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# Utility of the Colorimetric Folin-Ciocalteu and Aluminum Complexation Assays for Quantifying Secondary Metabolite Variation among Wild Sunflowers

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**Abstract:** Secondary metabolites serve multiple functions in plants, and play a key role in many ecological processes. Accordingly, the quantification of such compounds is central to addressing many questions in plant science. Alongside precision analytical methods like gas and liquid chromatography-mass spectrometry, there exists a substantial niche for inexpensive and rapid spectrophotometric approaches if their usefulness in a system can be demonstrated. This study seeks to examine the utility of two commonly used colorimetric methods – the Folin-Ciocalteu assay and the aluminum complexation assay – for quantifying variation in leaf phenolic and flavonoid content among members of the genus *Helianthus*, the sunflowers. Among species known a priori to vary substantially in both the diversity and relative concentrations of secondary metabolites, both assays detect substantial variation among species. Moreover, total phenolic content as assessed by the Folin-Ciocalteu assay correlates positively with concentrations of multiple individual phenolic compounds as quantified by high performance liquid chromatography-mass spectrometry, indicating that the Folin-Ciocalteu describes variation in sunflower phenolic content. Additionally, the diversity of flavonoids known from *Helianthus* include a number of those known to be sensitive to the aluminum complexation assay, indicating that this assay may also be a useful descriptor of relative variation in sunflower flavonoid content. In total, both the Folin-Ciocalteu and aluminum complexation assays appear to capture useful, if coarse, variation in secondary metabolites among *Helianthus* species, and seem useful as rapid low-cost methods for exploratory

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research, preliminary analyses, and potentially useful for high-throughput phenotyping within wild or cultivated sunflower with proper calibration.

**Keywords:** colorimetry, flavonoids, *Helianthus*, phenolics, sunflower

## Introduction

Quantifying variation in plant secondary metabolites is central to understanding many phenomena in plant biology, including interactions with herbivores, pollination and fruit dispersal, pathology and disease resistance, tolerance of the abiotic environment, and many other key processes (Schemske and Bradshaw, 1999; Dobson and Bergstrom, 2000; Lattanzio *et al.*, 2006; Treutter, 2006; Sanchez *et al.*, 2008; Agrawal and Weber, 2015). The most precise methods for quantifying plant secondary metabolites, for instance gas or liquid chromatography coupled with mass spectrometry, are both relatively expensive and time-consuming. Many applications do not require this level of analytical detail, including exploratory or preliminary screening analyses, and yet other questions seek relative quantification of broad classes of compounds. For such purposes, an array of spectrophotometric methods exist for the estimation of various classes of secondary metabolites, based on either innate absorbance spectra of compounds (e. g., carotenoids, anthocyanins), or based on the colorimetric reactivity of characteristic functional groups with a particular reagent (e. g., phenolics, flavonoids, alkaloids, tannins).

This study seeks to evaluate the utility of two common colorimetric assays for documenting variation in secondary metabolites in sunflowers: the Folin-Ciocalteu assay for phenolics (Singleton *et al.*, 1999; Ainsworth and Gillespie, 2007), and the aluminum complexation assay for flavonoids (Pełkal and Pyrzynska, 2014). The Folin-Ciocalteu assay employs a reagent consisting of mixed heteropolyphosphotungstate-molybdates which undergo reduction to blue ionic forms in the presence of phenolics and other reducing groups, yielding a blue coloration with maximum absorbance around 760 nm (Singleton *et al.*, 1999). This assay is widely used in studies of plant ecology and physiology (e. g., Ehlenfeldt and Prior, 2001; Riipi *et al.*, 2002; Sumbele *et al.*, 2012; Oguro and Sakai, 2014), as well as food science (e. g., Kim *et al.*, 2003; Lee *et al.*, 2003; Park *et al.*, 2008; Elfalleh *et al.*, 2012). The aluminum complexation assay is based on the formation of a complex between Al(III) ions and the 5-hydroxy-4-keto and 3-hydroxy-4-keto system as well as *o*-dihydroxy groups (Bohm, 1998; Malešev and Kuntić, 2007), yielding a yellow coloration with peak absorbance between 400 and 430 nm (Pełkal and Pyrzynska, 2014). This assay is often used

in studies of food and agricultural phytochemistry (e. g., Kim *et al.*, 2003; Lee *et al.*, 2003; Park *et al.*, 2008; Elfalleh *et al.*, 2012).

Both of these assays are rapid, require only inexpensive reagents, and can be used to process a very large number of samples in parallel. While these two assays are utilized in a wide variety of applications and have been repeatedly tested and improved upon since their development, it remains important to examine their utility in a given system before widespread implementation. Here we evaluate the utility of both assays across a diverse group of species of the genus *Helianthus*.

## Materials and methods

Dried, ground leaf tissue was obtained from 24 wild sunflower species grown in the common garden study of Mason and Donovan (2015). In brief, seeds were obtained from wild populations across North America as well as from germplasm accessions maintained by the United States Department of Agriculture National Genetic Resources Program, and grown under high-resource conditions in a two-year greenhouse experiment. This common garden was used to phenotype leaf ecophysiology (Mason and Donovan, 2015), as well as leaf defenses and secondary metabolite variation using high performance liquid chromatography-mass spectrometry (Mason *et al.*, 2016). Harvesting of leaves was standardized by ontogeny due to the variation present among species in growth rate and life history, with the most recently fully-expanded leaf sampled from all replicates of a given species on a single day once all replicate plants had produced at least four fully expanded leaf pairs (Mason and Donovan, 2015). Tissue was dried at 60 °C in a forced air drying oven and ground with a ball mill into a fine powder.

For the present study, approximately 50 mg dried powdered leaf tissue was extracted with 1.25 ml methanol, allowed to incubate for 48–96 h at approximately 2 °C in the dark (as recommended by Ainsworth and Gillespie, 2007), centrifuged to remove detritus, and analyzed with both the Folin-Ciocalteu and aluminum complexation assays (Singleton *et al.*, 1999; Pękal and Pyrzyńska, 2014). For the Folin-Ciocalteu assay, 0.1 ml leaf extract was diluted with 0.9 ml water, to which 0.1 ml commercial Folin-Ciocalteu reagent (MP Biomedicals, Solon, OH, USA) was added along with 0.9 ml water. After five minutes of equilibration, 1.0 ml of 7 % (w/v) Na<sub>2</sub>CO<sub>3</sub> was added, along with 0.4 ml water. After 30 min to allow for color formation (determined with preliminary analyses as an optimum length of time for color development), absorbance was recorded at 766 nm with a visible-near infrared spectrometer (Vernier, Beaverton, OR, USA). Absorbance values

were converted to caffeic acid equivalents per gram of dry leaf mass using a standard curve made with purified caffeic acid (CAS#331-39-5, Acros Organics, Geel, Belgium). For the aluminum complexation assay, 0.2 ml leaf extract were diluted with 1.8 ml water, to which 0.2 ml of 10% (w/v)  $\text{AlCl}_3$  and 1.0 ml water were added. After 10 min to allow color formation (determined with preliminary analyses), absorbance was recorded at 426 nm. Absorbance values were converted to quercetin equivalents per gram of dry leaf mass using a standard curve made with purified quercetin dihydrate (CAS#117-39-5, Acros Organics, Geel, Belgium). Both protocols were adjusted to increase the initial dilution of raw leaf extracts when high phenolic or flavonoid contents resulted in absorbance values above the maximum limits of the spectrometer.

Maximum likelihood ancestral state reconstructions of total phenolics and total flavonoids were performed using the *fastAnc* function in the R package *phytools* (Revell *et al.*, 2012) on the most recent phylogeny of the genus *Helianthus* (Stephens *et al.*, 2015). *Phoebanthus tenuifolius*, the sole diploid member of the sister genus to *Helianthus* was used as the outgroup for all analyses (Mandel *et al.*, 2014). Phylogenetic generalized least squares (PGLS) regression on species means was used to test for correlated evolution between total phenolics and total flavonoids (Martins and Hansen, 1997), implemented in the R package *ape* (Paradis *et al.*, 2004). Conventional least squares regression on species means was used to test for correlations between the results of these colorimetric assays and concentrations of 22 common non-volatile secondary metabolites in diploid wild sunflowers as previously quantified by high-performance liquid chromatography and reported in the HeliaMet database (Mason *et al.*, 2016).

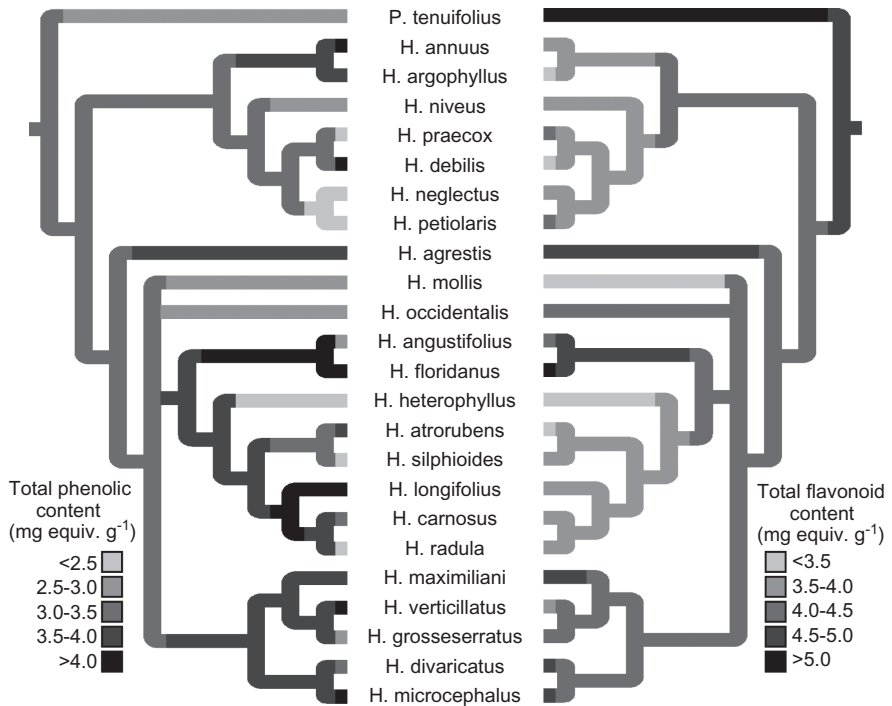
## Results

Mean concentrations of total phenolics as assessed by the Folin-Ciocalteu assay varied over five-fold among species, from as low as  $1.44 \text{ mg g}^{-1}$  of caffeic acid equivalents in the desert annual *H. neglectus* to as high as  $8.20 \text{ mg g}^{-1}$  in the rock outcrop basal rosette perennial *H. longifolius* (Table 1). Mean concentrations of total flavonoids as assessed by aluminum complexation varied much less among species, from  $3.26 \text{ mg g}^{-1}$  of quercetin equivalents in the erect woodland perennial *H. atrorubens* to  $5.66 \text{ mg g}^{-1}$  in the outgroup *P. tenuifolius* (Table 1). Among species, there was no significant macroevolutionary correlation between total phenolics and total

**Table 1:** Total phenolic content as assessed by the Folin-Ciocalteu assay and total flavonoid content as assessed by the aluminum complexation assay for the 24 species assessed in this study. Sample size ( $N$ ) reflects the number of individual plants assayed, with mean  $\pm$  standard error presented, as calculated over all individuals of each species. Total phenolic content is presented in caffeic acid equivalents, while total flavonoid content is presented in quercetin equivalents.

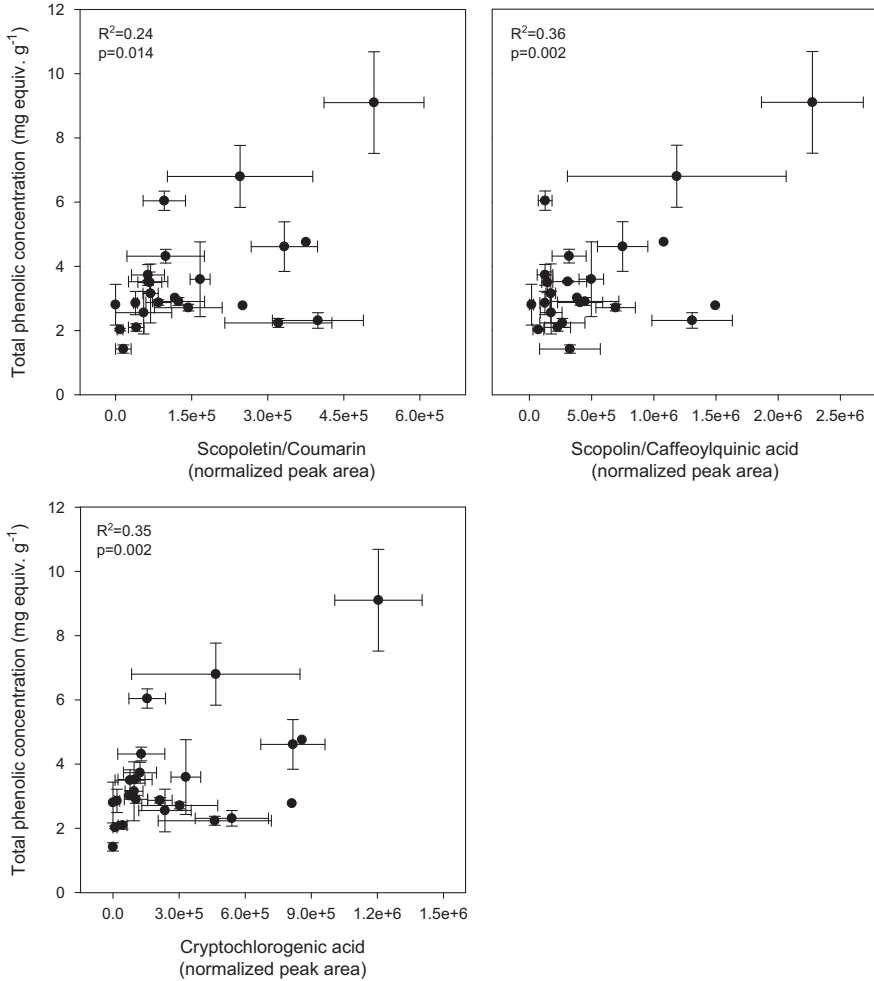
Species	$N$	Folin-Ciocalteu Total phenolic content (mg equiv. $g^{-1}$ )	Aluminum Complexation Total flavonoid content (mg equiv. $g^{-1}$ )
<i>H. agrestis</i>	16	3.61 $\pm$ 2.08	4.65 $\pm$ 0.79
<i>H. angustifolius</i>	1	2.78	4.41
<i>H. annuus</i>	42	4.30 $\pm$ 1.40	3.73 $\pm$ 0.61
<i>H. argophyllus</i>	23	3.52 $\pm$ 0.88	3.44 $\pm$ 0.54
<i>H. atrorubens</i>	15	3.64 $\pm$ 2.60	3.26 $\pm$ 0.48
<i>H. carnosus</i>	4	3.09 $\pm$ 1.31	3.93 $\pm$ 0.51
<i>H. debilis ssp. tardiflorus</i>	10	5.96 $\pm$ 1.90	3.40 $\pm$ 0.68
<i>H. divaricatus</i>	6	3.33 $\pm$ 0.89	4.53 $\pm$ 2.02
<i>H. floridanus</i>	10	6.51 $\pm$ 4.04	5.07 $\pm$ 0.62
<i>H. grosseserratus</i>	11	2.70 $\pm$ 0.87	4.18 $\pm$ 1.43
<i>H. heterophyllus</i>	12	2.34 $\pm$ 1.38	3.27 $\pm$ 0.54
<i>H. longifolius</i>	7	8.20 $\pm$ 3.32	3.75 $\pm$ 0.22
<i>H. maximiliani</i>	5	3.53 $\pm$ 0.39	4.92 $\pm$ 1.00
<i>H. microcephalus</i>	11	4.54 $\pm$ 2.56	4.81 $\pm$ 0.96
<i>H. mollis</i>	17	2.84 $\pm$ 0.48	3.39 $\pm$ 0.39
<i>H. neglectus</i>	12	1.44 $\pm$ 0.29	3.70 $\pm$ 0.49
<i>H. niveus ssp. tephrodes</i>	10	2.82 $\pm$ 0.72	3.64 $\pm$ 0.60
<i>H. occidentalis ssp. occidentalis</i>	10	2.78 $\pm$ 0.49	4.10 $\pm$ 0.84
<i>H. petiolaris ssp. petiolaris</i>	5	2.03 $\pm$ 0.74	4.13 $\pm$ 0.76
<i>H. praecox ssp. runyonii</i>	3	2.04 $\pm$ 0.59	4.48 $\pm$ 0.68
<i>H. radula</i>	28	2.30 $\pm$ 1.61	3.51 $\pm$ 0.61
<i>H. silphoides</i>	35	2.29 $\pm$ 1.20	3.71 $\pm$ 0.68
<i>H. verticillatus</i>	12	4.76 $\pm$ 1.09	3.97 $\pm$ 0.77
<i>Phoebanthus tenuifolius</i>	2	2.81 $\pm$ 1.10	5.66 $\pm$ 0.63

flavonoids (PGLS,  $p = 0.507$ ), and these compounds were found to be evolutionarily labile, with repeated back-and-forth evolution of high and low concentrations of both metrics across the tree (Figure 1).



**Figure 1:** Maximum likelihood ancestral state reconstruction of total leaf phenolic and flavonoid contents across *Helianthus*, performed using the *fastAnc* function in the R package *phytools* (Revell *et al.*, 2012). Note that these two traits are not evolutionarily correlated (phylogenetic generalized least squares regression,  $p = 0.507$ ).

When compared to the 22 most common secondary metabolites in the HeliaMet database, total phenolics as assessed by the Folin-Ciocalteu was found to be significantly positively correlated with concentrations of multiple individual phenolic compounds as quantified by high-performance liquid chromatography-mass spectrometry (Mason *et al.*, 2016). Three of the most strongly correlated compounds were identified as (1) cryptochlorogenic acid (by authentic standard), (2) either scopoletin or coumarin (by mass-to-charge ratio), and (3) either scopolin or caffeoylquinic acid (by mass-to-charge ratio), all with  $R^2$  values between 0.24 and 0.36 (Figure 2). None of the 22 most common secondary metabolites in the HeliaMet database were significantly correlated with total flavonoids as assessed by aluminum complexation, though none of these compounds were identified or putatively identified to be flavonoids.



**Figure 2:** Correlations between total leaf phenolic content as assessed by colorimetric spectrophotometry versus quantification of three prominent sunflower phenolics by high performance liquid chromatography-mass spectrometry (HeliaMet database; Mason *et al.*, 2016). The identity of one of these three compounds is known for certain, as cryptochlorogenic acid was confirmed with an authentic standard in that study. The other two compounds were putatively identified based on mass-to-charge ratios as one of two possible compounds each (scopoletin or coumarin, scopolin or caffeoylquinic acid) using the METLIN database (Smith *et al.*, 2005).

## Discussion

The positive relationship between total phenolics as assessed by the Folin-Ciocalteu assay and concentrations of three major phenolic compounds indicates that this rapid, inexpensive colorimetric assay is a good proxy for estimating total phenolic content across *Helianthus*. The moderate  $R^2$  values found with single compounds are somewhat surprising, as these compounds individually reflect only one of dozens to hundreds of phenolic compounds present in sunflower leaf tissue. Individual components of such diverse mixtures of phenolics would be expected to correlate rather poorly with the reactivity of the whole, unless such compounds make up a large proportion of each mixture, such compounds are disproportionately reactive in the assay, or the concentrations of various individual compounds are positively correlated with one another. All three explanations may be the case in sunflowers, as several of these phenolics have been shown to be disproportionately abundant in sunflowers (Weisz *et al.*, 2009), certain phenolics are known to be more reactive in the Folin-Ciocalteu assay than others (Singleton *et al.*, 1999), and shared biosynthetic pathways may result in correlated production of multiple phenolic compounds (Dixon *et al.*, 2002; Chen and Lubberstedt, 2009). Regardless of the cause, the Folin-Ciocalteu assay appears a useful estimate of total phenolics in sunflowers, especially for applications where time or cost are a major factor, including exploratory analyses or high-throughput phenotyping. However, because the Folin-Ciocalteu assay is not inherently specific to phenolic compounds (rather, many other readily oxidized compounds will also react), care should be taken when applying the assay to new groups of species or tissue types that may have a higher abundance of such interfering compounds than is typical for leaves (Ainsworth and Gillespie, 2007).

With respect to flavonoids, the utility of the aluminum complexation assay in sunflowers remains unclear. Previous work has shown that this assay is only sensitive to some classes of flavonoids, specifically flavonols and certain flavones like luteolin, but not to flavanones or flavan-3-ols (Pękal and Pyrzyńska, 2014). Additionally, as flavonoids are a subclass of phenolics, the Folin-Ciocalteu assay is known to be highly reactive to many flavonoids, including the flavonols and flavan-3-ols which have multiple free phenolic hydroxyl groups capable of reaction, but not the flavones or flavanones, which generally lack them (Singleton *et al.*, 1999). As such, the aluminum complexation and Folin-Ciocalteu assays are partially overlapping in their sensitivities and may give conflicting results requiring cautious interpretation.



The diverse flavonoids found in wild sunflowers have been long studied, originally for use in biochemical systematics, with species of *Helianthus* and *Phoebanthus* having between approximately three and fourteen flavonoids (Schilling and Mabry, 1981; Schilling, 1983; Schilling and Rieseberg, 1985; Schilling *et al.*, 1987; Spring *et al.*, 2015). These include two flavonols (kaempferol and quercetin) and a wide variety of flavones (e. g., acerosin, apigenin, gardenin, hispidulin, hymenoxin, jaceosidin, luteolin, nepetin, nevadensin, xanthomicrol). While the two flavonols found in *Helianthus* are known to be reactive in the aluminum complexation assay (Pękal and Pырzyska, 2014), some of the flavones are (e. g. luteolin) while others are not (e. g. apigenin). This indicates that the aluminum complexation assay is likely to be a good estimate of relative flavonol content, but variable in its utility as a quantification of ‘total flavonoid content’ depending on the relative abundance of reactive and non-reactive flavones present in a given species.

Overall, both the Folin-Ciocalteu and aluminum complexation assays appear to capture coarse variation in secondary metabolites among *Helianthus* species, and may be able to fill a useful niche as rapid low-cost methods for exploratory research and preliminary analyses. For instance, these assays would be useful for verifying the existence of phenotypic variation or the efficacy of treatments in manipulative experiments before embarking on more expensive and precise gas or liquid chromatography, or potentially even for high-throughput phenotyping applications in systems where variation is already well-described with such methods.

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## References

- Agrawal, A.A., Weber, M.G., 2015. On the study of plant defence and herbivory using comparative approaches: How important are secondary plant compounds. *Ecology Letters* 18: 985–991.

- Ainsworth, E.A., Gillespie, K.M., 2007. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. *Nature Protocols* 2: 875–877.
- Bohm, B.A., 1998. *Introduction to Flavonoids*, Amsterdam, The Netherlands: Harwood Academic Publishers.
- Chen, Y., Lübberstedt, T., 2010. Molecular basis of trait correlations. *Trends in Plant Science* 15: 454–461.
- Dixon, R.A., Achnine, L., Kota, P., Liu, C.-J., Reddy, M.S.S., Wang, L., 2002. The phenylpropanoid pathway and plant defence – a genomics perspective. *Molecular Plant Pathology* 3: 371–390.
- Dobson, H.E.M., Bergstrom, G., 2000. The ecology and evolution of pollen odors. *Plant Systematics and Evolution* 222: 63–87.
- Ehlenfeldt, M.K., Prior, R.L., 2001. Oxygen radical absorbance capacity (ORAC) and phenolic and anthocyanin concentrations in fruit and leaf tissues of highbush blueberry. *Journal of Agricultural and Food Chemistry* 49: 2222–2227.
- Elfalleh, W., Hannachi, H., Tlili, N., Yahia, Y., Nasri, N., Ferchichi, A., 2012. Total phenolic contents and antioxidant activities of pomegranate peel, seed, leaf and flower. *Journal of Medicinal Plants Research* 6: 4724–4730.
- Kim, D.O., Jeong, S.W., Lee, C.Y., 2003. Antioxidant capacity of phenolic phytochemicals from various cultivars of plums. *Food Chemistry* 81: 321–326.
- Lattanzio, V., Lattanzio, V.M., Cardinali, A., 2006. Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. *Phytochemistry: Advances in research* 66: 23–67.
- Lee, K.W., Kim, Y.J., Lee, H.J., Lee, C.Y., 2003. Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine. *Journal of Agricultural and Food Chemistry* 51: 7292–7295.
- Malešev, D., Kuntiđ, V., 2007. Investigation of metal-flavonoid chelates and the determination of flavonoids via metal-flavonoid complexing reactions. *Journal Serbian Chemical Society* 72: 921.
- Mandel, J.R., Dikow, R.B., Funk, V.A., Masalia, R.R., Staton, S.E., Kozik, A., Michelmore, R.W., Rieseberg, L.H., Burke, J.M., 2014. A target enrichment method for gathering phylogenetic information from hundreds of loci: An example from the Compositae. *Applications in Plant Sciences* 2: 1300085.
- Martins, E.P., Hansen, T.F., 1997. Phylogenies and the comparative method: A general approach to incorporating phylogenetic information into the analysis of interspecific data. *The American Naturalist* 149: 646–667.
- Mason, C.M., Bowsher, A.W., Crowell, B.L., Celoy, R.M., Tsai, C.J., Donovan, L.A., 2016. Macroevolution of leaf defenses and secondary metabolites across the genus *Helianthus*. *New Phytologist* 209: 1720–1733.
- Mason, C.M., Donovan, L.A., 2015. Evolution of the leaf economics spectrum in herbs: Evidence from environmental divergences in leaf physiology across *Helianthus* (Asteraceae). *Evolution* 69: 2705–2720.
- Oguro, M., Sakai, S., 2014. Difference in defense strategy in flower heads and leaves of Asteraceae: Multiple-species approach. *Oecologia* 174: 227–239.
- Paradis, E., Claude, J., Strimmer, K., 2004. APE: Analyses of phylogenetics and evolution in R language. *Bioinformatics* 20: 289–290.

- Park, Y.S., Jung, S.T., Kang, S.G., Heo, B.G., Arancibia-Avila, P., Toledo, F., Drzewiecki, J., Namiesnik, J., Gorinstein, S., 2008. Antioxidants and proteins in ethylene-treated kiwi-fruits. *Food Chemistry* 107: 640–648.
- Pękał, A., Pyrzynska, K., 2014. Evaluation of aluminium complexation reaction for flavonoid content assay. *Food Analytical Methods* 7: 1–7.
- Revell, L.J., 2012. phytools: An R package for phylogenetic comparative biology (and other things). *Methods in Ecology and Evolution* 3: 217–223.
- Riipi, M., Ossipov, V., Lempa, K., Haukioja, E., Koricheva, J., Ossipova, S., Pihlaja, K., 2002. Seasonal changes in birch leaf chemistry: Are there trade-offs between leaf growth and accumulation of phenolics? *Oecologia* 130: 380–390.
- Sanchez, D.H., Siahpoosh, M.R., Roessner, U., Udvardi, M., Kopka, J., 2008. Plant metabolomics reveals conserved and divergent metabolic responses to salinity. *Physiologia Plantarum* 132: 209–219.
- Schemske, D.W., Bradshaw, H.D., 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proceedings of the National Academy of Sciences* 96: 11910–11915.
- Schilling, E.E., 1983. Flavonoids of *Helianthus* Series *Angustifolii*. *Biochemical Systematics and Ecology* 11: 341–344.
- Schilling, E.E., Mabry, T.J., 1981. Flavonoids of *Helianthus* Series *Corona-Solis*. *Biochemical Systematics and Ecology* 9: 161–163.
- Schilling, E.E., Panero, J.L., Storbeck, T.A., 1987. Flavonoids of *Helianthus* Series *Microcephali*. *Biochemical Systematics and Ecology* 15: 671–672.
- Schilling, E.E., Rieseberg, L.H., 1985. Foliar flavonoid aglycones of *Phoebanthus*. *Biochemical Systematics and Ecology* 13: 403–404.
- Singleton, V.L., Orthofer, R., Lamuela-Raventos, R.M., 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods in Enzymology* 299: 152–178.
- Smith, C.A., O'Maille, G., Want, E.J., Qin, C., Trauger, S.A., Brandon, T.R., Custodio, D.E., Abagyan, R., Siuzdak, G., 2005. METLIN: A metabolite mass spectral database. *Therapeutic Drug Monitoring* 27:747–751.
- Spring, O., Pfannstiel, J., Klaiber, I., Conrad, J., Beifuß, U., Apel, L., Aschenbrenner, A-K., Zipper, R., 2015. The nonvolatile metabolome of sunflower linear glandular trichomes. *Phytochemistry* 119: 83–89.
- Stephens, J.D., Rogers, W.L., Mason, C.M., Donovan, L.A., Malmberg, R.L., 2015. Species tree estimation of diploid *Helianthus* (Asteraceae) using target enrichment. *American Journal of Botany* 102: 910–920.
- Sumbele, S., Fotelli, M.N., Nikolopoulos, D., Tooulakou, G., Liakoura, V., Liakopoulos, G., Bresta, P., Dotsika, E., Adams, M.A., Karabourniotis, G., 2012. Photosynthetic capacity is negatively correlated with the concentration of leaf phenolic compounds across a range of different species. *Aob Plants*. DOI: 10.1093/aobpla/pls025.
- Treutter, D., 2006. Significance of flavonoids in plant resistance: A review. *Environmental Chemistry Letters* 4: 147–157.
- Weisz, G.M., Kammerer, D.R., Carle, R., 2009. Identification and quantification of phenolic compounds from sunflower (*Helianthus annuus* L.) kernels and shells by HPLC-DAD/ESI-MSn. *Food Chemistry* 115: 758–765.