IR-4 Project

NATIONAL EDUCATION CONFERENCE February 3-5, 2026

Method Adaptations from Reference to Working Method

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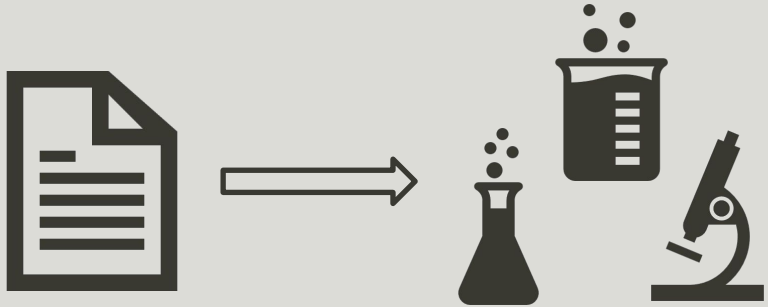


Discussion Outline

- Before Starting: Cheat Sheets and Planning
- Efficient R&D: Extraction, SPE elution profiles, gradients, etc.
- Matrix-Matched Standards: When/how to use, pros and cons
- Roundtable Discussion

Cheat Sheets and Planning

- **A condensed source of information for the analyst that includes:**
    - Project # and Protocol requirements (e.g., fort levels)
    - All analytes with structures and relevant physical-chemical properties (more on this later)
    - Reference method steps, LLMV, low calibration standard, etc.
    - Instrumentation information (if available)
- **Putting in the work up front can save a number of headaches in R&D and streamline projects**

Project Breakdown

1. What are we looking at?
2. Information gathering
3. What does the Reference Method do?
4. Build/adapt the method
5. Iterate for success

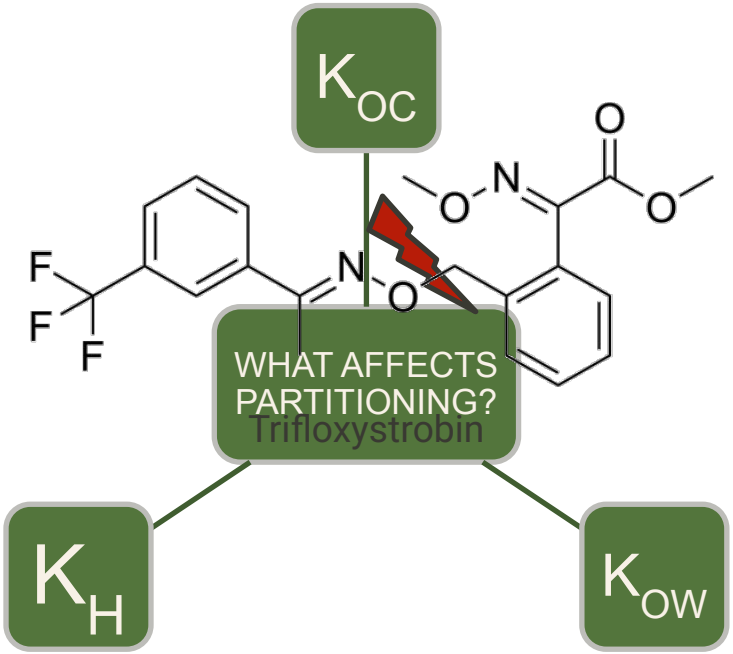
1. What are we looking at?

- Identify parent/metabolites specified in protocol
- What are the fortification levels?
- What are the crop fractions to test?

2. Information Gathering

- Use NIH/NIST/SDS/CoA to determine relevant properties
- Some information may also be given in reference methods (available on eQA) or found through a broader literature search (e.g., Google Scholar).
- But what constitutes a “useful” property for IR-4 analysts?

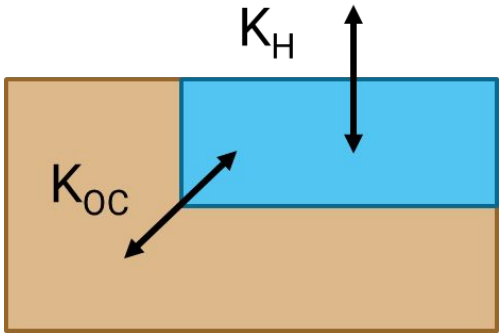
Physical-Chemical Properties



- Consider compound structure
- Functional groups
  - Electron donating/withdrawing
- Overall polarity
- Structure affects on MS fragmentation?
- What fragments are we likely to observe?

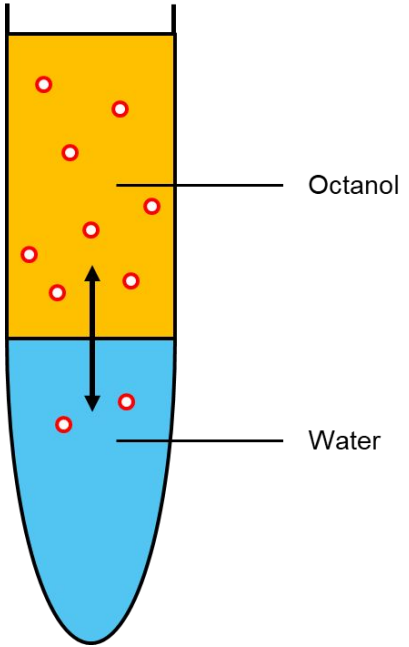
K_{OC} and K_H – Tools for Tracking Analytes

- K_{OC} – Organic carbon normalized soil-water partition coefficient for organic compounds
- K_H – The air-water partitioning coefficient
- Having access to these parameters can tell us:
  - Where to expect the majority of analyte
  - Volatility and sorption
- In the context of IR-4, these coefficients can be used to understand behavior under an evaporation step (K_H), relative hydrophilicity/expected residues (K_{OC} along with K_{OW}), and potential paths to manipulate analyte partitioning



K_{OW} – Lipophilicity vs Hydrophilicity

- Distribution of analyte between a two-phase system of water and octanol at equilibrium at a fixed temperature
  - Also denoted *P*
- Defined as the ratio of equilibrium concentration of analyte in octanol to concentration in water
  - $K_{OW} = \frac{[A]_{Octanol}}{[A]_{Water}}$
- Acts as a quick measure of baseline polarity and ease of extraction from specific matrices



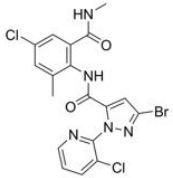
3. What does the Reference Method do?

- Take some time to list out the steps followed in the reference method:
  - Stocks/forts solvents
  - Any stability info?
  - Extraction protocol/crop and solvent amounts
  - How low did they go?
    - Low calibration standard, LLMV, etc.

4. Build/Adapt the Method

- Where does the reference method fall short of what you need?
  - Example: A Caneberry method you need to adapt for Mint
- What things can be improved right away?
  - Addition of cleanups, less crop needed, solvent:crop ratio increase?
- ***Remember: extractions cannot be reduced or substantially changed, only added to. Always consult the protocol, SD, and your LRD before making any changes!***

Example: Chlorantraniliprole/Hemp



PR 13000: Chlorantraniliprole/Hemp

Chlorantraniliprole (CAP)

MW: 483.1 g/mol

Log(Kow) = 2.76

Solubility (g/L): 0.001 (water), 1.71 (MeOH), 3.4 (acetone), 1.14 (ethyl acetate), 2.48 (dichloromethane)

LC-MS Data:

Orbitrap: ESI (+) mode, 283.92233, 450.9379, 130.0054

Orbitrap: ESI (-) mode, Precursor 479.9647, Products 201.96214, 78.91905, 144.94107

IR-4 Protocol:

Analyze for CAP only: Seed (covers hearts, meal, flour), Oil, Flower Buds, Fiber, CBD extracts

Fortification Levels = 0.01, 1.0, 10 ppm

Tentative:

2.00 g sample, soak 5 mL water 20 mins. Extract 20 mL ACN, Geno 5 min/1000RPM. Spin, decant, repeat with 20 mL ACN. Bring to 50 mL with ACN.

Loading 2.5 mL of extract is equivalent to 0.1 g crop. At 0.01ppm level, gives final concentration of 1 pg/µL in 10 mL final volume. Should have plenty of room to go lower.

Reference Method:

- 10 g sample, soak 20 mins in 20mL Milli-Q, extracted using 80 mL ACN and homogenizer (2 mins/50% speed). Spin 3000RPM/10min. Decant.
- Add 100 mL ACN to sample, two-minute blend 50% speed, spin 3000RPM/10min. Decant.
- Bring to 200 mL with ACN
- Aliquot 5.0 mL into 50 mL centrifuge tube, dilute to 20 mL with Milli-Q.
- SPE = Dual SAX (2.0g) over Oasis HLB (1.0g) conditioned with Milli-Q.
- Load entire 20mL aliquot onto cartridges, rinse centrifuge tube with 25 mL of 30% ACN in water, add after load passes frit, elute and discard. Use full vacuum to dry the cartridges.
- Remove SAX, wash Oasis HLB with 10 mL hexane, discard. Dry cartridge using vacuum 5 mins.
- Elute CAP using 40 mL ACN. Use vacuum to start flow, but turn off once flowing.
- Evaporate to dryness using rotary evaporator at 35°C.
- Reconstitute in 5 mL ACN. Vortex 30 seconds, sonicate 5 minutes.
- Adjust volume to 10 mL using Milli-Q. Filter into LC vial using 13mm PTFE filter. Extract is stable approx. 72 hours.
- Cal Curve Range: 0.15 – 5.0 pg/µL

Efficient R&D: Extraction

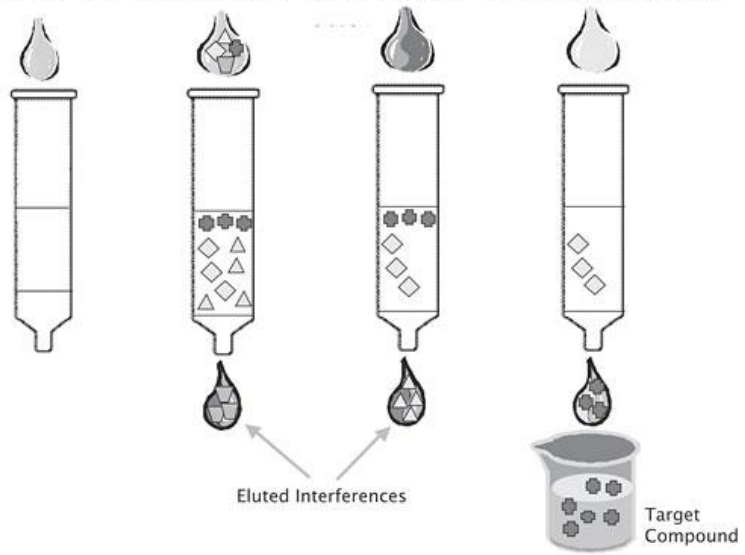
- Reference method gives us guidance on the best extraction system, but can it be fine-tuned for efficiency?
- What are the easiest things to check?
  - Addition of Sequential Extractions
  - Extraction Time
  - Extraction Volume
- How can we test each factor?

Efficient R&D: Cleanup

- What cleanup options are proposed in reference method?
- Do the matrices tested resemble ours, or are they vastly different (e.g., hops vs. blueberry)?
- Consider available cleanups and how you will use them:
  - Filter out matrix components and let analytes pass through?
  - Retain analytes, wash away matrix, and selectively elute?

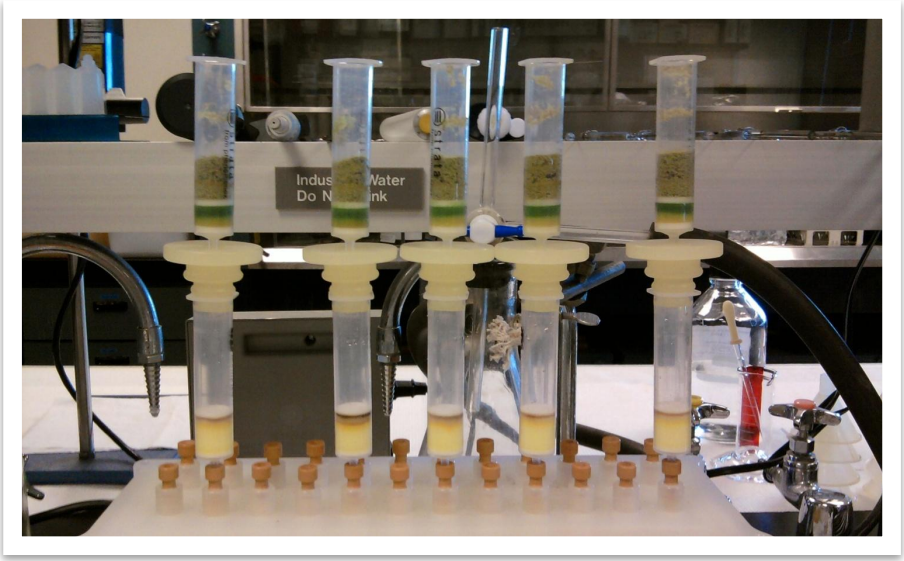
Solid Phase Extraction (SPE)

- In simplest case, used for **filtration**, we allow analytes to pass freely while retaining interfering compounds on packing
- With more complicated matrices, we prefer **retention** of analytes on packing, washing with solvent to selectively elute interferences before analytes



Considerations

- Partitioning of analyte between mobile and stationary phases
- Select stationary phase with similar properties as analyte (retention) or similar properties to matrix contaminants (filtration)
- Manipulation of mobile and stationary phases can maneuver analytes as desired

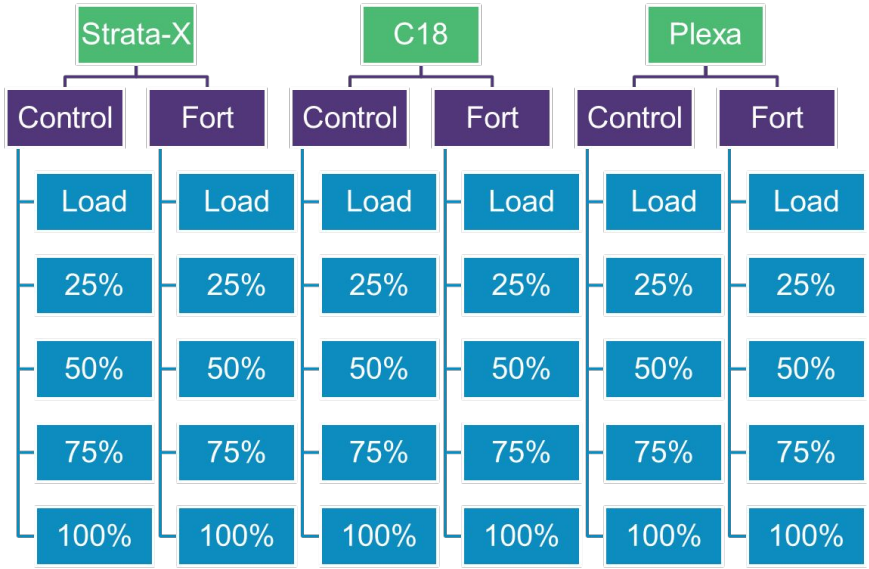


SPE Selection Chart

Your Sample Matrix is:	Aqueous (high water content)			Organic (fatty, waxy, resinous, dried material, low water content)
Recommended Retention Mechanism:	Reverse Phase	Ion Exchange		Normal Phase
Analyte Characteristics:	Moderately polar to non-polar compounds	Weak cations / anions	Strong cations / anions	Polar to moderately polar compounds
Recommended SPE Phase:	C_{18} C_8 -Ph -CN Carbon	WCX NH_2 PSA	SCX SAX	Florosil Alumina (A/B) Si NH_2 Diol CN PSA

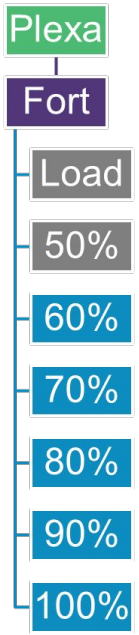
- Easy to use
- Flexible system
- Can run several samples at once
- Solvent and glassware use greatly reduced

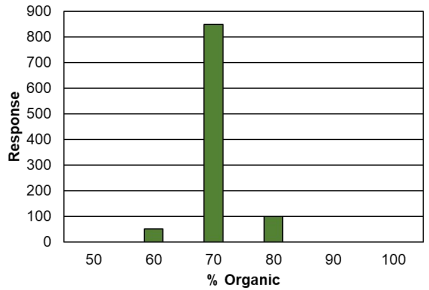
SPE: Rough Elution Profiles



- To maximize efficiency, test multiple SPE profiles simultaneously and allow instrument to run overnight
- Prepare a solvent fortification in the same solvent you will be loading, fortify high to enable dilution prior to analysis. Elute all cuts with 1 CV (column volume)
- Run Controls alongside, can show you if any interferences elute in each cut

SPE: Fine Tuning





- Findings from Previous: We see strongest analyte retention on Plexa, with no elution until 60% organic, small peak left in 80% organic
- Control looks clear of interferences
- Considerations: What does our final solvent need to be? Can we elute with less volume of a higher organic %, then dilute? Or do we need a greater volume alongside an evaporation step?
- Based on the chart above, where would you set the next layer of cuts?

SPE: Fine Tuning

- One option: choose the highest % organic at which we see the last of the analytes eluting, and take sequential cuts of smaller volumes at that percentage
- Assuming 1 CV = 6 mL, we are testing up to 2 CV total volume
- If we see that additional volume is required to elute everything, and we don't want to evaporate, we can try a smaller volume of 90% or 100% organic
- However, higher organic content may also allow additional matrix contaminants through, so care must be taken to balance convenience with extract cleanliness

Efficient R&D: Instrument Considerations

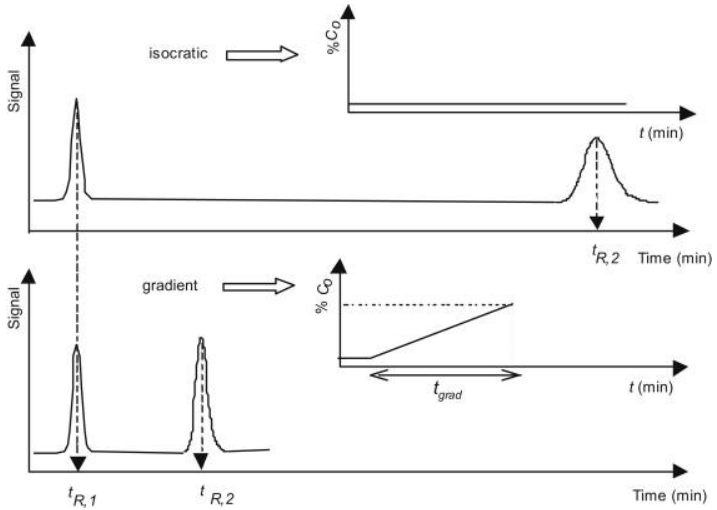
- Which instrumentation will be used: GC-MS or LC-MS?
- If using GC-MS, is solvent exchange required based on reference method?
  - NO WATER
  - Is your solvent choice the best for injection/instrument conditions?
- If using LC-MS, how complex must clean up be?
- Keep an eye on column/ion source fouling, must maintain instrument sensitivity at or below LLMV for analytes.

LC-MS/MS Gradient Development

- With well-established chemistries and more modernized methods using LC-MS/MS, chromatography changes are often focused on fine tuning rather than generation from scratch
- Necessary in cases where additional analytes are added (metabolites), or when matrix contaminants remain after cleanup that affect sensitivity/peak shape

Why Use a Gradient?

- Optimized separation of analytes
- Optimized peak shape
- Potentially shorter run times
- Higher %B can be used as a column washout to prevent late eluting peaks from fouling subsequent runs
    - This can minimize instrument downtime



Gradients and Dwell Volume

- **Need to know rate of mobile phase change:**
  - Change of % B / Gradient Time
  - Changing this rate is the primary way to dial in resolution with gradients

- **Dwell/Delay volume:**
  - The volume from the gradient formation (pump mixer) to the column
  - Calculating your precise mobile phase composition at a select time requires knowing this volume, since you're actually being shown what is at the pump, not what is on the column
  - Binary pumps have lower dwell volumes than quaternary pumps, since mixing occurs after the pumps at high pressures in binary systems

Calculating Dwell Volume

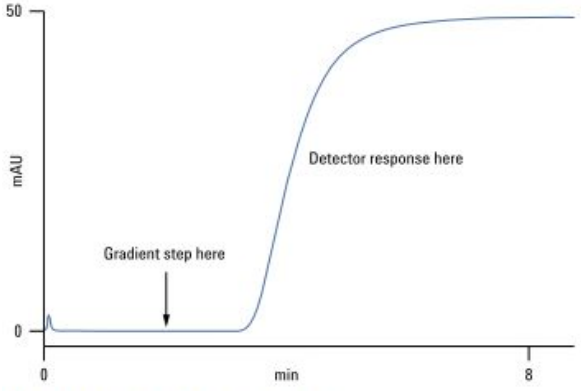


Figure 30. *Calculating dwell (delay) volumes, step one*

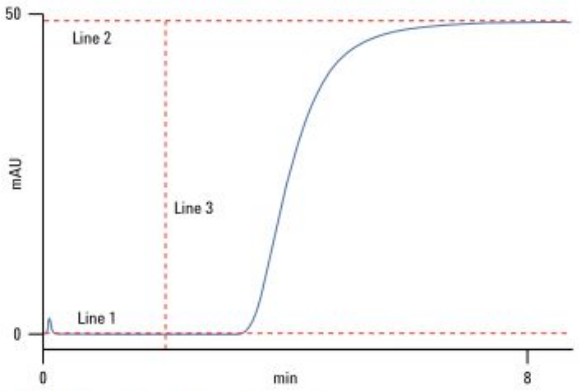


Figure 31. *Calculating dwell (delay) volumes, step two*

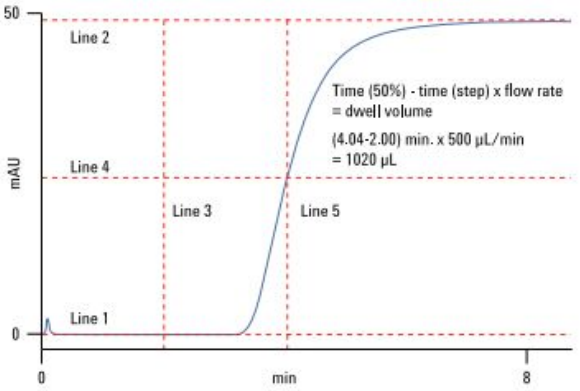


Figure 32. *Calculating dwell (delay) volumes, step three*

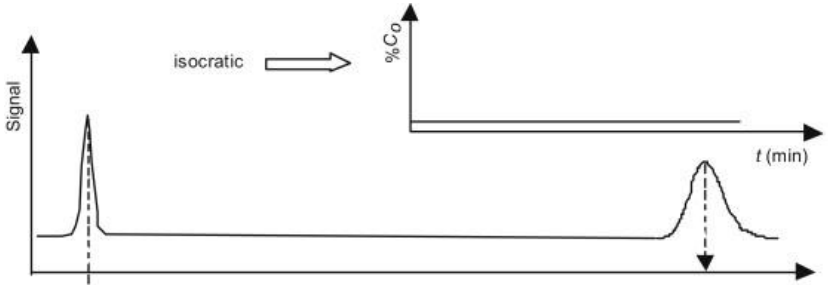
Source: Agilent Technologies, LC Handbook

Tips for Efficient Gradient Development

- Scouting gradients are a good starting point:
  - 5-95 %B with 1 minute of gradient per cm column length recommended
- Use dwell volume to get dwell time at desired flow rate, subtract that from your last eluting peak, and plug that into your gradient table to determine what your precise %B composition was at that time.
- Round up a bit, and that can be the high end of your gradient**. Adjust time to keep your %B/time rate the same to keep the same resolution. Can also play with the rate a bit to alter resolution.

Gradient Development: Discussion

- Old reference method uses isocratic run at 60%B, but you notice the second peak looks a bit ugly. How can we improve?
- What if the isocratic run had both peaks coming out on top of each other?



Matrix-Matched Standards

- A powerful tool available to help:
  - Normalize for inter-field variability
  - Correct for suppression/enhancement that cannot be remedied by cleanups
  - Increase consistency of analytical runs (ensuring all injections are of the same composition/contain the same amount of matrix)

Considerations

- First, ensure that the working method results in the cleanest possible final extract
  - Are there other cleanups available?
  - Can a smaller sample aliquot be used?
- Also confirm that matrix effects are the cause of altered recoveries:
  - Run concurrent recoveries with neat standards and MM standards
  - Differences of > 20% warrant use of MM standards
  - If significant (> 20%) differences in matrix effects exist between fields, then separate MM standards must be prepared for each field, proving this requires surveying all controls
- **Always consult LRD and SD for approval prior to official use outside of R&D**

Roundtable Discussion

- Share your tips and tricks with us!
  - What do you fall back on when developing a working method?
  - Are they crop specific? (e.g., hop cones will usually require an NH_2 cleanup)
- What do you see as the biggest challenge in managing your projects?
- Are there specific resources you would find beneficial?

Thank you!

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IR-4 Project