Analyzing Difficult Projects: Ethofumesate/Sugar Beet

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Reference Method*

- Extraction via soxhlet
- Remove ethofumesate via liquid/liquid partition
- Cleanup of extract for ethofumesate florisil SPE
  - Analyze using GC-FPD
- Combine aqueous extract and solids for further extraction of incurred residues of metabolites using acid hydrolysis
  - Liquid/Liquid Partition cleanup
  - Acetylation of NC 8493 to NC 8906
  - Sample Cleanup using florisil SPE
    - Analyze using GC/FPD

Issues:
1. GC-FPD vs. LC-MS/MS
2. Extraction and cleanup of ethofumesate
3. Separate extraction for metabolites
4. SPE cleanup
5. Additional cleanup for roots samples
6. Time consuming and labor intensive method
1. GC/FPD vs. LC-MS/MS

- Did not have a GC/FPD in the lab
- LC-MS/MS allowed for smaller sample size, which led to less solvent usage
- Eliminated acetylation step for NC 8493
- Did not have to use MPDMS

Ethofumesate (NC 8438)
2-ethoxy-2,3-dihydro-3,3-dimethyl benzofuran-5-yl methane sulphonate

NC 8493
2,3-dihydro-2-hydroxy-3,3-dimethyl benzofuran-5-yl methane sulphonate

NC 9607
2,3-dihydro-3,3-dimethyl-2-oxo-benzofuran-5-yl methane sulphonate
2. Extraction and cleanup of ethofumesate

- Extraction Via Soxhlet
- Liquid/Liquid Partition to separate ethofumesate from metabolites
- Florisil SPE Cleanup
- Analyze on GC-FPD
Large Number of Samples

- 13 field trials including a decline study
- 272 field samples (roots and tops)
Soxhlet Vs Shaker
Reference Method Using Soxhlet*: Extract overnight 25 g of sample with 200 mL of acetone (at least 8 hrs.)

Add 150 mL of water to the extract

Evaporate off all acetone

Liquid – liquid partition: Add 2.5 mL of 3 M KOH to the aqueous extract and 100 mL of hexane and shake for 1 minute (3X)

Combine the hexane layer and evaporate off all hexane at 50°C

Overnight extraction with maximum 8 samples per set

IR4 working method using shake, soak, and filter:

Weigh 5.0 g of the frozen sample into an 8 oz. jar and add 40 mL of acetone

Place on a platform shaker and shake for 4 hrs. at 200 rpm and let sit overnight at room temperature

Filter the sample and make up to 50 mL with acetone

Overnight extraction with up to 20+ samples per set with significantly less solvent used

Bonus: Peaceful sleep!


Bonus: Peaceful sleep!
**Ethofumesate Sample Cleanup**

**Reference method***:

- Dissolve residue in 3 mL of hexane
- Condition the *silica gel cartridge* with 3 column volume of hexane
- Load the extract into the silica gel cartridge and rinse the cartridge with 5 mL of 40:60 dichloromethane:hexane (v/v)
- Elute ethofumesate with 10 mL of 90:10 dichloromethane:hexane (v/v)
- Evaporate the eluant to dryness and dissolve the residues in ethyl acetate (*An internal standard was used for analysis of ethofumesate, 4-methyl-1,2-phenylenedimethane sulphonate (MPDMS))
- GC/FPD (12 minute run)

**IR4 working method**:

- Take 1 mL aliquot and dilute with 1 mL of water
- Condition a *Strata-X SPE* cartridge with 1CV of acetone followed by 1 CV of water
- Load the sample onto the cartridge. Rinse the cartridge with 10 mL of water and then with 5.0 mL of methanol: water (50:50, v/v).
- Elute with 1 CV of methanol: water (90:10, v/v)
- Evaporate the extract to approximately 2 mL at 45°C and dilute the sample to an initial volume with methanol
- LC-MS/MS (9 minute run)

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* *Analytical Method for the determination of Ethofumesate and major metabolites in grass and sugar beets (Improved Method)*, J. D. Manley, M. D. Reeve, and P. J. Snowdon, Dated 2nd April, 1986.
3. Separate extraction for metabolites

Reference method*:

 Extraction via soxhlet
  ↓
 Liquid/Liquid Partition
  ↓
 Acid Hydrolysis
  ↓
 Liquid/Liquid Partition Cleanup
  ↓
 Acetylation of NC8493
  ↓
 Florisil SPE Cleanup
  ↓
 Analyze on GC-FPD

To extract conjugated metabolites NC 8493 and NC 9607

*“At-Harvest Ethofumesate derived residues in or on sugar beet roots and tops following sequential application of Nortron SC and Betamix at the highest recommended pre-emergence plus post-emergence rate combination, USA 1993”, AgrEvo Study Number B-93R-03 dated April 25, 1995.
Hydrolysis of NC 8493 and NC 9607:

**Reference method**:  
- 150 mL of aqueous extract → Remove the acetone from extract by evaporating
- Add 2.5 mL of 3 M KOH to the aqueous extract and 100 mL of hexane and shake for 1 minute (3X)
- Not included in the reference method
- Add 150 mL of HCL → Place in a water bath at 80°C for 2.5 hrs.
- Make up to 400 mL → Filter sample, add make up to a known volume with water

**IR4 working method**:  
- Add 30 mL of water → Add 0.4 ml of 3 M KOH to the aqueous extract and 20 mL of hexane and shake for 1 minute (2X)
- Add the solids to the aqueous layer and blend for 2 minutes at 11,000 rpm
- Add 30 mL of HCL → Make up to 100 mL

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**Main difference between methods** was amount of solvents used, proportional to the smaller sample size.

**At-Harvest Ethofumesate derived residues in or on sugar beet roots and tops following sequential application of Nortron SC and Betamix at the highest recommended pre-emergence plus post-emergence rate combination, USA 1993**, AgrEvo Study Number B-93R-03 dated April 25, 1995.
Hydrolysis of NC 8493 and NC 9607:

- Removal of ethofumesate prior to acid hydrolysis was very important because it converted to NC 8493 during acid hydrolysis.

- Unexpected challenge was the loss of NC 9607 during hydrolysis step.
  - Ran an experiment with samples at 30 minute intervals.
  - Recoveries for NC 9607 after 60 minutes in acid were ~70%. After 90 minutes recoveries were 40-60%.
  - For incurred residues, the samples needed a minimum of 2.5 hours.
Florisil SPE cartridges did not work. Tried different sizes, phases and combinations of phases including alumina, silica, C18, Strata-X, Cucarb, NH2.
In the end we had to pack our own florisil columns. Florisil was activated in a ~100°C oven overnight.
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5. Additional Cleanup for Roots Samples

After acid hydrolysis, the roots extracts were a dark amber color. This added additional cleanup challenges for NC 9607.

NO PROBLEM!

BIG PROBLEM! (for NC 9607 only)
We added a silica SPE cleanup after florisil.

From our previous R&D, we knew that silica SPE would not work for cleanup of both metabolites, so we had to split the sample.
6. Time consuming and labor intensive method

- Overnight extraction for one compound
- Separate extraction for metabolites
- Separate analytical runs for parent and metabolites. For roots samples, we injected samples for each compound separately
- Glassware intensive
Working together, we were able to

- Run 2 sets per week, with ~20 samples per set.
- One analyst completed parent analysis while the other moved on to metabolite extraction.

The whole project including R&D, analysis, and ASR took almost a year.
Fabiola’s next project:

Nikolas Trong Zuno-Nguyen
Thank You!