quercetagetin strongly influences the light absorption properties of this compound. The aglycone quercetin used in the present study is UV reflective most likely because it lacks the methoxyl group in position 6 present in quercetagetin. Schlangen *et al.* isolated kaempferol and its derivatives solely from the distal, UV reflective, portions of ray florets. In the present study kaempferol also was found to be UV reflective.

Overall, this biochemical study of the ray and disk florets revealed a complex chemical composition. The role of flavonoids in the formation of the target pattern that attracts honeybees is well established in the literature (Harborne and Smith, 1978; McCrea and Levy, 1983; Harborne, 1994; Winkel-Shirley, 2001; Schlangen *et al.*, 2009). This study identified luteolin and pelargonidin in disk floret extracts, which has not yet been reported before in the scientific literature. Luteolin and pelargonidin in the disk

florets contribute to, but are not solely responsible for, the dark UV absorbing portion of the target pattern of the sunflower inflorescence. Additional studies on the ray floret flavonoids and carotenoid pigments should be completed in order to better and completely understand the chemistry of sunflower target pattern.

CHAPTER V

CONCLUSIVE REMARKS

Plant floral pigments play a very important role in the visual attraction of pollinators. Visual floral cues are vital to the process of cross-pollination, which increases the genetic diversity and seed set in plants. Most flowering plants reward pollinators with offerings of pollen and nectar and pollinators contribute to cross-pollination of those plants. Specific pigments are synthesized and compartmentalized in specific floral areas that form target patterns that are recognized by insect pollinators in the UV-radiation spectrum and interpreted as a signal for the presence of nourishment. Pollinator vision and floral target patterns evolved as a biosemiotic relationship (Chittka and Menzel, 1992).

During its evolution, the sunflower (*H. annuus, Asteraceae*), have developed morpho-anatomical and biochemical adaptations to maximize pollination success and seed set, mainly through cross-pollination by honeybees. The corolla morphology and pigmentation of the ray and disk flowers in the sunflower inflorescence help form a target pattern under UV radiation. Bees recognize the target pattern and, in the process of collecting rewards, cross-pollinate the disk florets in the pistillate stage of development. The morpho-anatomical co-adaptations of *H. annuus* and *A. mellifera* were studied with light, scanning electron (SEM), and confocal laser scanning (CLSM) microscopes. The adaxial epidermis of the ray and disk florets is made up of specialized conical cells that

reflect light. The staminate stage in the development of the disk florets show the five fused anthers extending through the open corolla. It is at this stage in the floral development that pollen and nectar rewards are offered to bee pollinators. During the pistillate stage of disk floret development, the style and stigma elongate emerging from the anther column and stigma opens its two lobes exposing the receptive short conical papillae for pollination. This study reports for the first time the presence of one to three rows of transitional papillae between the short and elongated papillae areas of the stigma, which may function in protecting the receptive stigmatic area from self-pollination during emergence of stigma from inside the anther column. Honeybees interpret *H. annuus* reward availability signals and utilize structural adaptations for collecting and transporting rewards, such as specialized mouth structures for sucking nectar, and combs, rakes, branched and unbranched hairs, and pollen baskets for collecting and transporting pollen. Based on the review of literature and performed morpho-anatomical analyses of sunflower ray and disk floret, this study presents a model of the cross-pollination of sunflower inflorescence by honeybees. The results of this first part of the study (Chapter III) enhance our knowledge of the mutualistic relationship between sunflowers and honeybees with possible practical applications in pollination biology.

The second part of the study focused on the chemical characterization of sunflower floret pigments that contribute to the formation of the target pattern as visualized by honeybee pollinators. Thin Layer and High Performance Liquid Chromatography of the ray and disk floret extracts identified flavonoid pigment fractions with relative mobility similar to those of luteolin and pelargonidin standards. The

structures of flavonoid pigments in the sunflower disk floret extracts were elucidated based on MS and MSMS spectroscopic analyses. The pigment analyses identified two flavonoids in the disk florets that have not been reported previously. Luteolin, a UV-absorptive flavone, contributes to the dark rings of the target pattern created by the disk florets. Pelargonidin, a UV-absorptive anthocyanidin, contributes to the brown-burgundy visible color of the disk florets, in addition to contributing to the dark rings of the target pattern created by the disk florets. The results of the second part of the study (Chapter IV) make a contribution to the metabolomics of the phenylpropanoid pathway in *H. annuus* in addition to enhancing our understanding of mutualism and biosemiotic relationship between flowering plants and pollinators.

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