

**Protocol:** *Flavonoid and phenolic profiling of leaves and flowers of the Iochrominae collected in the field*

**Written by:** Andrea E. Berardi

**Home lab:** Smith lab, University of Colorado Boulder ([www.iochroma.info](http://www.iochroma.info))

**Host lab:** Winkel lab, Virginia Tech Department of Biology and the Virginia Tech Mass Spectrometry Incubator (<http://www.biochem.vt.edu/facilities/msi/index.html>)

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### **Rationale and background:**

The goal of the exchange was to perform metabolomic analyses of several species in the Iochrominae, an Andean clade of colorful flowering shrubs in the Solanaceae. In particular, we were interested in the flavonoids in floral and leaf tissue, and whether there were distinct patterns of flavonoid expression among floral color morphs across the phylogeny. Another goal of the research exchange was to develop a reliable flavonoid profiling technique that allowed for tissue collection with silica. Many researchers do not have access to a lyophilizer (the best method for tissue drying for subsequent metabolomics) in the field, but silica has its own mass spectral signature. We therefore incorporated silica gel into our technique and were able to control for it in our MS analyses.

### **Protocol preparation and comments:**

- All reagents and solvents should be MS grade.
- All plastics, glassware, and beads must be washed with MS-grade ethanol and dried in a hood before use, except for pipette tips and autosampler vials.
- When flavonoid extracts are in solution, they should generally be kept out of extended exposure to light and heat. Thus, keep ice and aluminum foil for covering at hand, and whenever samples are not in use, they should be kept covered on ice or in a -20C environment.

### **Procedure:**

- 1) Collect tissue and submerge in small silica beads.
- 2) Carefully scrape off as many silica gel beads as possible - try to get rid of all traces. A small paintbrush may be useful. \*
- 3) Weigh the cleaned, dry tissue on a balance, and carefully transfer to a 2 mL screw-cap polypropylene tube with 8 stainless steel beads.
- 4) Secure the tubes in racks, and shake the tubes in a paint shaker at top speed at 1-minute intervals until the tissue is a fine powder. Over-shaking may warm the tissue, which is not ideal. **Figure 1.**
- 5) Centrifuge at 14000 rpm for 5 minutes to pellet the dried tissue at the bottom of the tube. The dried tissue can get sticky in the tubes and fly out when the lid is opened.
- 6) Add 18 ul of flavonoid extraction buffer per mg of dry tissue, and vortex to mix. **Figure 2.**
- 7) Centrifuge samples at 14000 rpm for 1 minute, so that all tissue is submerged in the extraction buffer.
- 8) Sonicate the tubes in a water bath for 10 minutes, avoiding overheating the samples.

- 9) Centrifuge samples at 14000 rpm for 10 minutes to create a dense pellet of tissue and stainless steel beads. **Figure 3.**
- 10) Aliquot 100 ul (carefully avoiding the pellet) into a 1.5 mL microcentrifuge tube for LC-MS. Keep on ice, and covered. Unhydrolyzed flavonoids (anthocyanins especially) are unstable in light and heat.
- 11) Dry the samples completely under nitrogen gas. **Figure 4, Figure 5.**
- 12) Resuspend samples in 100 ul MS sample buffer.
- 13) Sonicate samples in a water bath for 10 minutes. Add ice to the sonicator if it tends to get warm.
- 14) Centrifuge at 14000 rpm for 10 minutes. This is to remove the sample of any further particulate matter. Here there may be dark particles that will not go into solution - this is largely chlorophyll, other insoluble lipids, and possibly leftover plant tissue. It is ok to let that pellet to the bottom of the tube. **Figure 6.**
- 15) Remove 50 ul of the supernatant, careful to leave the pellet undisturbed, into an autosampler vial. **Figure 7.**
- 16) Create a “master mix” for each tissue; aliquot 2 ul from each leaf sample into one “leaf master mix” vial and 2 ul from each flower sample into one “flower master mix” vial. These serve as references for total tissue compounds as well as for targeted MS2 analysis.
- 17) Remaining extracts should be dried down for future use and storage.
- 18) Standard chemicals should be run along with unknown samples as references for retention time and mass spectra. We ran 1:100 dilutions of 1 mg/ml suspensions of standard chemicals (resuspended in MS sample buffer). A list of standards used follows the protocol.

\*One sample should be prepared as above from an extraction of the silica beads used to dry the tissues, and the cleaned supernatant run on the mass spec in positive and negative mode.

### **UPLC-MS Analysis:**

#### *UPLC conditions:*

- We used a Waters Acquity I-class UPLC coupled with a Synapt-G2S-HDMS (QTOF-ESI) fitted with a BEH Acquity C18 reversed phase column (2.1 x 50 mm, 1.7  $\mu$ m) for sample analysis. The binary solvent system was A: water with 0.1% formic acid and B: acetonitrile with 0.1% formic acid.
- The autosampler was refrigerated at 8C.
- A 15 minute gradient was established with the following conditions with a sample chamber 8°C, column temperature 35°C, flow rate 0.2 ml/min, an injection volume of 1ul:
  - 0-1 min 99% A
  - 1-7 min 99-70% A
  - 7-12 min 70-5% A
  - 12:30-13 min hold at 5% A
  - 13-14:30 min 99% A

#### *Mass Spec conditions* (listed as +/- where conditions were different between polarity modes):

- We used a Synapt-G2S-HDMS (ESI-QTOF) in high-resolution, MSe mode with coupled to a Waters Acquity I-class UPLC with the following settings:

Mass scanning: 50 Da to 1800 Da  
Capillary voltage: 3.0/2.2 kV  
Cone voltage: 40  
Source offset: 80  
Source temperature: 120C  
Desolvation temperature: 350  
Desolvation gas flow: 500 L/hr  
Cone gas flow: 50 L/hr  
Nebulizer gas flow: 6 Bar  
Low energy – 4 transfer/ 2 trap  
High energy positive/negative ramp 10-45 transfer/ 2 trap  
Scan time 0.185 seconds (cycle time 0.2) for both high and low energy scans.  
Lockmass – leucine enkephalin infused at 5ul/min scanned every 20 seconds with a 0.1 scan time (0.2 cycle time).  
Data was collected in centroid mode.

- 1) Randomize samples and program a “blank” run every few samples (“blank” vials filled with MS sample buffer).
- 2) For the MS analysis, run each sample (including each master mix) in both positive [M+H] and negative [M-H] mode and in technical duplicate. (See MS analysis parameters above)
- 3) Run targeted MS2 in positive mode on the flower and leaf master mixes for particular flavonoid masses of interest to assist in compound identification. We chose positive mode because of the more reliable response of the positively charged flavylum ion in anthocyanins.

Masses [M+H] for targeted MS2 on common flavonoid aglycones:

271.06	Pelargonidin, apigenin
287.06	Cyanidin, luteolin, kaempferol
301.07	Peonidin
303.05	Delphinidin, quercetin, tricetin (rare flavone)
317.07	Petunidin
319.05	Myricetin
331.08	Malvidin

*MS2 conditions:*

Polarity: ES+  
Capillary voltage: 3.0 kV  
Cone voltage: 65  
Source offset: 80  
Source temperature: 120C  
Desolvation temperature: 350  
Desolvation gas flow: 500 L/hr  
Cone gas flow: 50 L/hr  
Nebulizer gas flow: 6 Bar  
Collision energy Ramp 10-45 transfer and 2 trap  
Scan time 0.5 seconds

Lockmass – leucine enkephalin infused at 5ul/min scanned every 20 seconds with a 0.3 scan time  
Data was collected in centroid mode.

### Data Analysis:

Note: you will have a lot of large files.

All file processing and data analysis was done in the mass spectrometry software package provided by Waters: MassLynx v4.1 SCN 851, MarkerLynx XS, and EZ-info 2.0 (Umetrics).

- 1) Import files into MarkerLynx, which aligns chromatograms and picked peaks as markers or “features”. This creates a table of data with aligned peaks for all of your samples with retention times, mass, and relative abundance. This can take a while.
- 2) Import the newly processed files using MarkerLynx and view chromatograms for compound identification.
  - a. Note: this protocol does not serve as a mass spec analysis protocol. There are many sufficient texts for the introduction to mass spec, and several good papers to reference for assistance in compound identification. Generally speaking, we compared unknown spectra to the spectra of standard chemicals, published spectra from literature searches, spectral searches, fragment and substructure searches against databases. The following websites are useful for masses and mass spectra.
    - Chemspider [www.chemspider.com](http://www.chemspider.com)
    - ReSpect for Phytochemicals [www.spectra.psc.riken.jp](http://www.spectra.psc.riken.jp)
    - MassBank [www.massbank.jp](http://www.massbank.jp)
    - KNApSAcK [www.kanaya.naist.jp/KNApSAcK](http://www.kanaya.naist.jp/KNApSAcK)
    - CAS SciFinder [www.cas.org/products/scifinder](http://www.cas.org/products/scifinder)
- 3) Use the Waters EZ-Info package to perform principal components analysis and to graph the results\*.

\*Special attention should be given to the extract of the silica beads. Our silica peaks came out at the end of the UPLC protocol and did not interfere with our compounds of interest. Therefore, we largely ignored the peaks, but were aware of potential silica adducts. Align the chromatograms of the silica sample and your true samples to determine whether the silica peaks interfere with your compounds of interest. **Figure 8.**

### Solutions:

- Ethanol, for cleaning materials
- *Flavonoid extraction buffer*: 99% methanol, 1% formic acid (v/v)
- *MS sample buffer*: 89.9 % water, 10% acetonitrile, 0.1% formic acid (v/v)
- *LC-MS Buffer A*: H<sub>2</sub>O + 0.1% formic acid (v/v)
- *LC-MS Buffer B*: Acetonitrile + 0.1% formic acid (v/v)

### Materials

- Stainless steel beads, 2.3mm diameter, BioSpec #11079123ss
- Silica beads, Flower Drying Art Silica Gel, #2610, Activa Products Inc.
- 2 mL screw-cap polypropylene vials (Gene Mate #C-3318-1, C-3326-1, BioExpress)

- 1.5 mL microcentrifuge tubes (Seal-Rite 1.5 ml microcentrifuge tube, natural #1615-5500, USA Scientific)
- Mass spec vials and pre-slit septa caps (MSCERT4000-36LVW, National Scientific)

### Machinery and Accessories

- Paint shaker, or Qiagen TissueLyser
- Water bath sonicator
- Microcentrifuge
- Nitrogen gas sample evaporator; we used a Thermo Scientific (Pierce) Reacti-Therm III without heating the blocks.
- Scale for weighing tissue samples
- Acquity C18 reversed phase column (2.1 x 50 mm, 1.7  $\mu$ M), Waters #186002350
- Tandem Waters Acquity I-class UPLC and Synapt-G2S-HDMS

### Chemical Standards

Flavonoid standard	Manufacturer	Catalog ID	CAS Number
Naringenin	Extrasynthese	1128 S	67604-48-2
Petunidin chloride	Extrasynthese	O942	1429-30-7
Cyanidin chloride	Extrasynthese	O909	528-58-5
Malvidin chloride	Extrasynthese	O913 S	643-84-5
Peonidin chloride	Extrasynthese	O906 S	134-01-0
Chrysin	Extrasynthese	1009	480-40-0
Delphinidin chloride	Extrasynthese	O904 S	528-53-0
Pelargonidin chloride	Extrasynthese	O912 S	134-04-3
Quercetin	Sigma	Q4951-100G	6151-25-3
Eriodictyol	Extrasynthese	1111 S	4049-38-1
Apigenin	Cayman Chemicals	10010275	520-36-5
Luteolin	Cayman Chemicals	10004161	491-70-3
Myricetin	Sigma	70050-25MG	529-44-2
Kaempferol	Sigma	60010-25MG	520-18-3
Isovitexin	Extrasynthese	1235 S	38953-85-4
Homoorientin (isoorientin)	Extrasynthese	1055 S	4261-42-1
Orientin	Extrasynthese	1054 S	28608-75-5
p-Coumaric acid	Sigma	C9008-5G	501-98-4
Chlorogenic acid hemihydrate	Sigma	25700-250MG-F	6001-76-9
Rutin hydrate	Sigma	R5143	207671-50-9
Cyanin chloride	Sigma	74397	2611-67-8

## Figures

Figure 1. Tissue ground to fine powder



Figure 2. After adding extraction buffer to ground tissue

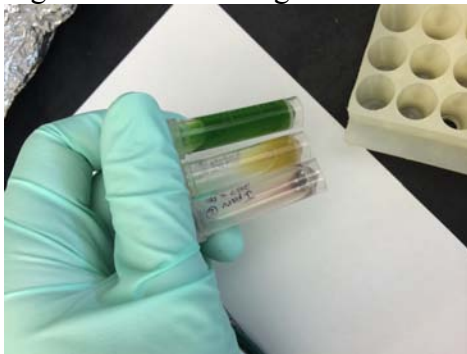


Figure 3. Tissue extracts after centrifuging, tissue and stainless steel balls pellet at bottom

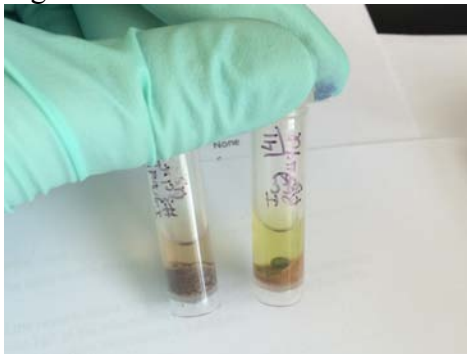


Figure 4. Supernatants of samples drying under nitrogen



Figure 5. Dried supernatants



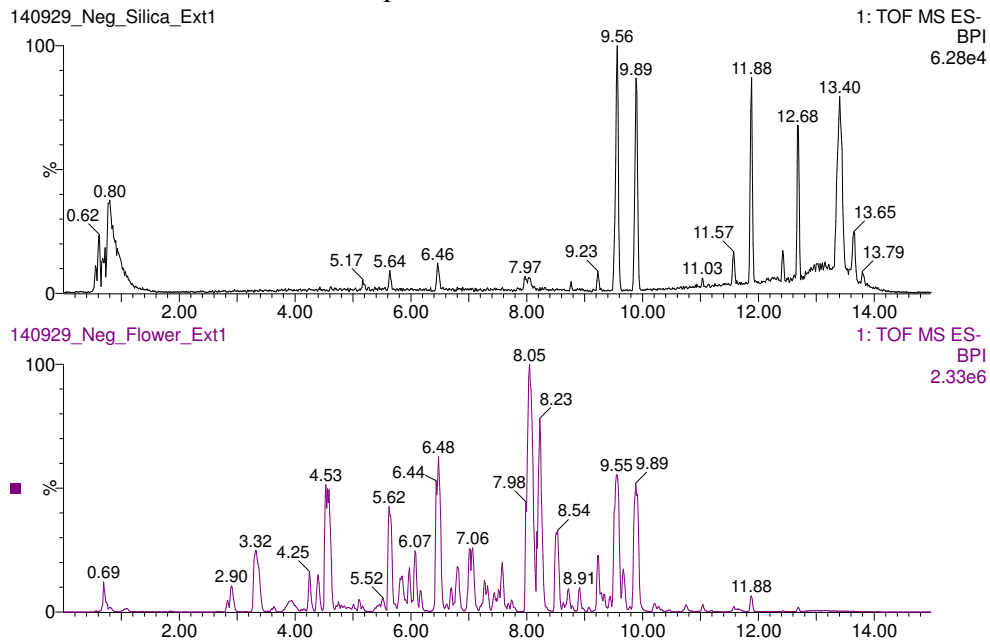
Figure 6. Example of chlorophyll and lipids that will not go back into solution after resuspension in MS extraction buffer.



Figure 7. Samples ready for LC-MS in autosampler vials



Figure 8. Here we show the MS results (negative mode) of the silica extract in the top panel and a flower extract in the bottom panel.



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