

HS-SPME-GC-MS-based untargeted metabolomics for kinetics tracking of natural herbicides' volatile residues: can we “footprint” the “Volatilome”??

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Perpignan, France

Summary

- Introduction: *Environmental Metabolic Footprinting*
- Context of the experiment
- Method development and application
- Results and discussion
- Conclusions and perspectives

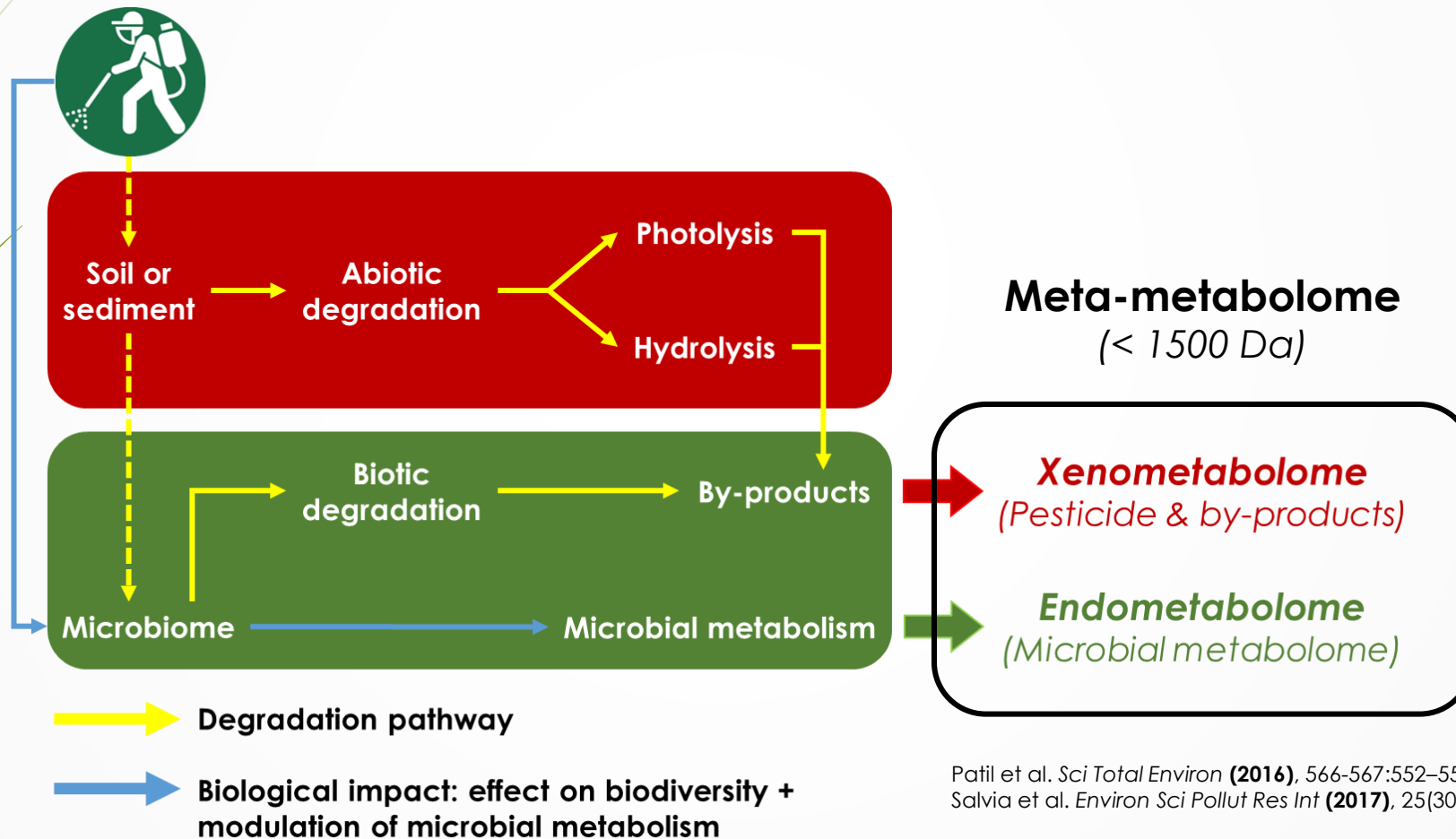
Introduction

- ▶ **Biopesticides** (*Biocontrol Agents*) are **emerging** in order to replace **chemical pesticides**
- ▶ Usually, Biocontrol Agents have **complex Chemical/Biochemical composition**
- ▶ **Classical protocols** are **non-adaptable** with this complexity
 - ✓ Development of **new approaches** is needed
 - ✓ Evaluating the environmental **fate** and **impact** is highly recommended with the emerging ecological problems



Introduction

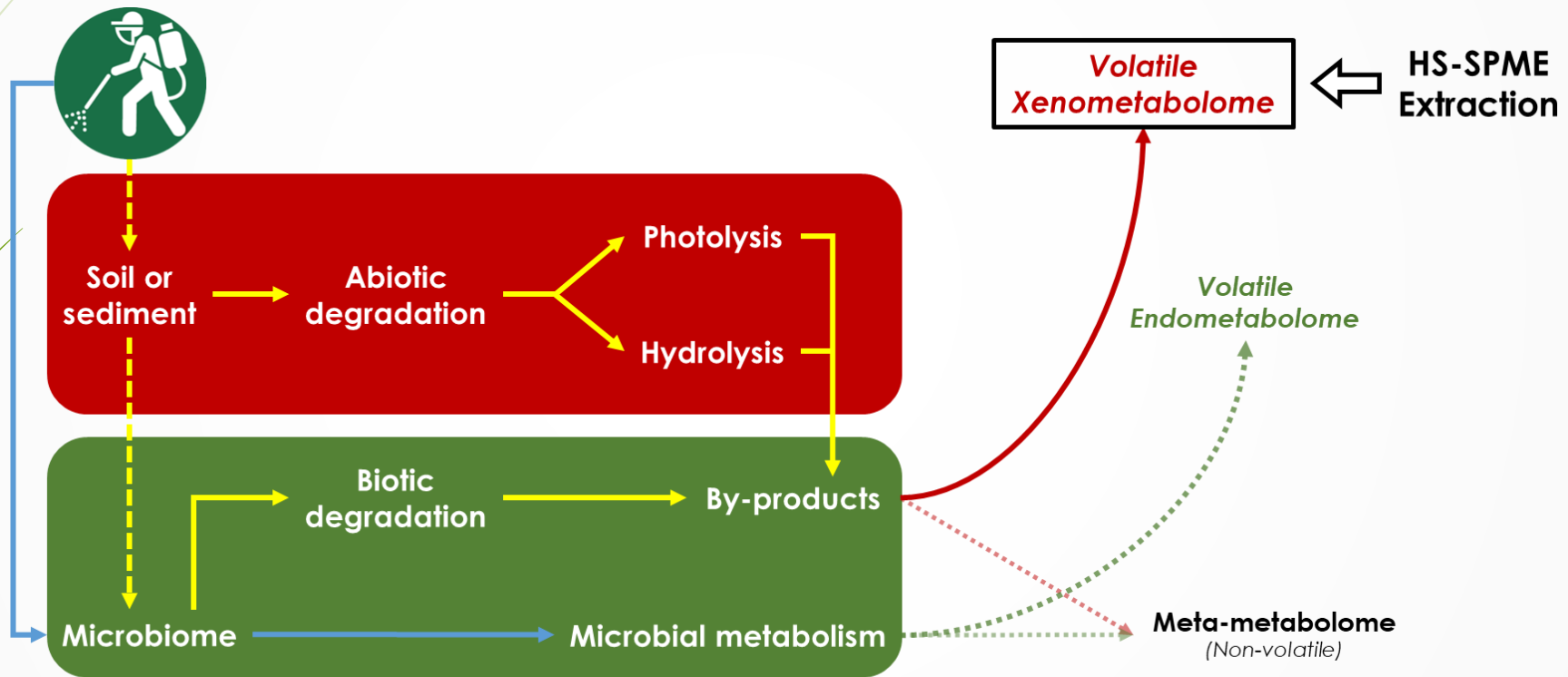
Environmental Metabolic Footprinting (EMF)



Patil et al. *Sci Total Environ* (2016), 566-567:552–558
 Salvia et al. *Environ Sci Pollut Res Int* (2017), 25(30):29841–29847

Introduction

Environmental Metabolic Footprinting (EMF)



→ Degradation pathway

→ Biological impact: effect on biodiversity + modulation of microbial metabolism

Ghossou et al. 12JSRFMF (2019). Clermont-Ferrand, France. (Poster)
Ghossou et al. 11JSRFMF (2018). Liège, Belgium. (Poster)

Introduction

Environmental Metabolic Footprinting (EMF)

Meta-metabolome

Extraction
SLE

LC-MS

Statistical
Analysis

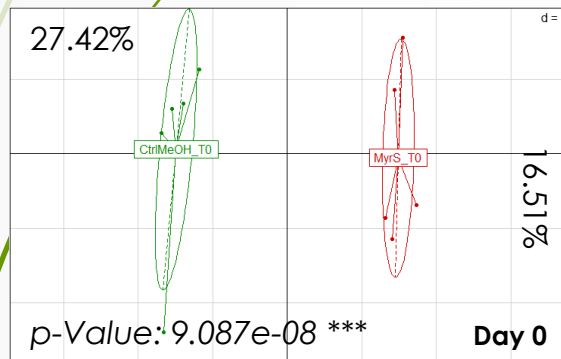
Meta-volatilome

Extraction
HS-SPME, DHS

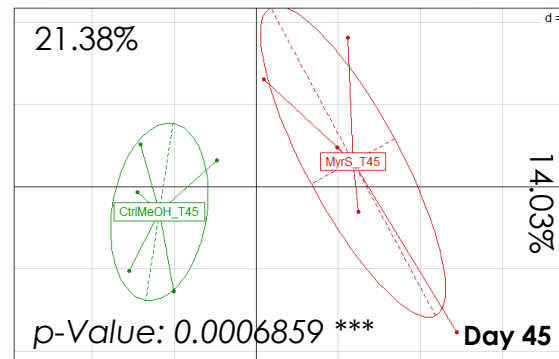
GC-MS

Statistical
Analysis

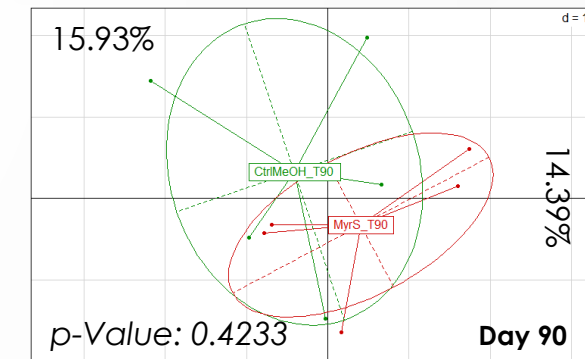
PCA / MANOVA



PCA / MANOVA



PCA / MANOVA



Patil et al. *Sci Total Environ* (2016), 566-567:552-558

Ghossou et al. *Biocontrol* 2018 (2018). Perpignan, France. (Poster)

Comparison of Meta-metabolomes of
Control and **Spiked** Samples

Introduction

Environmental Metabolic Footprinting (EMF)

Meta-metabolome

Extraction
SLE

LC-MS

Statistical
Analysis

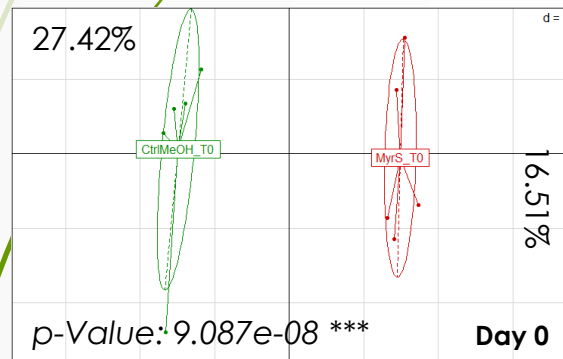
Meta-volatilome

Extraction
HS-SPME, DHS

GC-MS

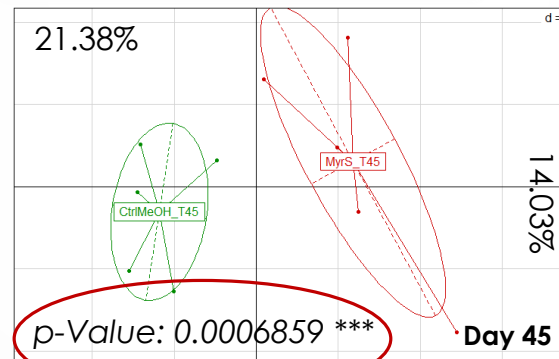
Statistical
Analysis

PCA / MANOVA

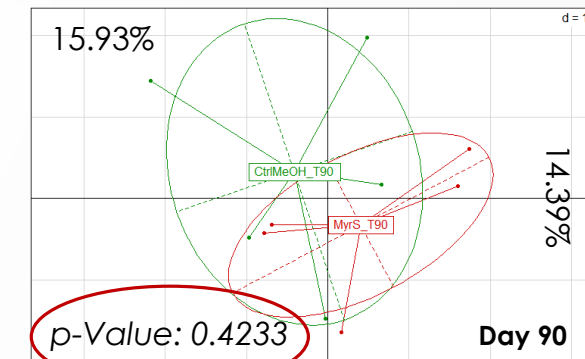


Patil et al. *Sci Total Environ* (2016), 566-567:552-558
Ghossou et al. *Biocontrol* 2018 (2018). Perpignan, France. (Poster)

PCA / MANOVA



PCA / MANOVA



“Resilience Time”

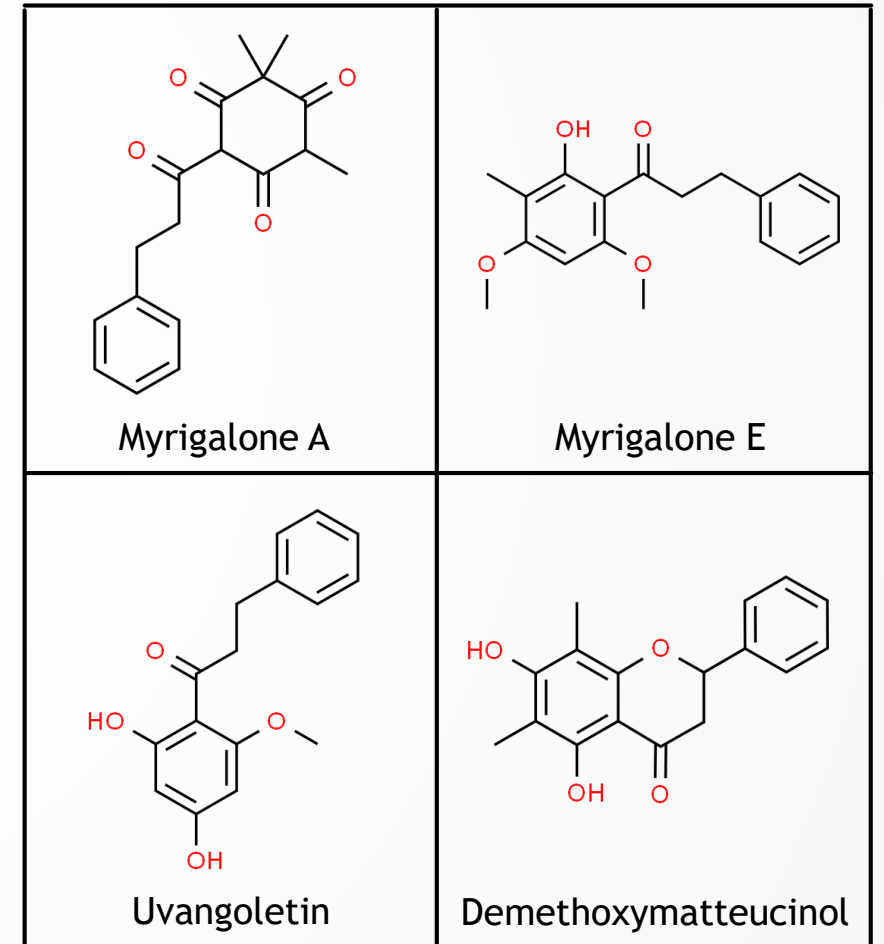
Context of the experiment

Studying **Triketone herbicides*** applied on **soil**

- Studied natural herbicide: **Myrica gale extract**
- Active compound: **Myrigalone A**
- **Allelochemical/ β -Triketone** herbicide
- *Myrica gale* extract: **Complex mixture** of molecules
- Several **unknown** compounds



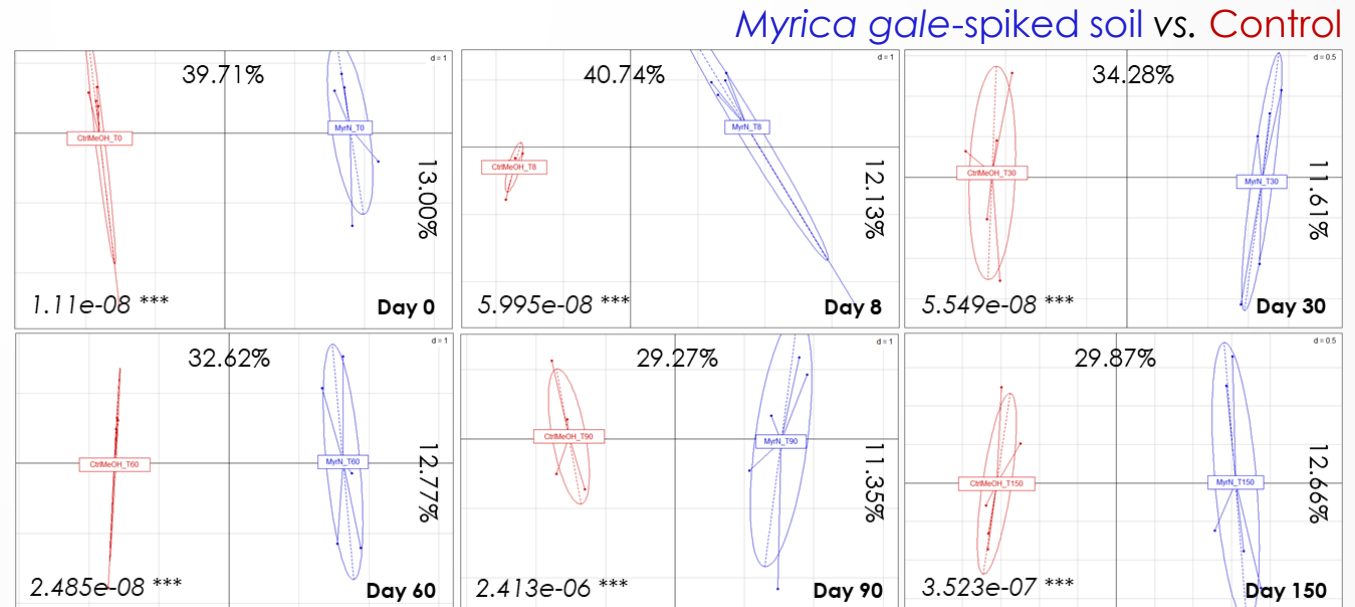
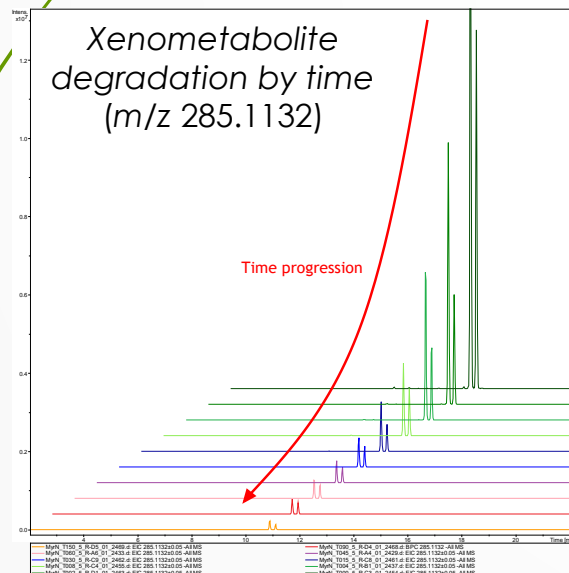
*: ANR TRICETOX project (finished)



Popovici et al. *Appl Environ Microbiol* (2010), 76(8):2451–2460

Context of the experiment

- A previous **kinetic study** was performed on **soil meta-metabolome** (LC-HRMS)
- The **pure Myrigalone A** and the **Myrica gale extract** were studied
- The **kinetics tracking** was applied for **150 days** with **10 time points**
- **Resilience** was **not detected** after **150 days** for the **Myrica gale-spiked soil**



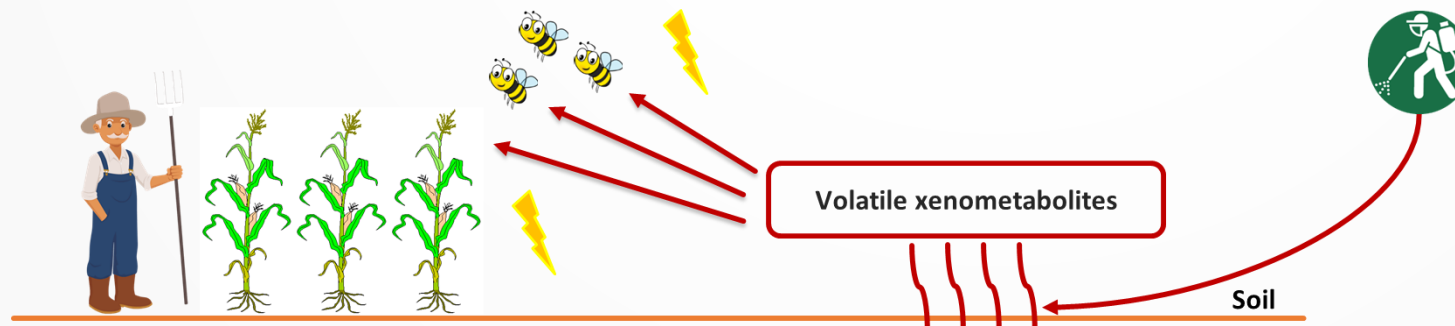
Context of the experiment

Can we “footprint” the “Volatilome”??

► What is the added value?

- ✓ Acquiring **complementary information** about **degradation pathways** of the pesticide in soil
- ✓ Following the **OECD Guidelines** for the Testing of Chemicals
- ✓ Estimating the **exposure** of **farmers, insects** and **plants** to pesticides and their degradation by-products

OECD (2002), Test No. 307: *Aerobic and Anaerobic Transformation in Soil*, OECD Guidelines for the Testing of Chemicals, Section 3, OECD Publishing, Paris
OECD (2002), Test No. 308: *Aerobic and Anaerobic Transformation in Aquatic Sediment Systems*, OECD Guidelines for the Testing of Chemicals, Section 3, OECD Publishing, Paris



Context of the experiment

Can we “footprint” the “Volatilome”??

► Aims:

- ✓ Set-up of a **simple** and **cheap** method
- ✓ Detection of **soil endogenous VOCs**
- ✓ Detection of **volatile xenometabolites** (pesticide + degradation by-products)

► Challenges:

- ✓ Establishing **sample design**
- ✓ Optimizing **HS-SPME extraction**
- ✓ Setting-up **GC-MS method**
- ✓ Developing **data processing workflow**

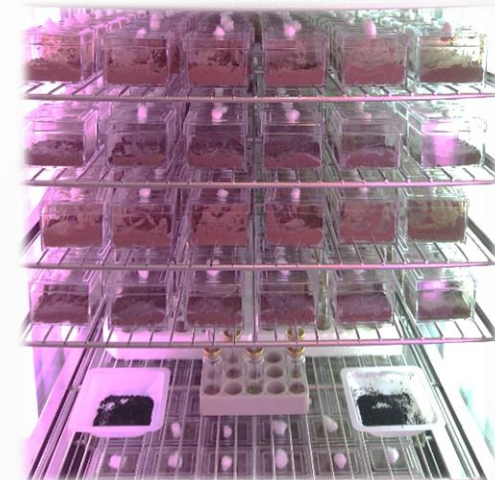


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Method development and application

Sample design: a living system

- ▶ **6 g of soil** (20% of humidity) in a **20 mL vial tube**
- ▶ Crimped caps with **2 needles** drilled in the septum to assure **aerobic conditions**
- ▶ Incubation in **climatic chamber** for at least 24 h in order to stabilize the biological system before spiking:
 - ✓ **24h day/night cycle: 28°C/18°C, 40%/65% RH, light/dark**
- ▶ Only 6 samples were prepared:
 - ✓ 3 replicates of **Untreated Control** “**UnTr**”
 - ✓ 3 replicates of **Pesticide-Spiked** “**MyrN**”
- ▶ The **used bio-pesticide**: the **Extract of *Myrica gale*** containing the **Myrigalone A** (10x field dose: 300 µg/g)



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Method development and application

HS-SPME: a simple non-destructive extraction

- ▶ Extraction conditions:
 - ✓ Fiber type: **DVB/CAR/PDMS**
 - ✓ Incubation time: **5 min**
 - ✓ Extraction time: **30 min**
 - ✓ Extraction temperature: **40 °C**
- ▶ Major advantages:
 - ✓ **Non-destructive** extraction
 - ✓ **Green** (no solvents or reagents needed)
 - ✓ **Automated** and **non-laborious**



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Method development and application

GC-MS System

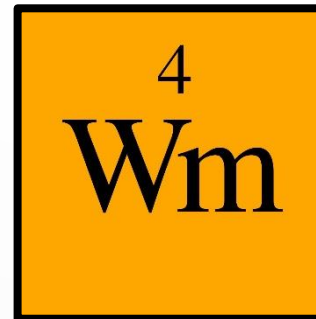
- Gas Chromatography:
 - ✓ System: **Focus GC** (Thermo)
 - ✓ Column: **DB-5MS** (30 m, 0.25 mm, 0.25 μ m)
 - ✓ Gradient: **40 °C to 230 °C**
 - ✓ Run time: **36 min/run**
- Mass Spectrometry:
 - ✓ System: **DSQ II Electron Impact Ionization-Single Quadrupole MS** (Thermo)
 - ✓ Full Scan range: **m/z 40-400**
 - ✓ Scan rate: **5 scans/sec**



Method development and application

Automated data processing

- Data processing:
 - ✓ Thermo Fisher **Xcalibur 3.0.63** (manual processing)
 - ✓ Bruker **Compass DataAnalysis 4.3** (manual processing)
 - ✓ **Workflow4Metabolomics** platform (automated processing)
 - ✓ **MetaMS** tool for GC-MS data processing (Integrated on W4M)
- Statistical Analysis:
 - ✓ **MetaboAnalyst** platform for several interesting statistical models and tests



Chong et al. *Nucleic Acids Res* (2018), 46:486–494
Giacomini et al. *Bioinformatics* (2015), 31 (9):1493–1495
Guitton et al. *Int J Biochem Cell Biol* (2017), 93:89–101
Wehrens et al. *J Chromatogr B* (2014), 966:109–116

Method development and application

Application: 38 days kinetic tracking

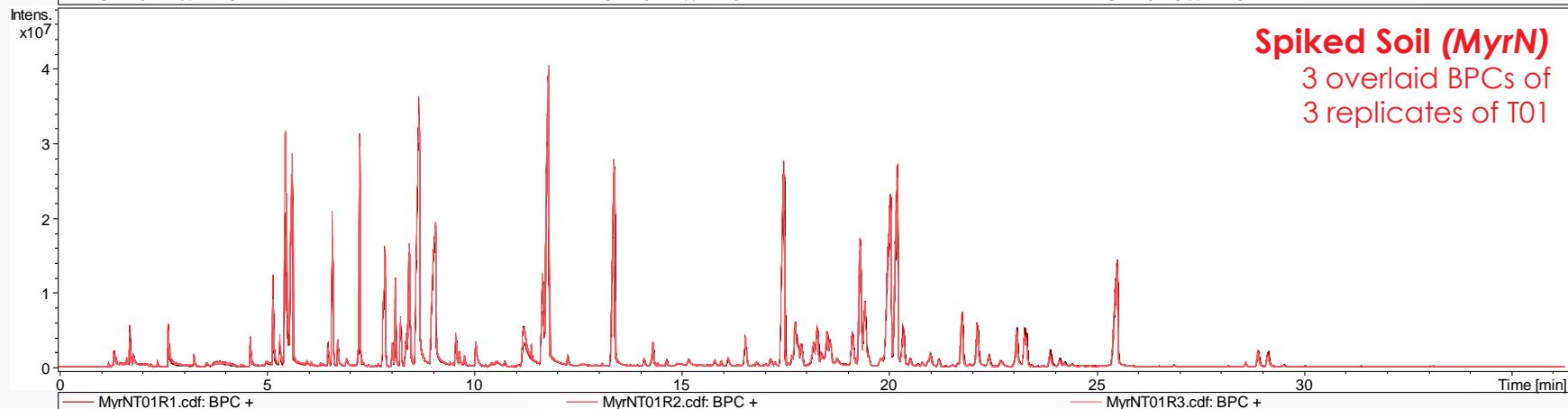
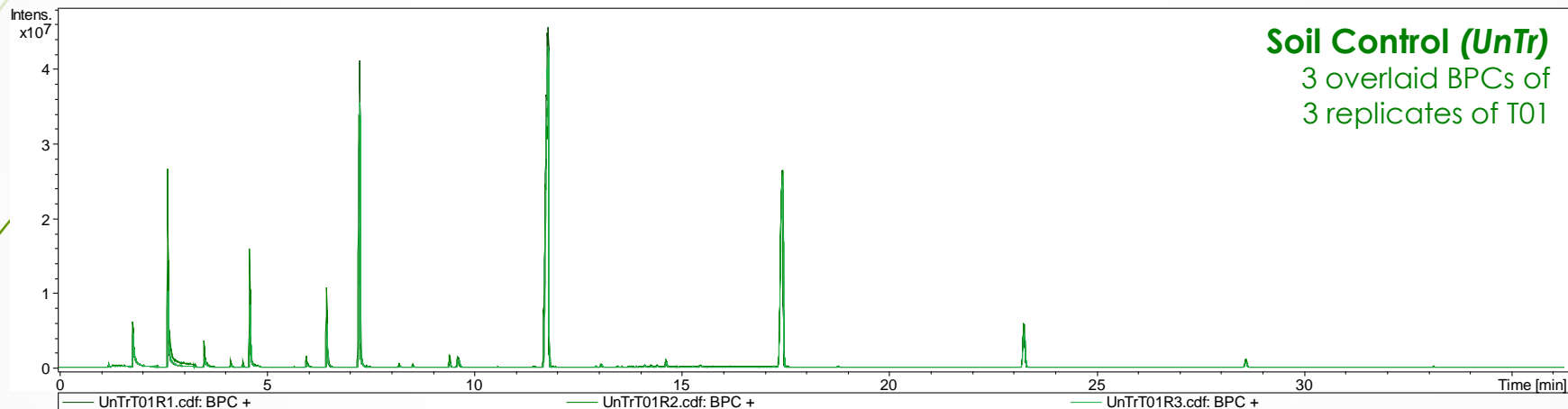
As a non-destructive extraction:

- The **kinetics tracking** is applicable on the **same sample**
- **8 extractions** were performed on the **same 6 samples**
- **8 kinetics time points** (days) were provided:
 - ✓ **Days 01, 02, 03, 04, 08, 17, 24, 38 after spiking**
(T01, T02, T03, T04, T08, T17, T24, T38)

Results and discussion

Xenometabolites detection

- Rich **volatile xenometabolome** profile was detected
- **Soil endogenous VOCs** were not detected → **Low matrix background**



Results and discussion

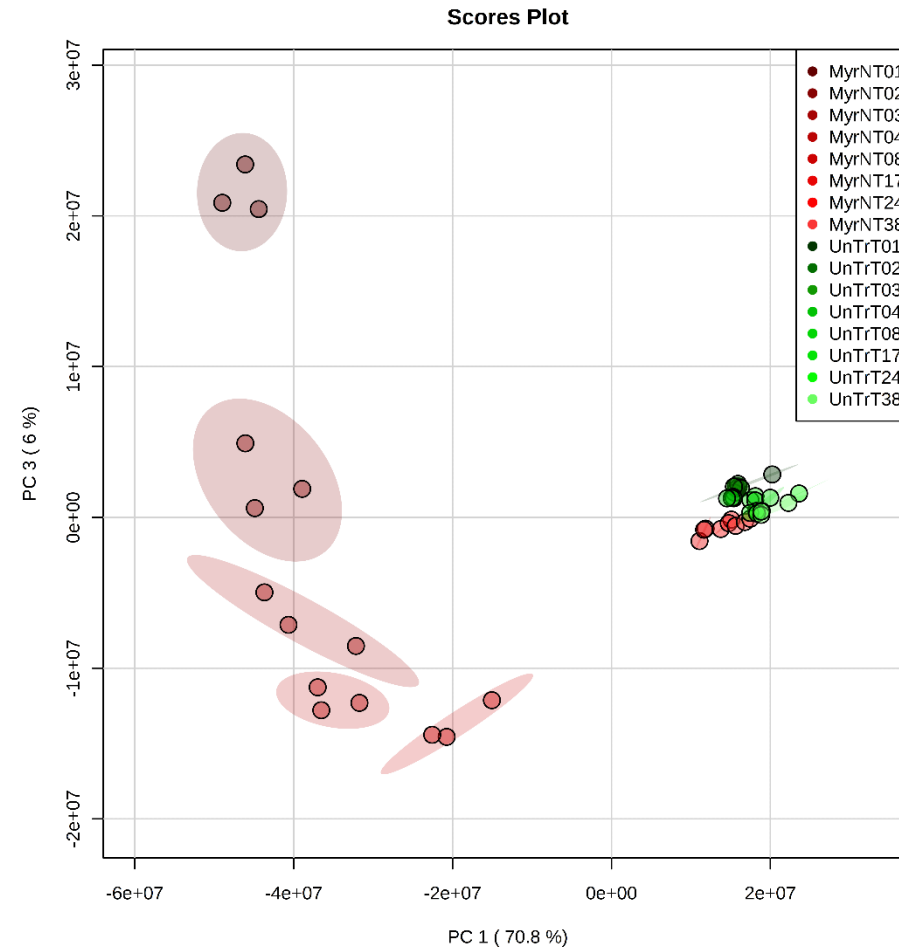
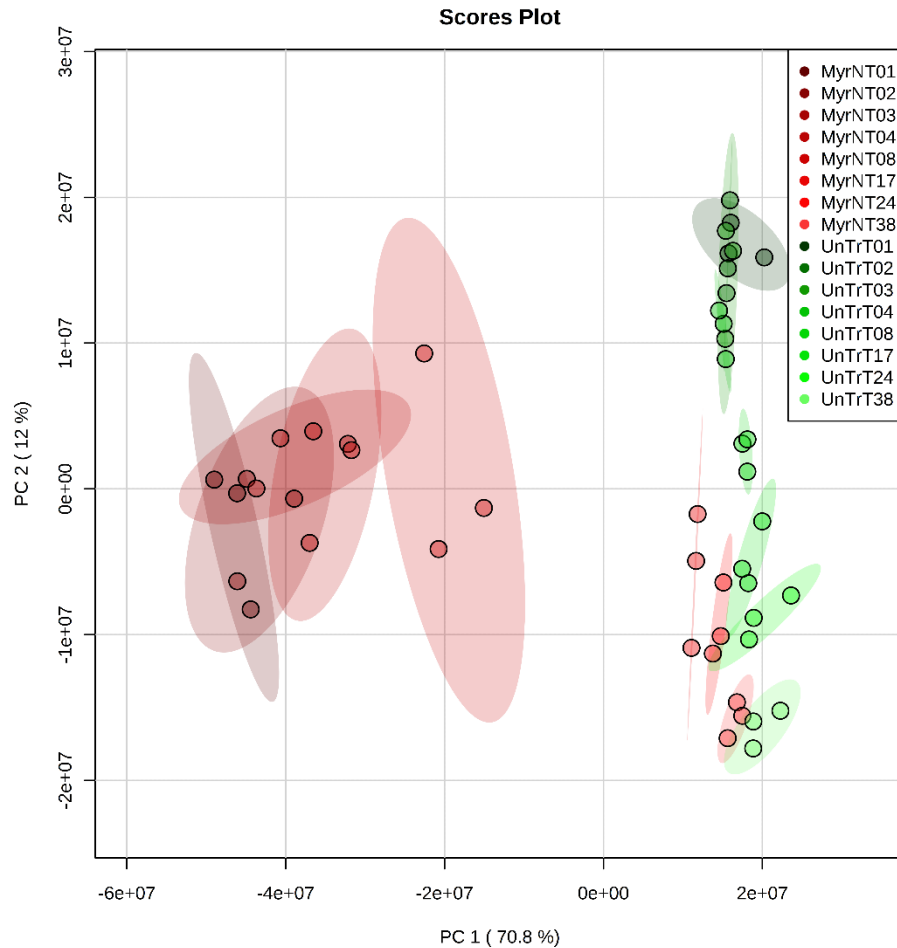
Method reproducibility

Compound peak	Inter-Sample (n=3) Area RSD (%)	Inter-Sample (n=3) RT SD (sec)	Inter-days (n=8) RT SD (sec)
RT05.67	4.37	0.35	1.73
RT11.83	5.78	0.00	N/A
RT20.07	4.67	0.35	N/A
RT25.53	3.53	0.35	3.53

- Method withdraw:
 - **Internal standards** or **QCs** can not be used or applied
- Proposed solution:
 - For **each kinetic point** (day), spike a soil on **the same day**, using the pesticide + IS, and **inject it in the sequence** as a “system QC”

Results and discussion

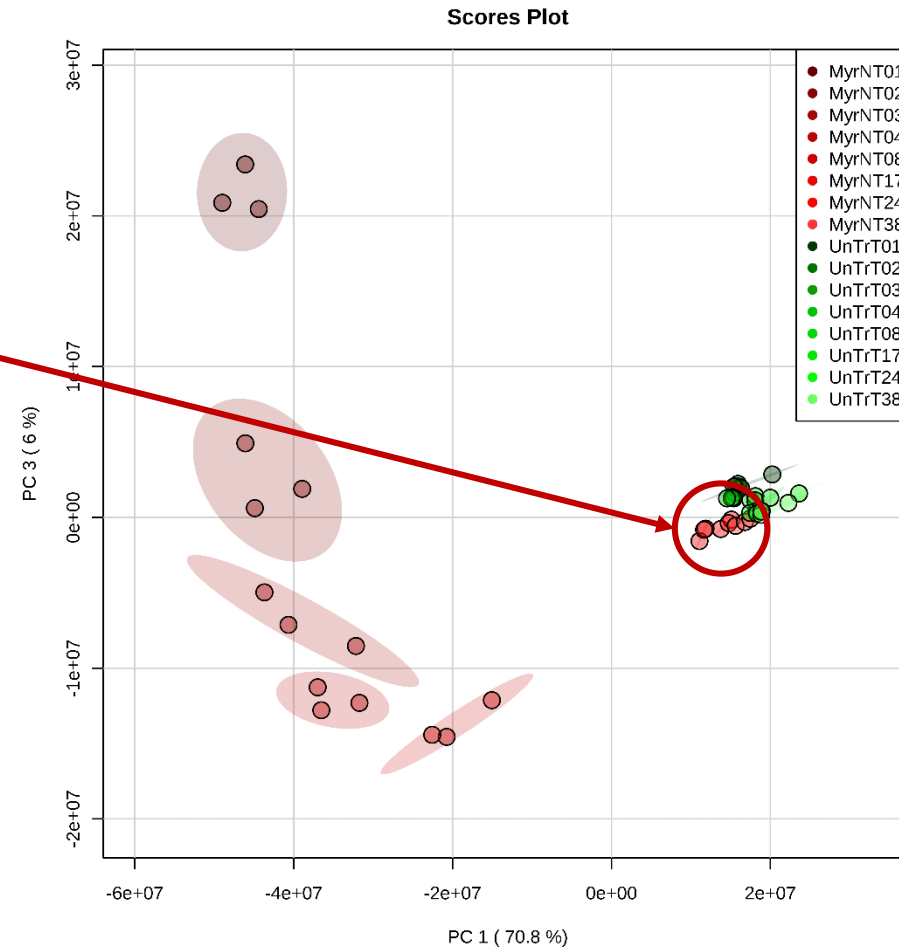
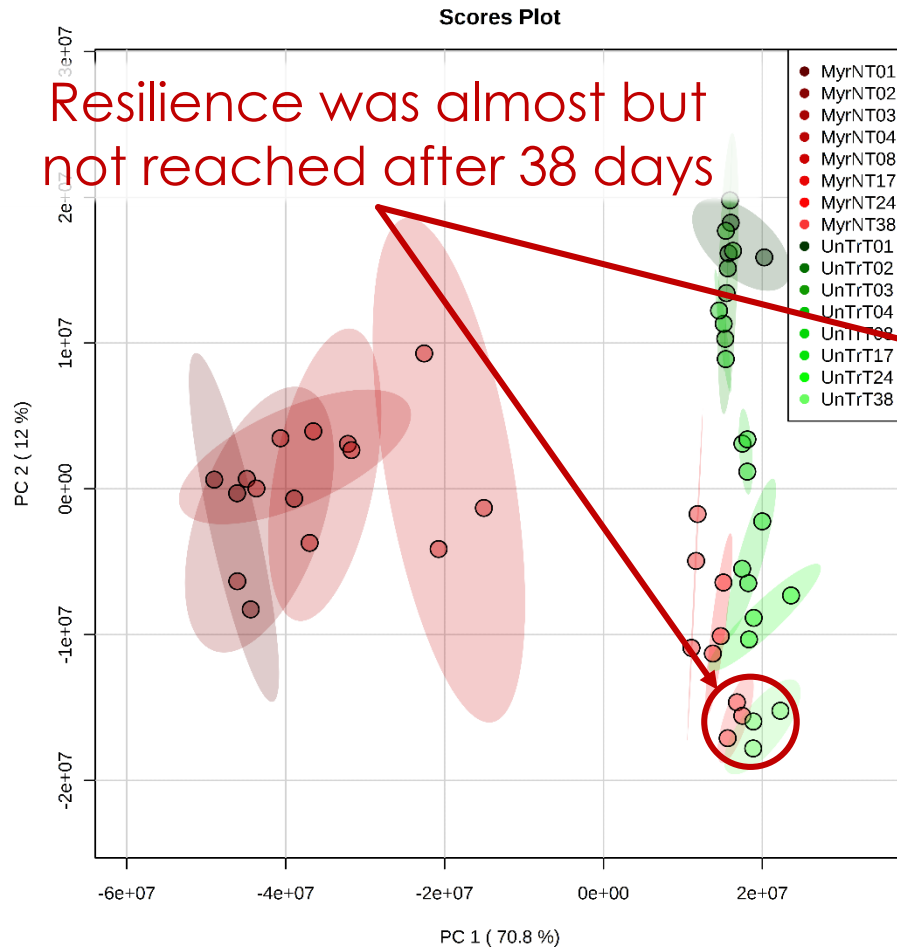
Principal Component Analysis



- MyrNT01
- MyrNT02
- MyrNT03
- MyrNT04
- MyrNT08
- MyrNT17
- MyrNT24
- MyrNT38
- UnTrT01
- UnTrT02
- UnTrT03
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- UnTrT24
- UnTrT38

Results and discussion

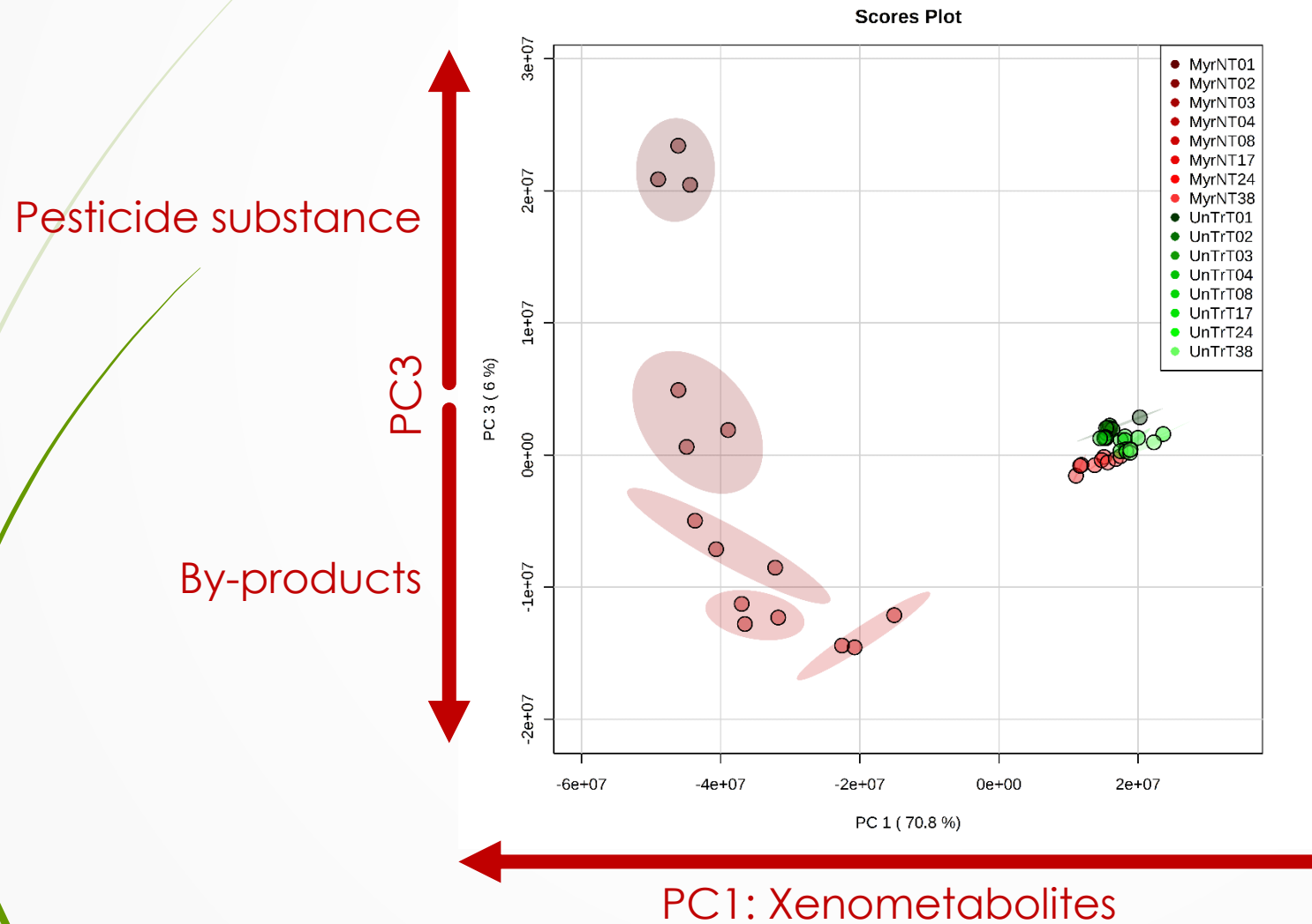
Principal Component Analysis



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Results and discussion

Principal Component Analysis



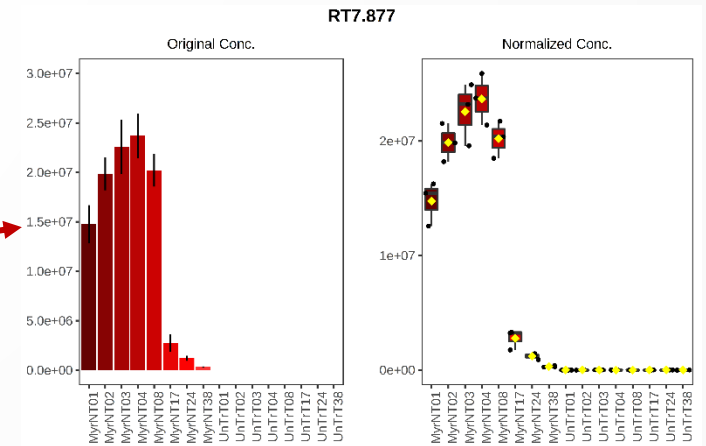
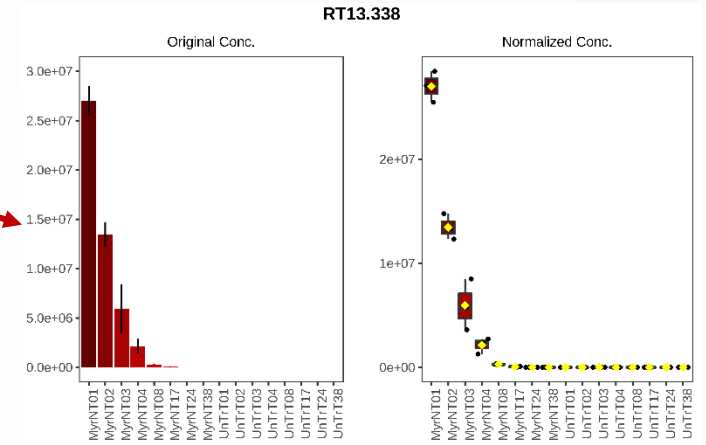
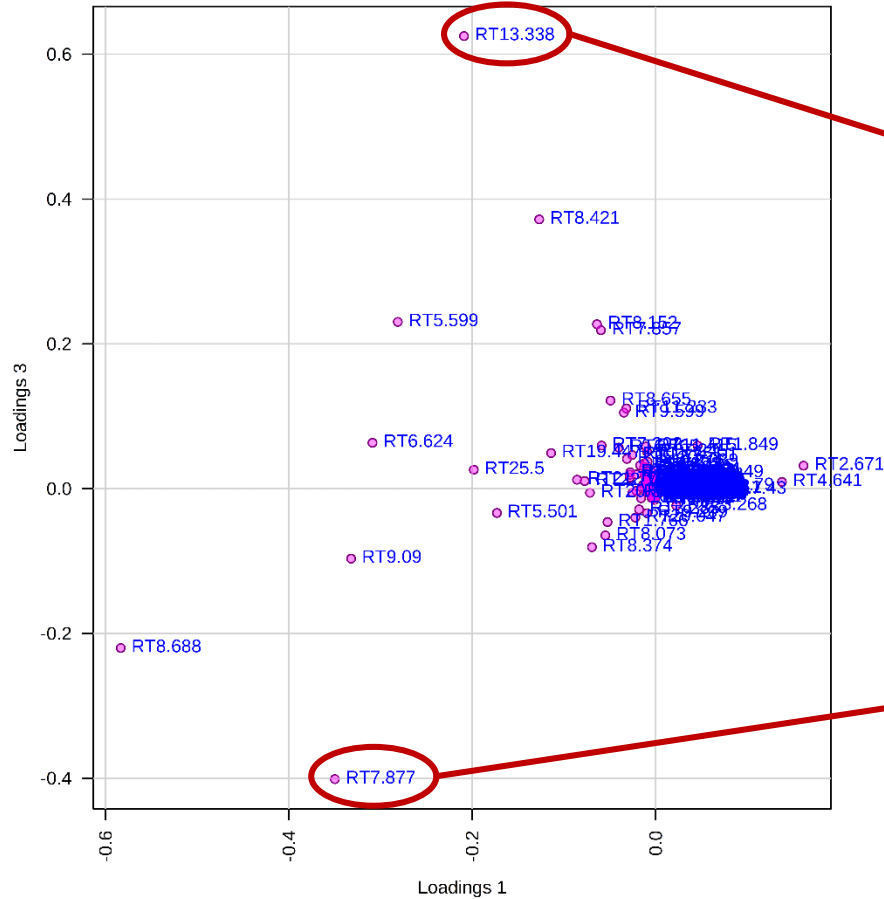
Results and discussion

Principal Component Analysis

Pesticide substance

PC3

By-products

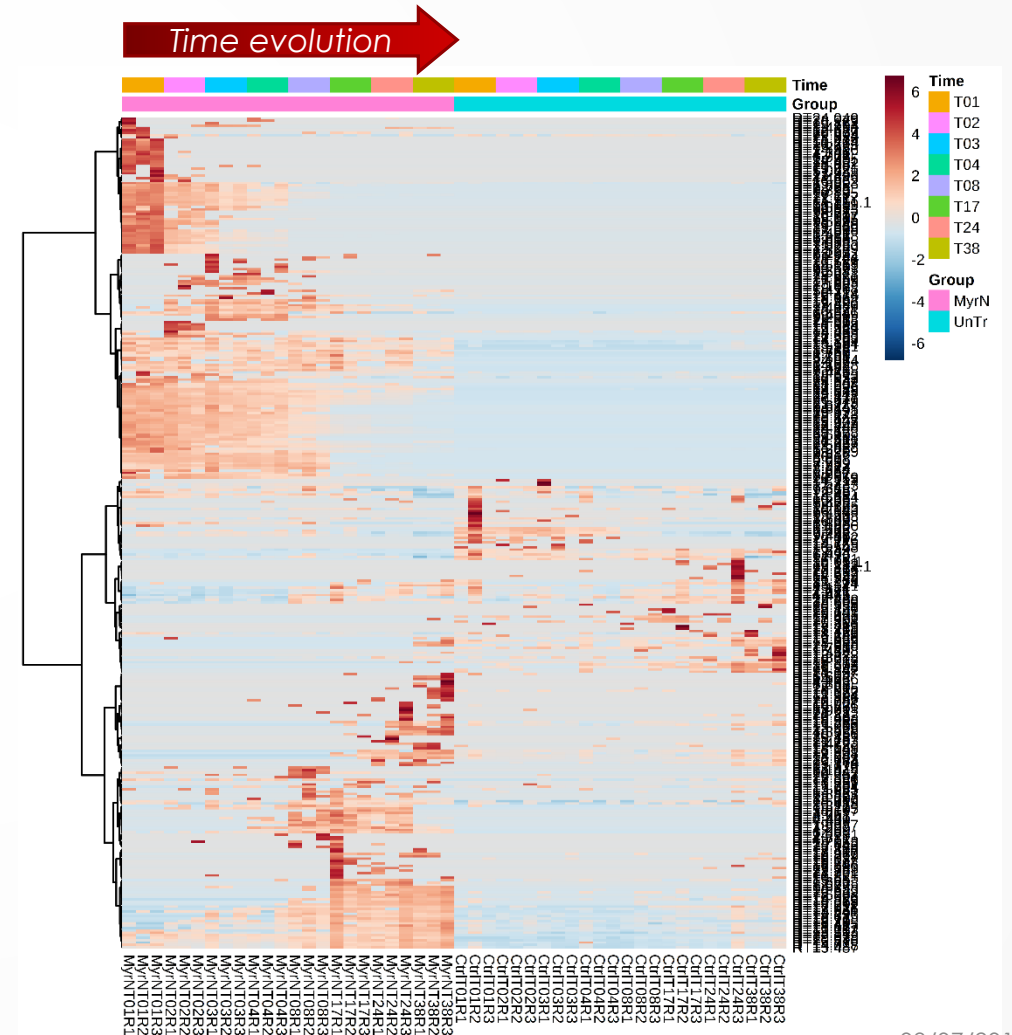


PC1: Xenometabolites

Results and discussion

“Fishing” of degradation by-products

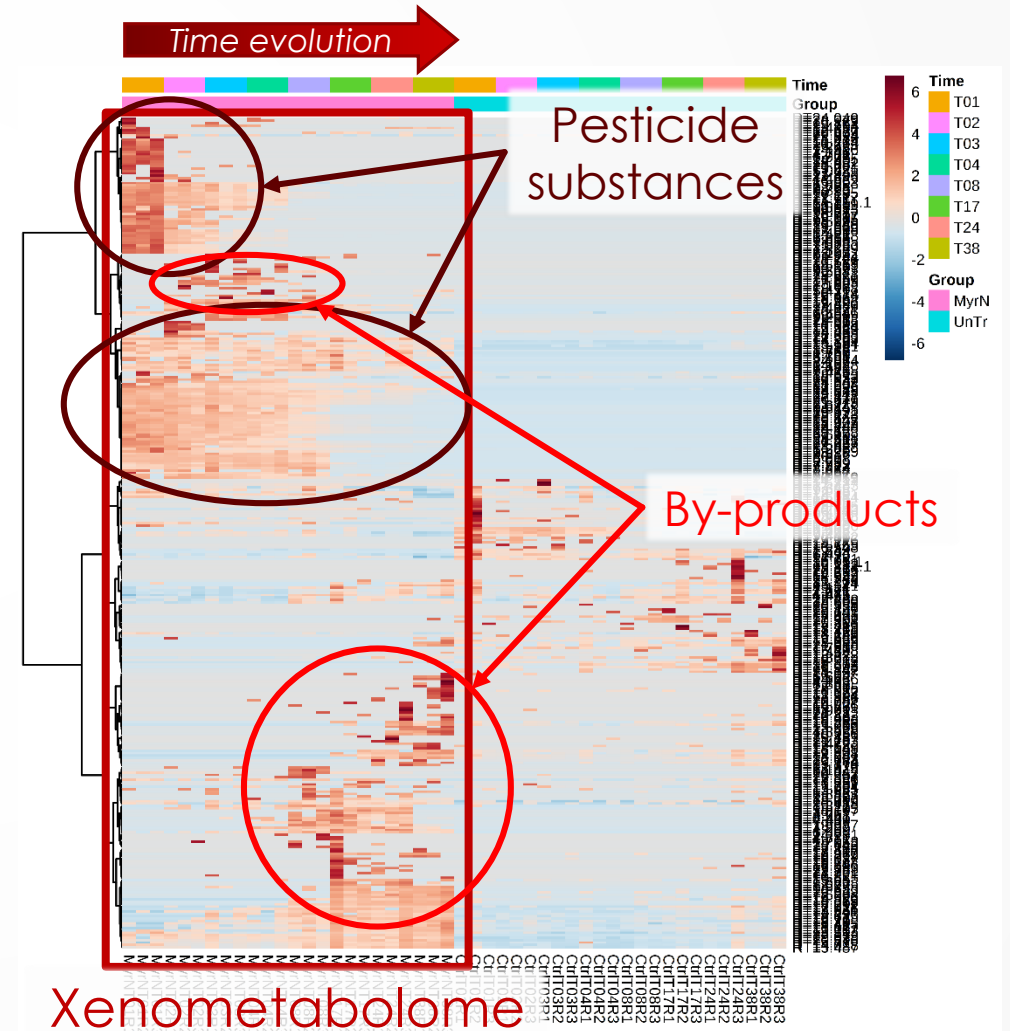
- ➔ Heatmap applied:
 - ✓ Distance Measure: **Pearson**
 - ✓ Clustering Algorithm: **Ward**
 - ✓ 2 factors time series: **MyrN vs. UnTr**



Results and discussion

“Fishing” of degradation by-products

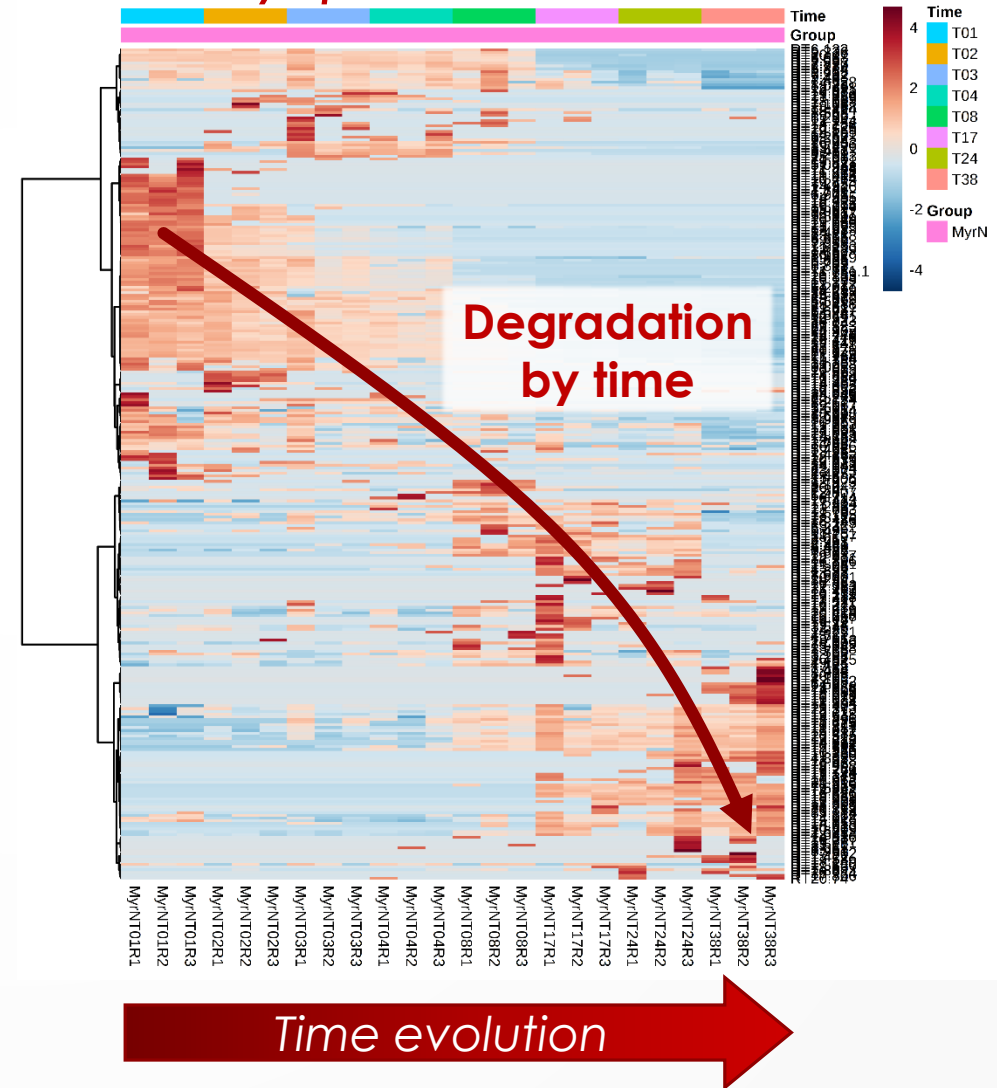
- Heatmap applied:
 - ✓ Distance Measure: **Pearson**
 - ✓ Clustering Algorithm: **Ward**
 - ✓ 2 factors time series: **MyrN vs. UnTr**
- **Xenometabolome** appeared clearly
- Discrimination between **pesticide substances** and **by-products**



Results and discussion

“Fishing” of degradation by-products

- Heatmap applied:
 - ✓ Distance Measure: **Pearson**
 - ✓ Clustering Algorithm: **Ward**
 - ✓ 1 factor time series: **MyrN**
- Plotting the **degradation** by **time evolution**

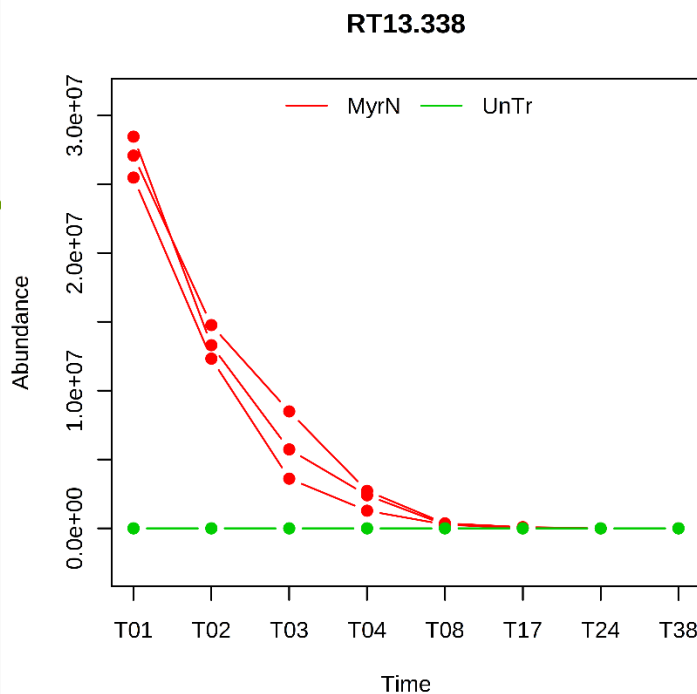


Results and discussion

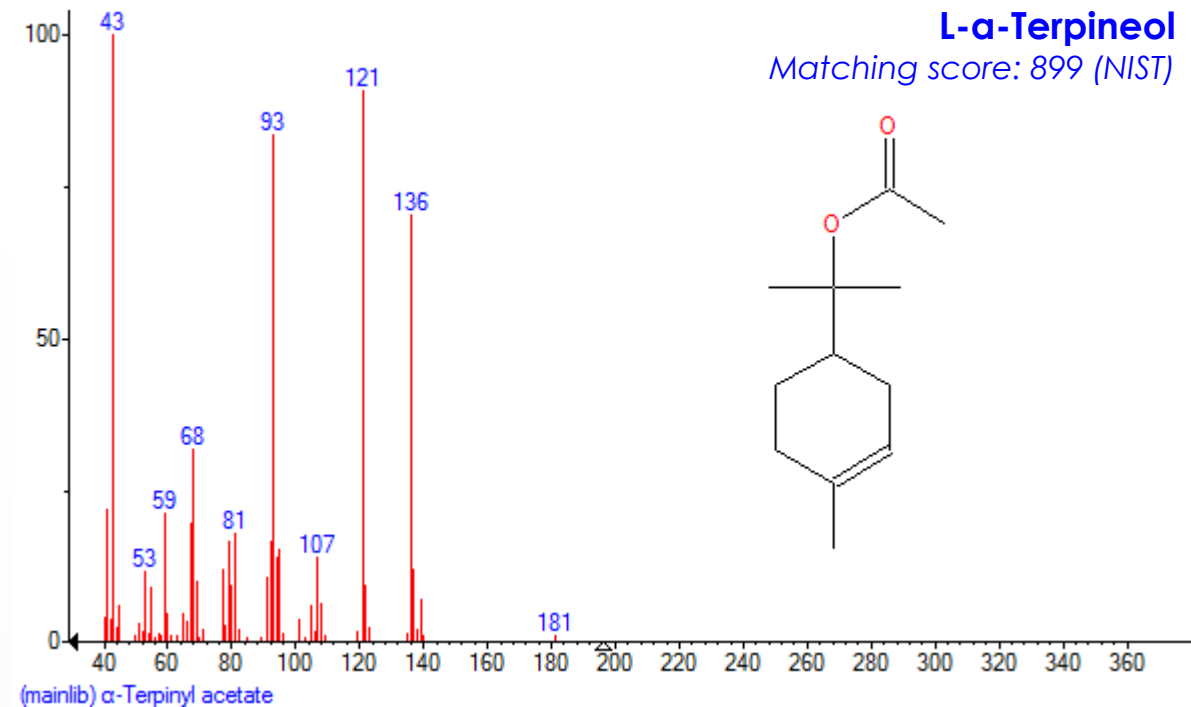
"Fishing" of degradation by-products

Kinetics tracking by time evolution:

► **Pesticide substances** and *Myrica gale* extract compounds



Oracz et al. *Plant Cell Physiol* (2012), 53(1):81–95

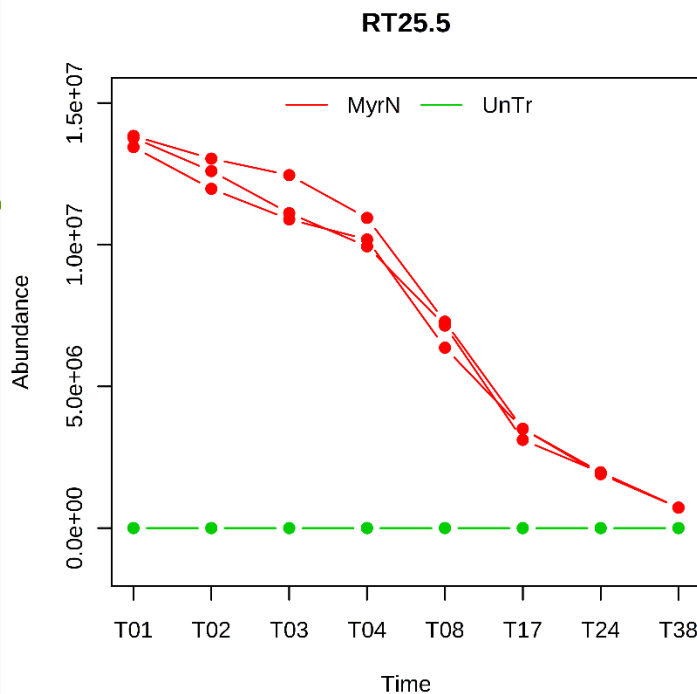


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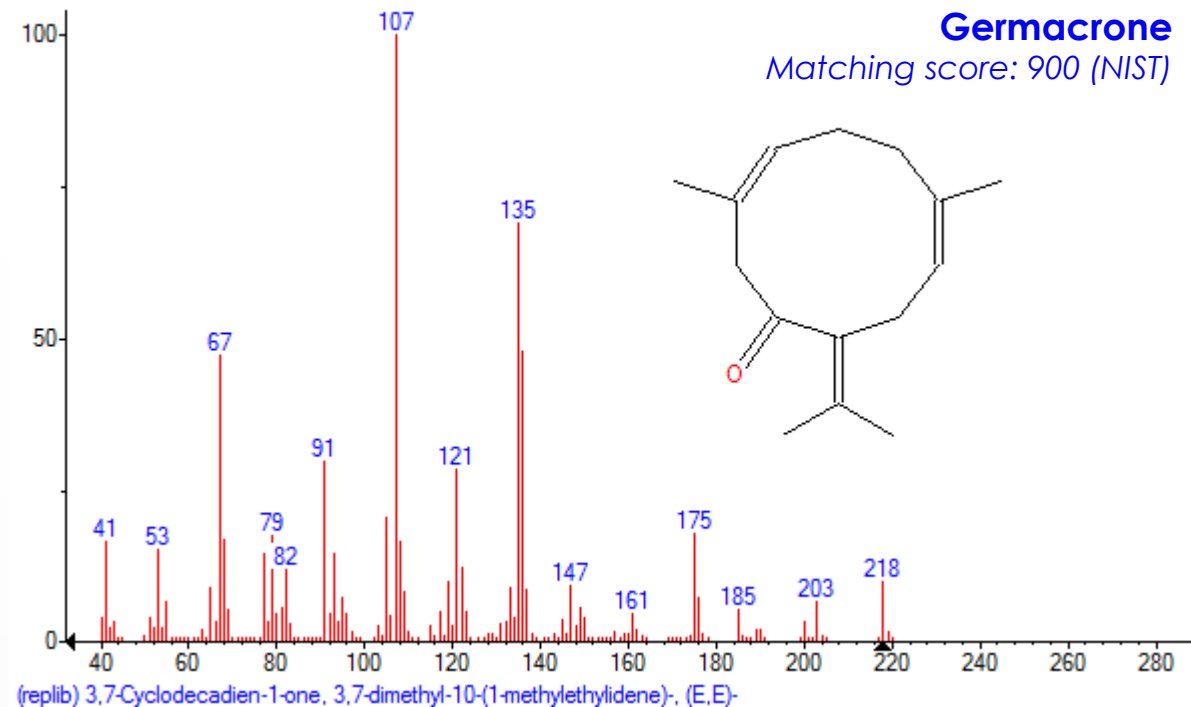
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Khaled et al. *J Agric Food Chem* (2019), In press

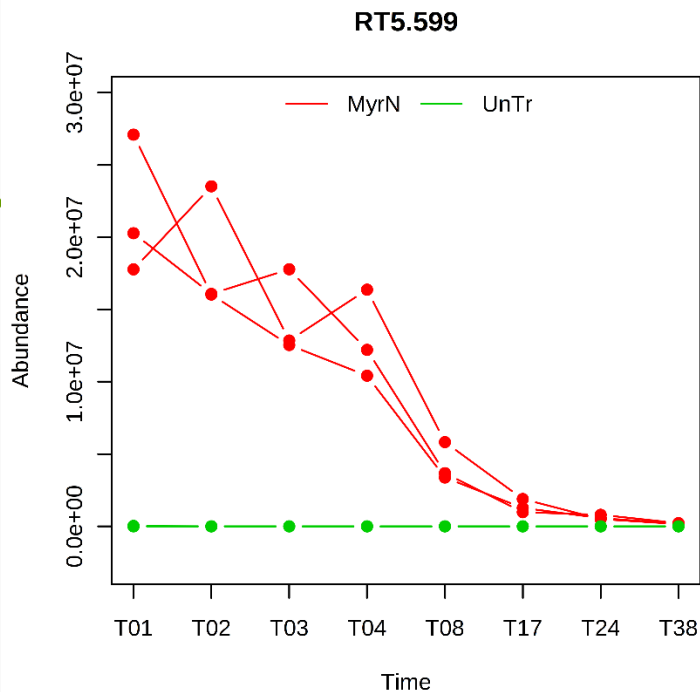


Results and discussion

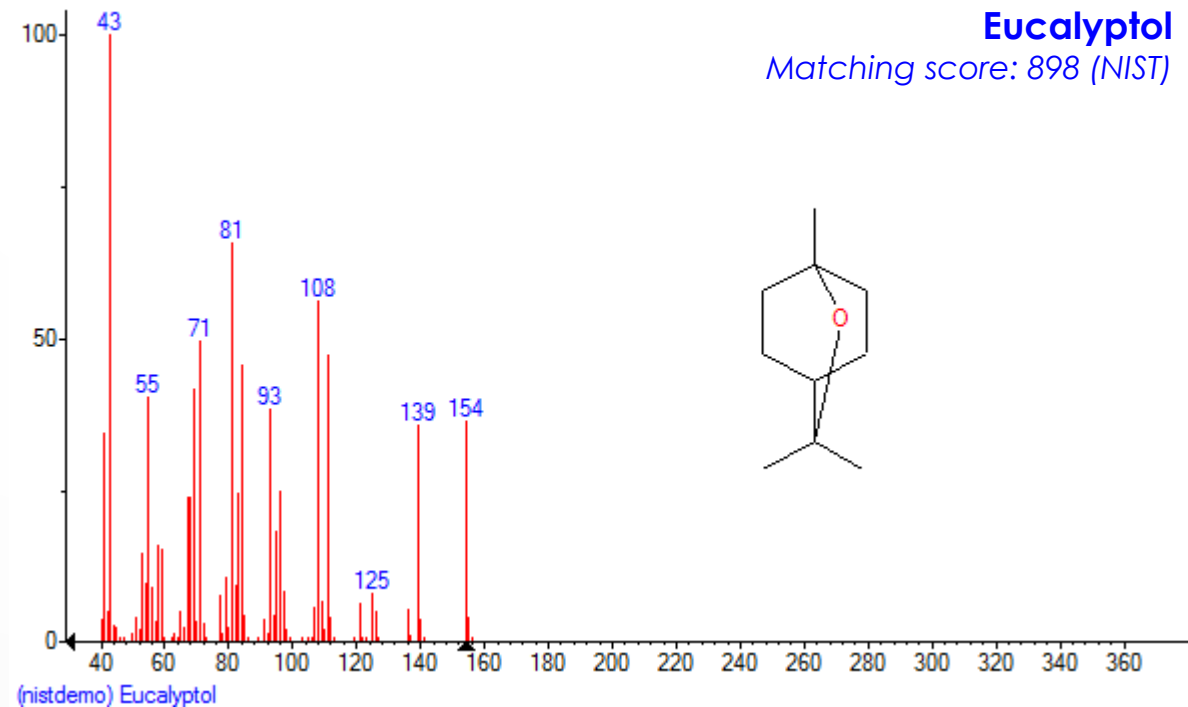
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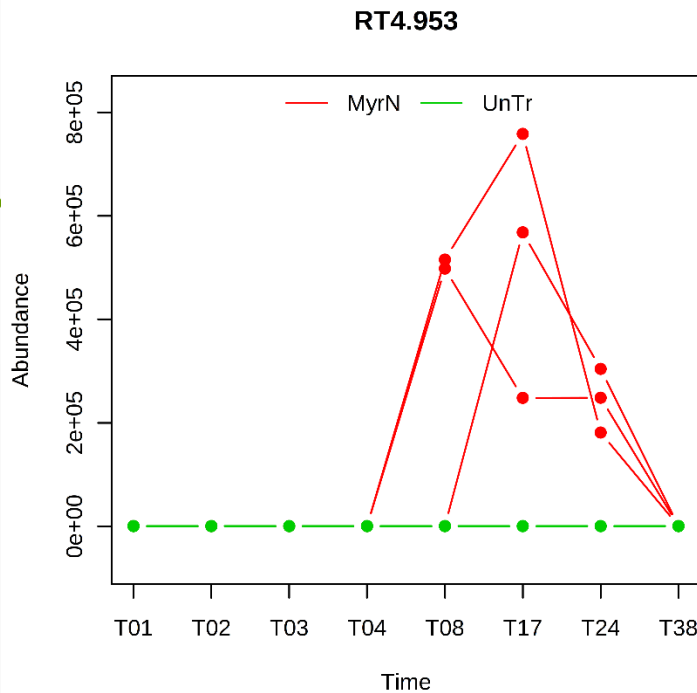


Results and discussion

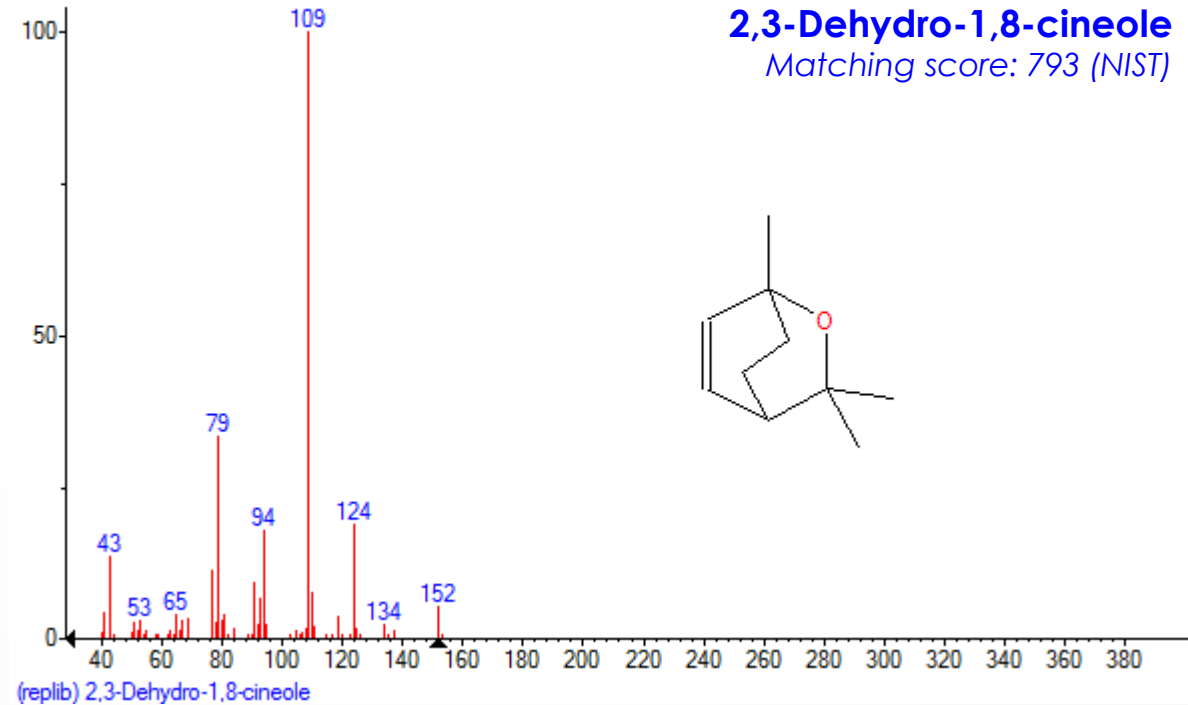
"Fishing" of degradation by-products

Kinetics tracking by time evolution:

► Potential **degradation by-products**



Khaled et al. *J Agric Food Chem* (2019), In press



Conclusions and perspectives

- ▶ **Conclusions for Headspace-Solid Phase Micro Extraction (HS-SPME) method:**
 - ✓ **Simple** and **rapid** design for **sample preparation**
 - ✓ **Small number of samples** is needed (material and matrix economization, less time-consuming experiment)
 - ✓ **Cheap, green,** and **automated** non-laborious extraction
 - ✓ **High reproducible** analysis with **low matrix background**
 - ✓ Kinetics tracking on the **same sample**: reducing **inter-sample bias risks**
 - ✓ **Endometabolome** was **not detected**, however

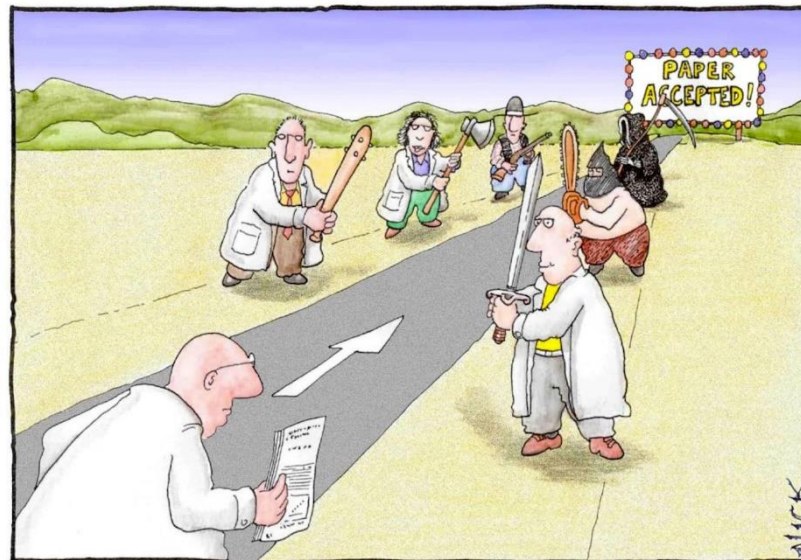
Conclusions and perspectives

- ➔ Main **advantages** of the GC-MS system:
 - ✓ **Cheap cost**
 - ✓ Good **reproducibility of Retention times** with the GC
 - ✓ **Retention Indices (RI)** are provided for **compound characterization**
 - ✓ **Electron Impact Ionization (EI)** provides **reproducible MS fragmentation patterns**
 - ✓ **Fast putative identification** of detected molecules *via MS spectra libraries (e.g. NIST)*



Conclusions and perspectives

- Further **data mining** and manual **cross-check** of results
- Preparing a **short communication article** to introduce the concept
- Preparation of a **bigger principal experiment** and introducing **new type of pesticides** (*i.e.* Beloukha[®]* and Glyphosate)
- Testing a new extraction technique: the **Dynamic Headspace** (DHS)



*: POCTEFA PALVIP project (*in course*)

Acknowledgments

Thank you for supporting this work



Workflow4metabolomics



CRIOBE USR 3278 Staffs:

Metabolomics & Environmental Chemistry team

Pr. Cédric Bertrand

Dr. Marie-Virginie Salvia

Delphine Raviglione, M.Sc.

Mathieu Lazarus, M.Sc.

Thank you for your attention !

Any questions ?



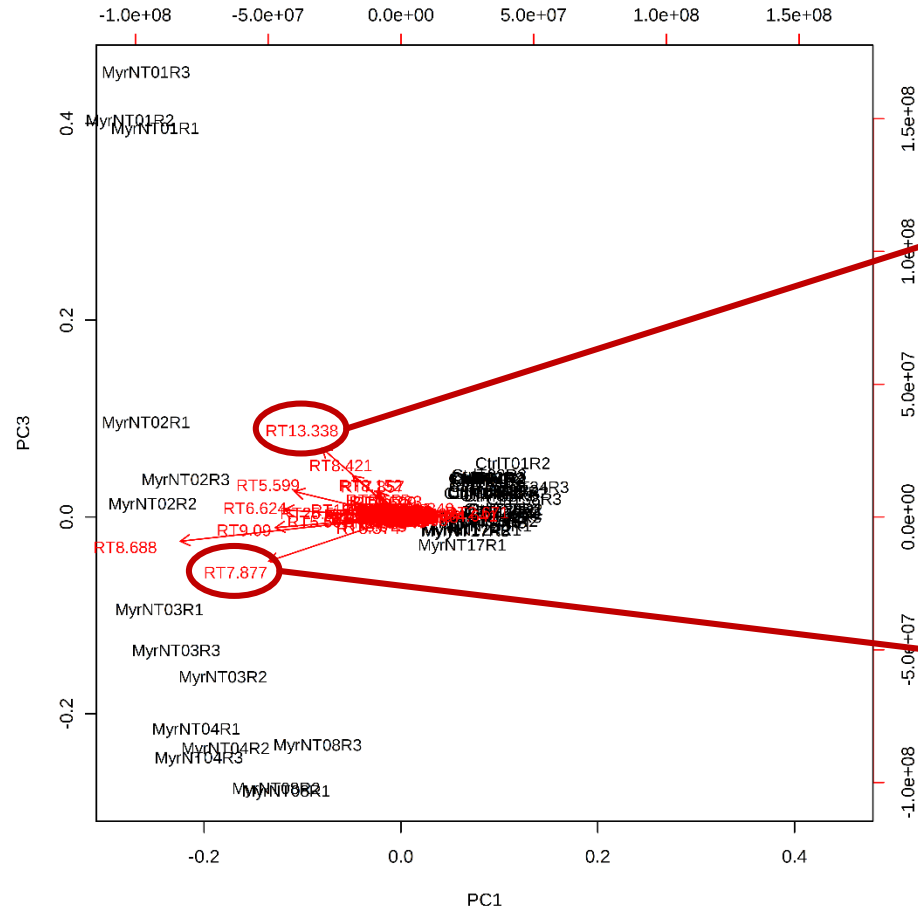
Appendix

Principal Component Analysis

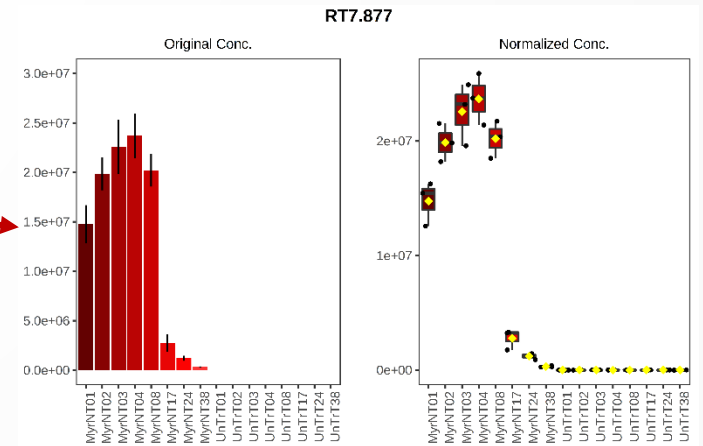
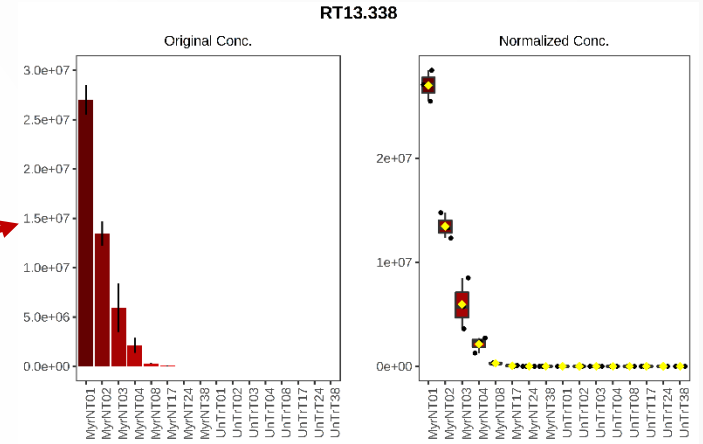
Pesticide substance

PC3

By-products



PC1: Xenometabolites

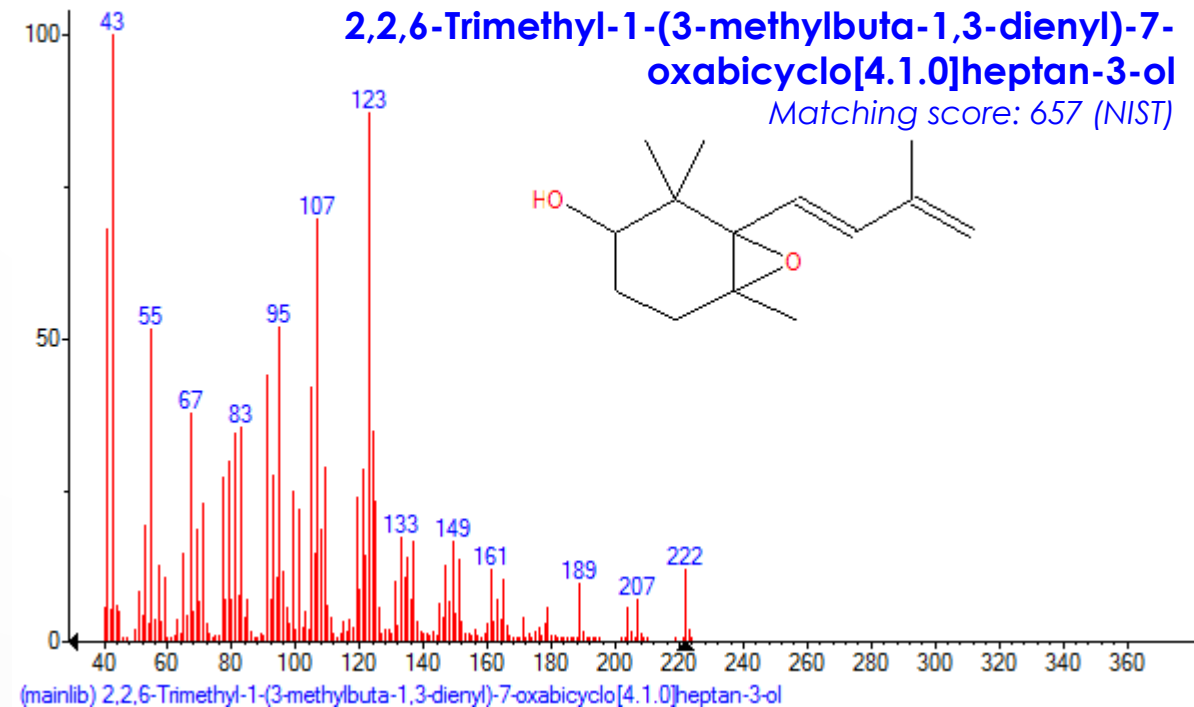
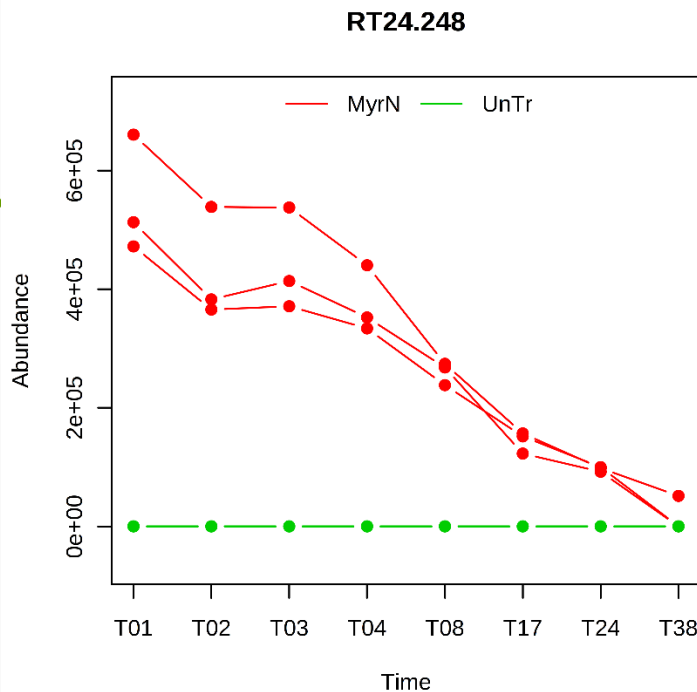


Appendix

"Fishing" of degradation by-products

Kinetics tracking by time evolution:

► **Pesticide substances** and *Myrica gale* extract compounds

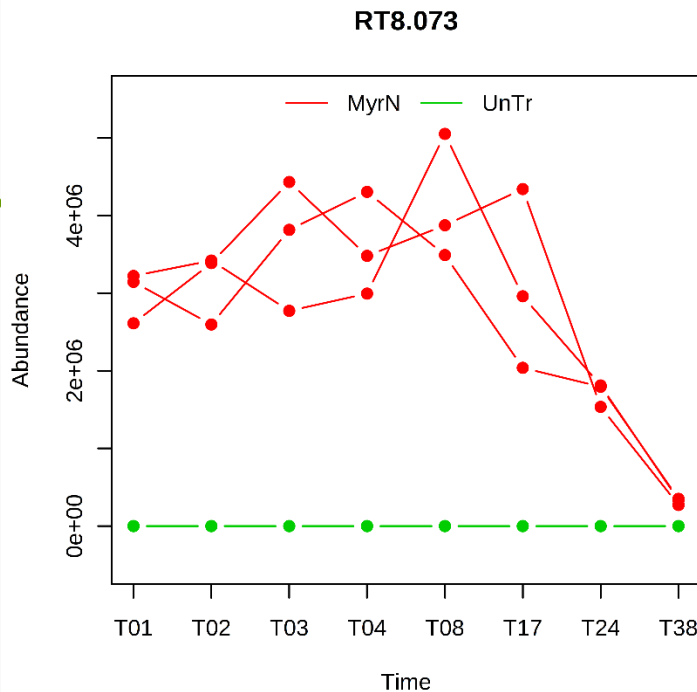


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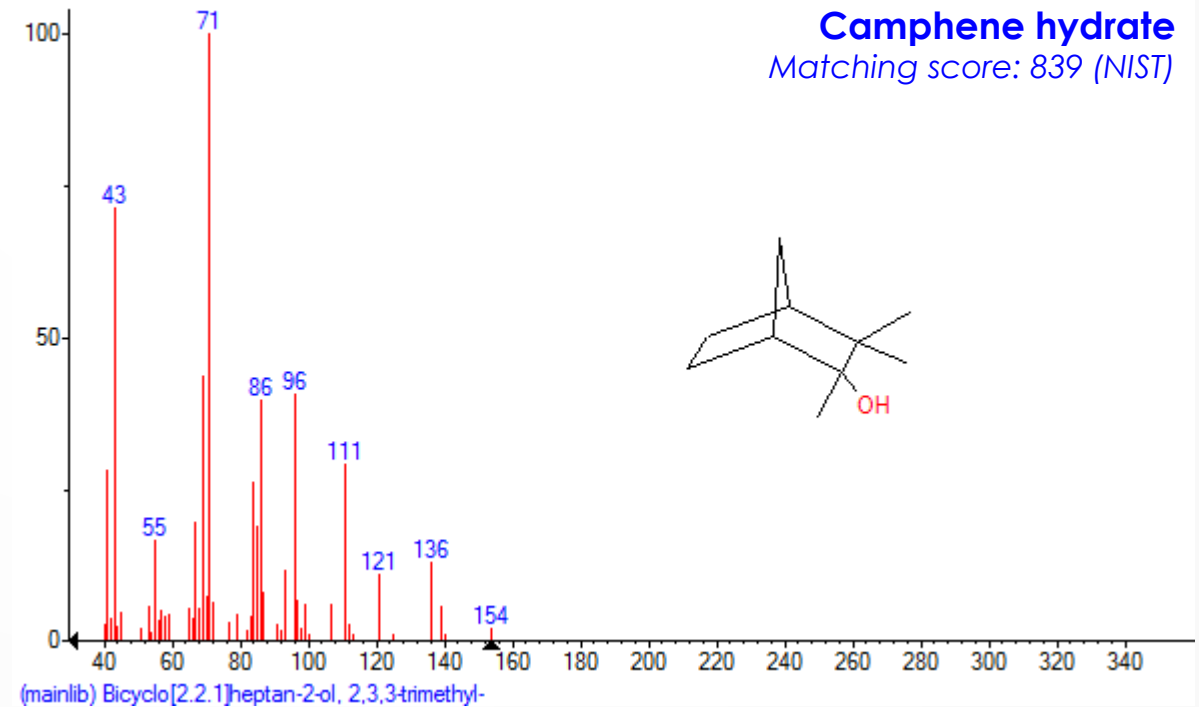
"Fishing" of degradation by-products

Kinetics tracking by time evolution:

► **Pesticide substances** or degradation **by-products?** Or **both?**



Khaled et al. *J Agric Food Chem* (2019), In press

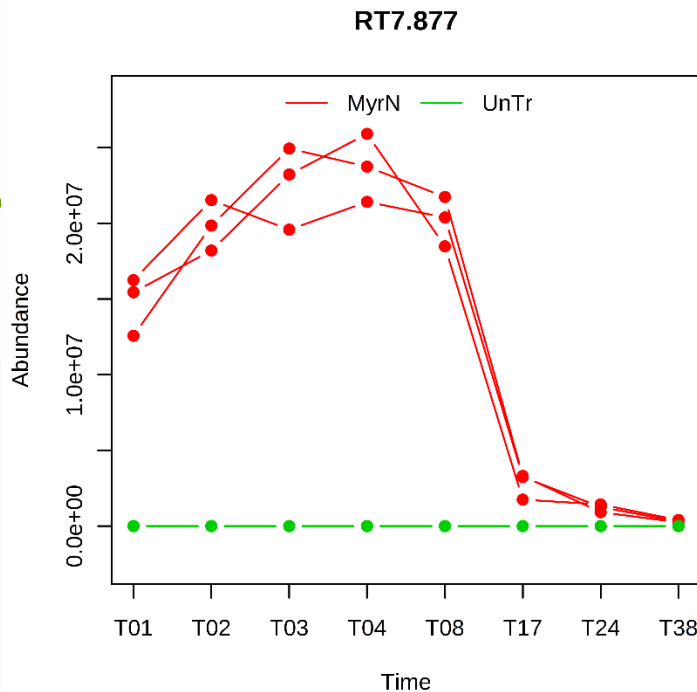


Appendix

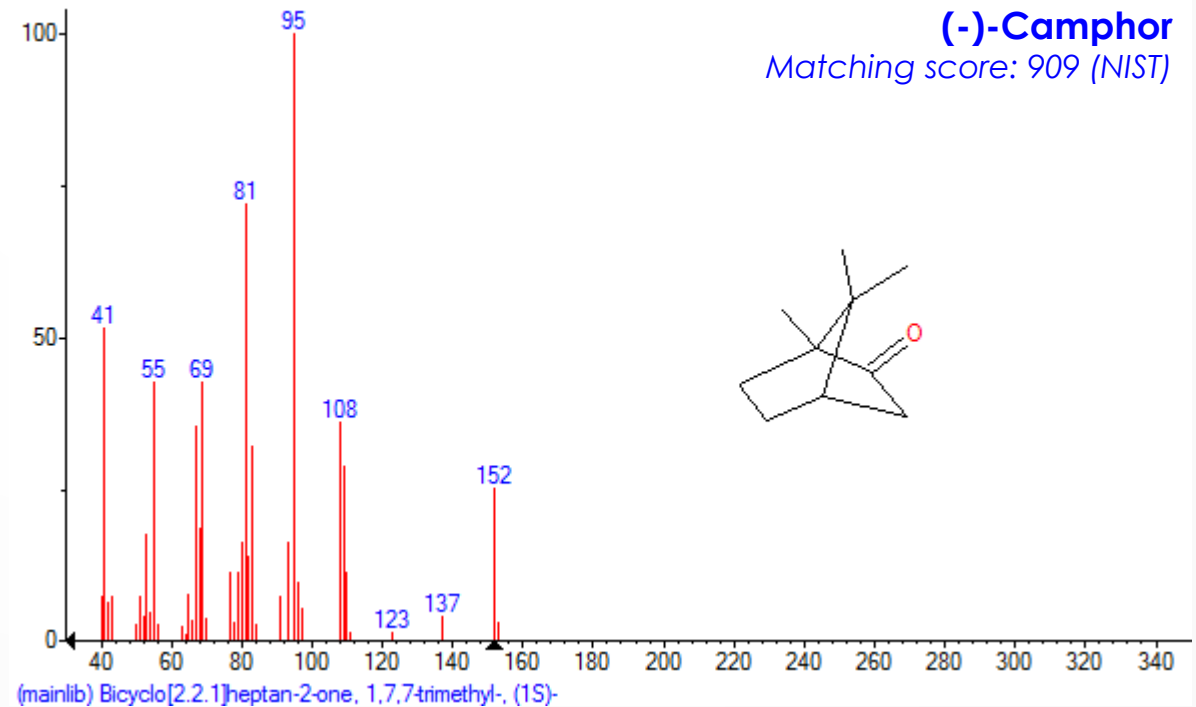
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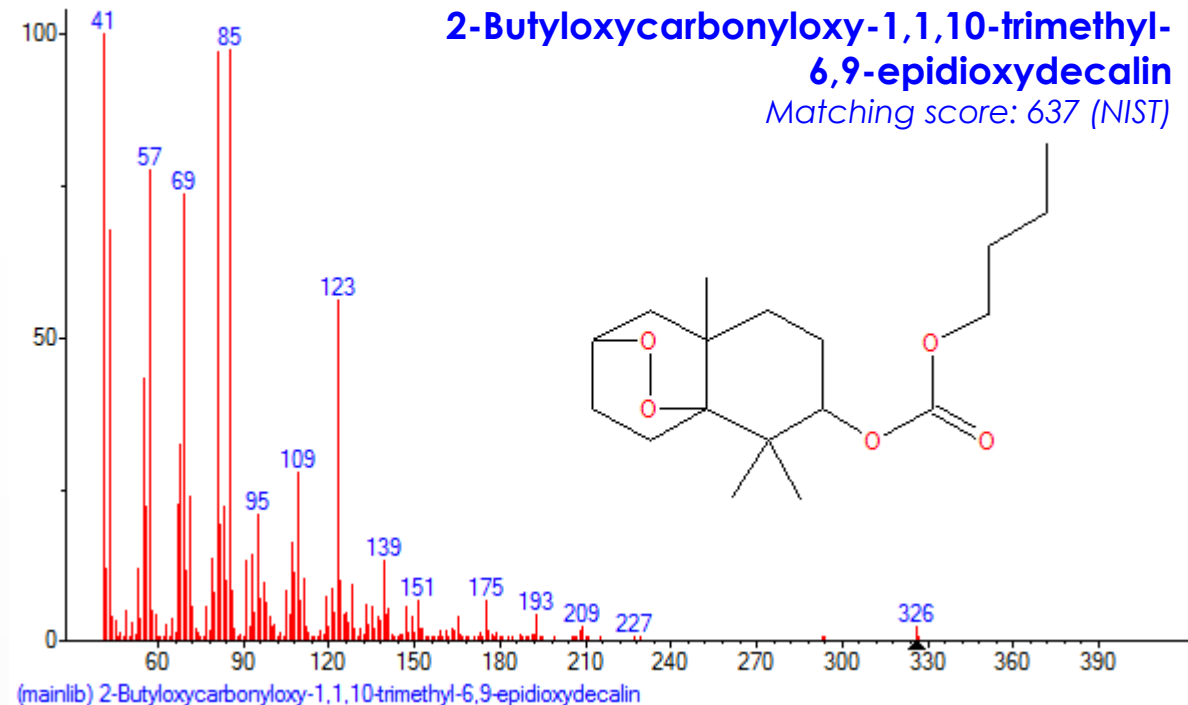
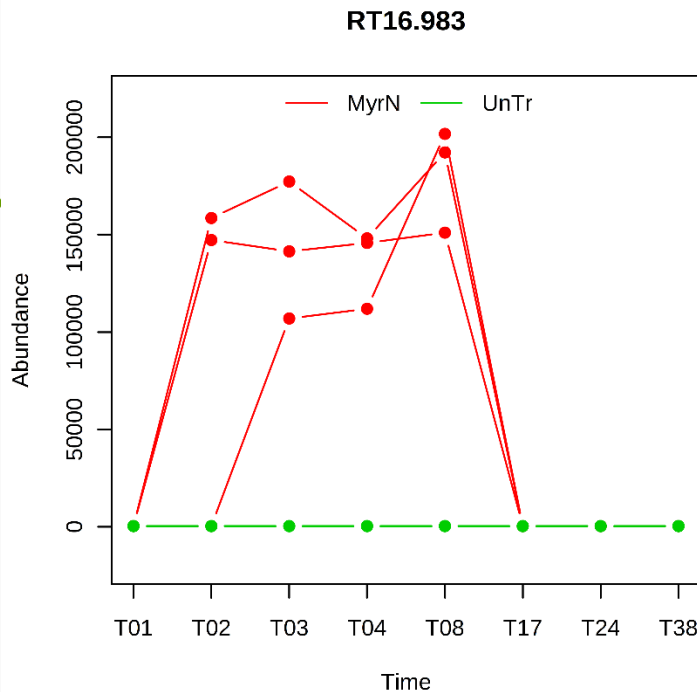


Appendix

"Fishing" of degradation by-products

Kinetics tracking by time evolution:

► Potential **degradation by-products**

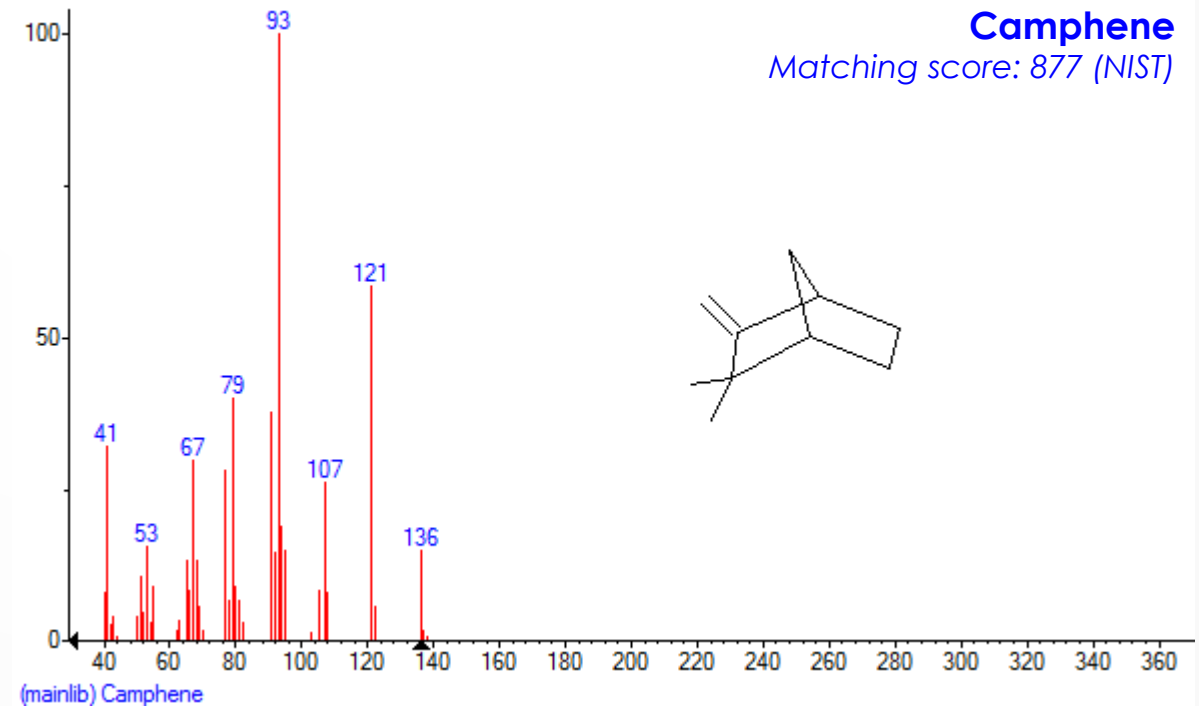
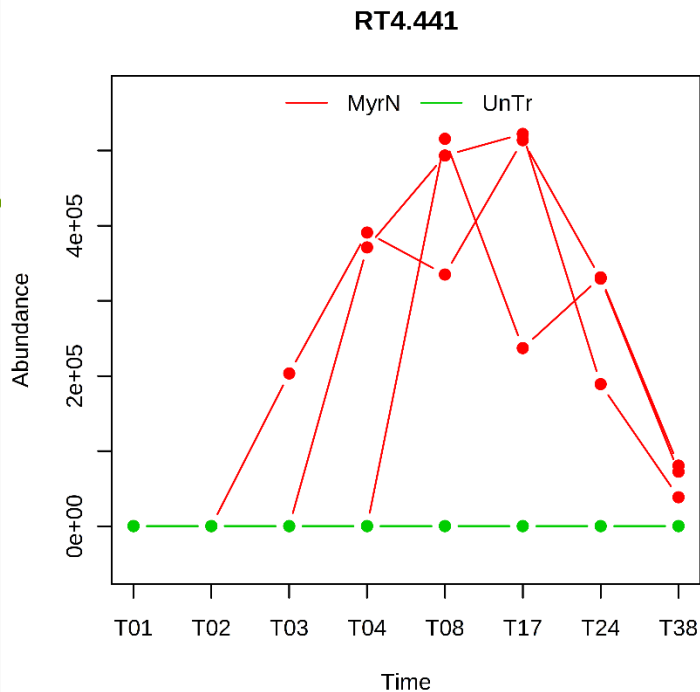


Appendix

"Fishing" of degradation by-products

Kinetics tracking by time evolution:

► Potential **degradation by-products**

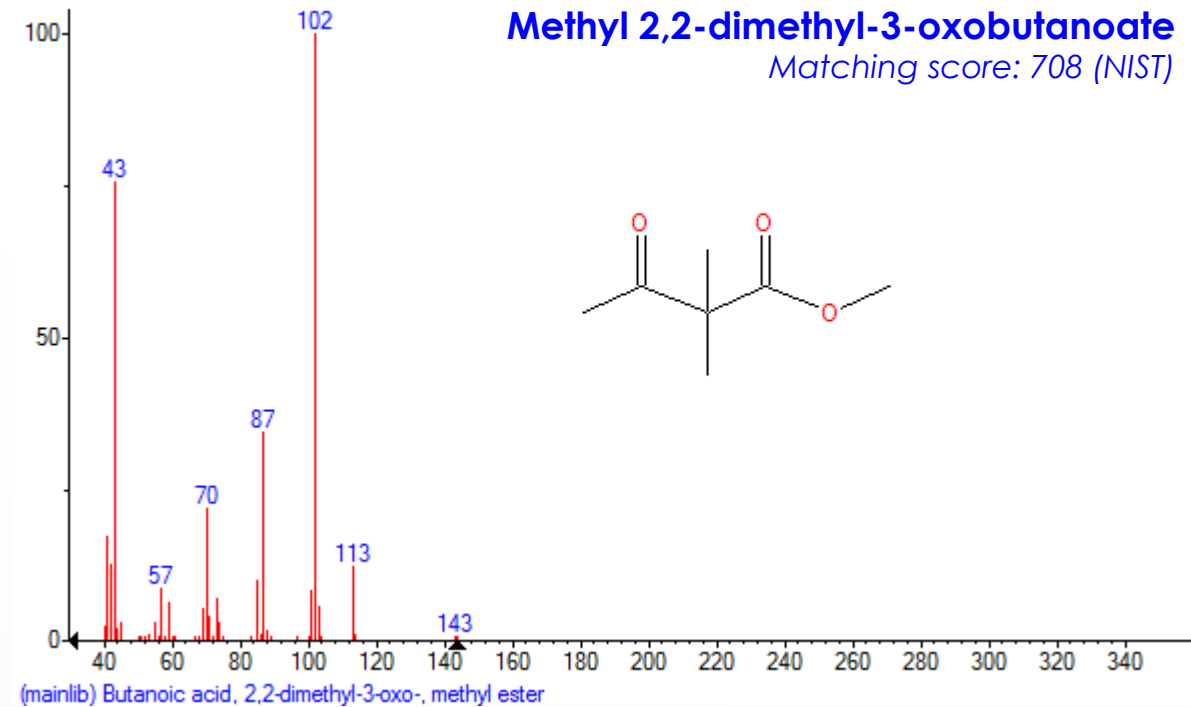
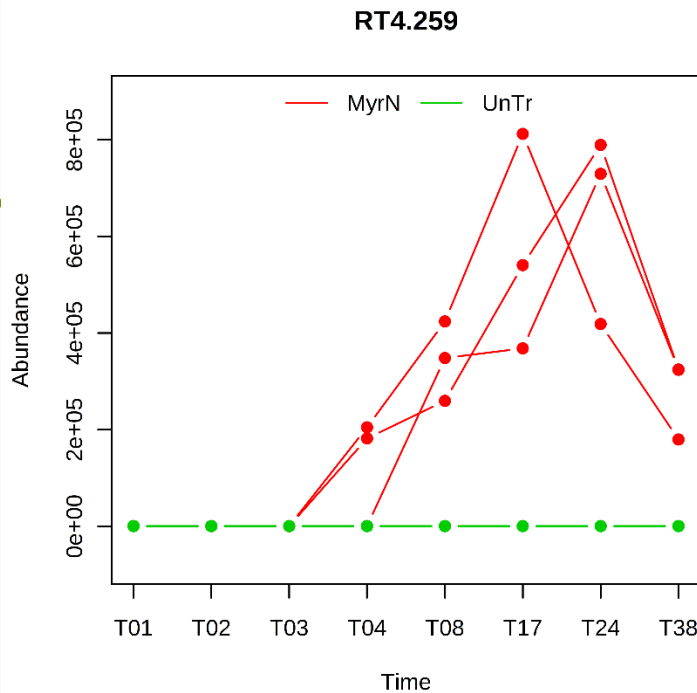


Appendix

"Fishing" of degradation by-products

Kinetics tracking by time evolution:

► Potential **degradation by-products**

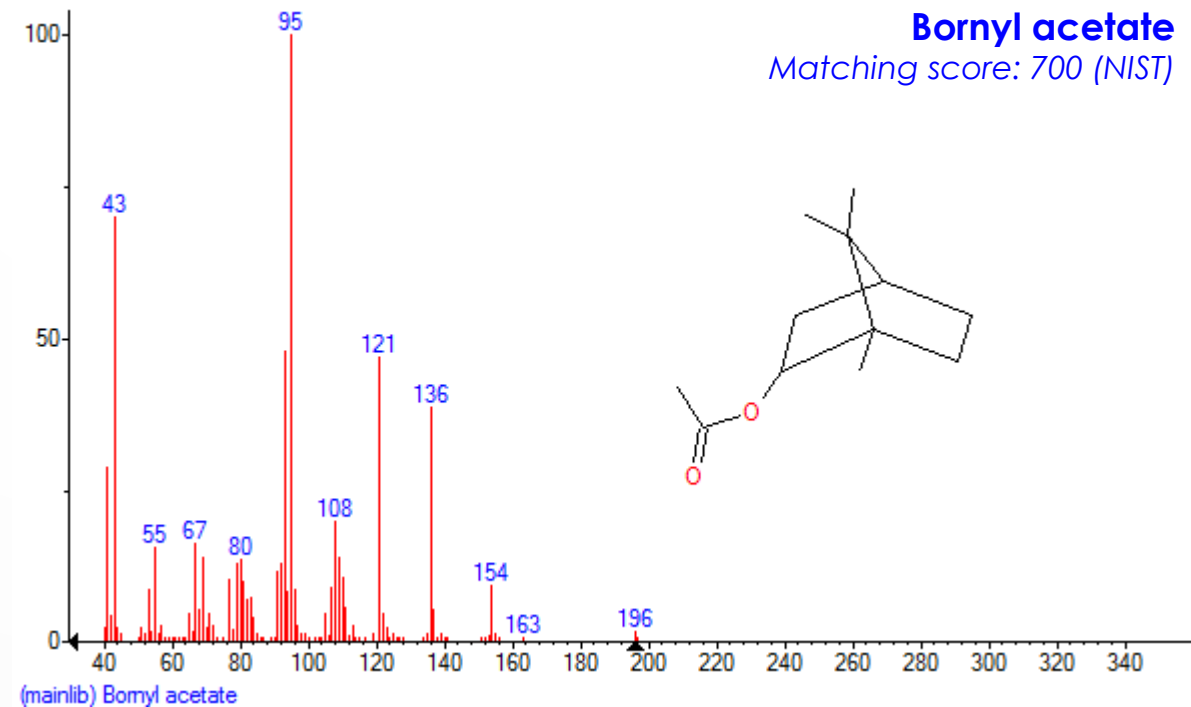
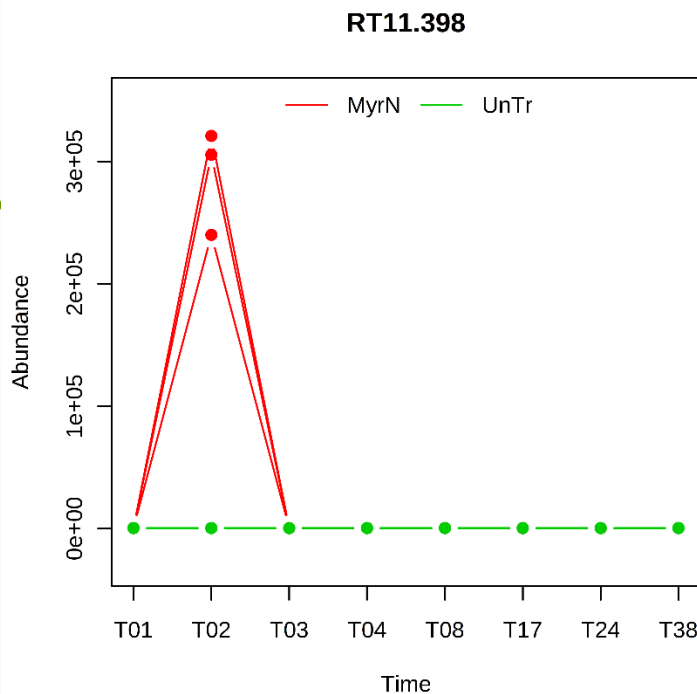


Appendix

"Fishing" of degradation by-products

Kinetics tracking by time evolution:

► Potential **degradation by-products**

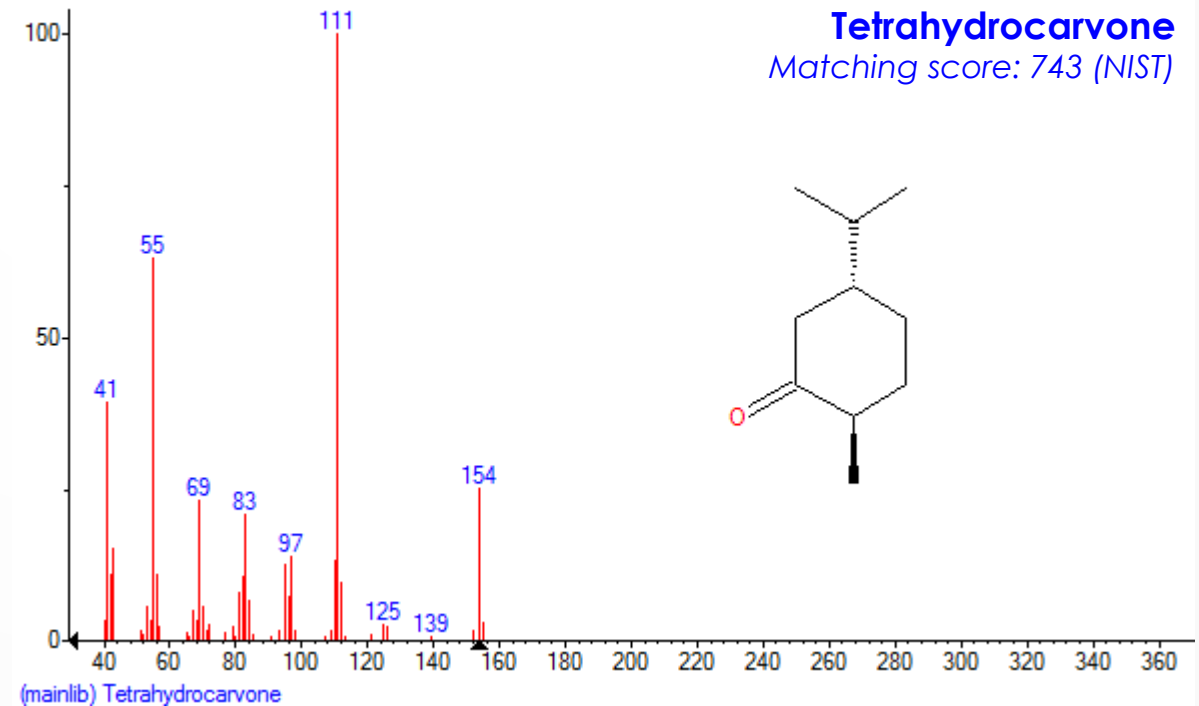
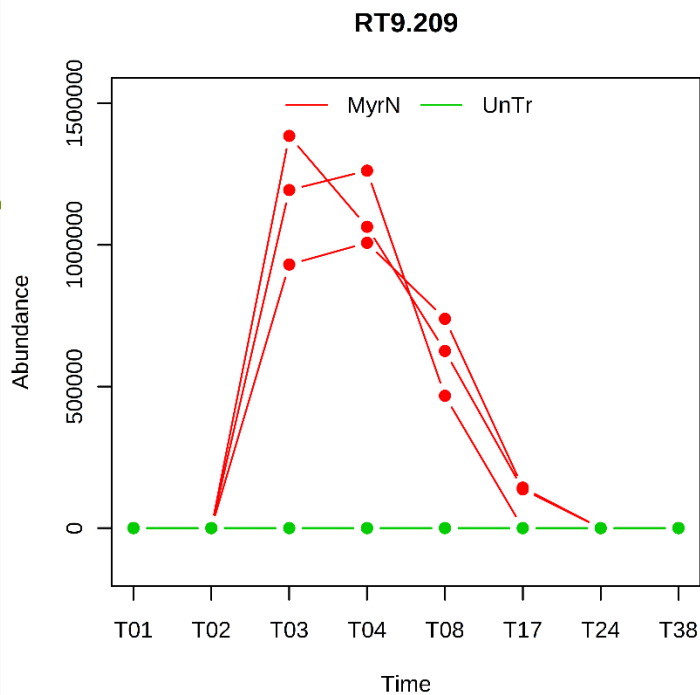


Appendix

"Fishing" of degradation by-products

Kinetics tracking by time evolution:

► Potential **degradation by-products**

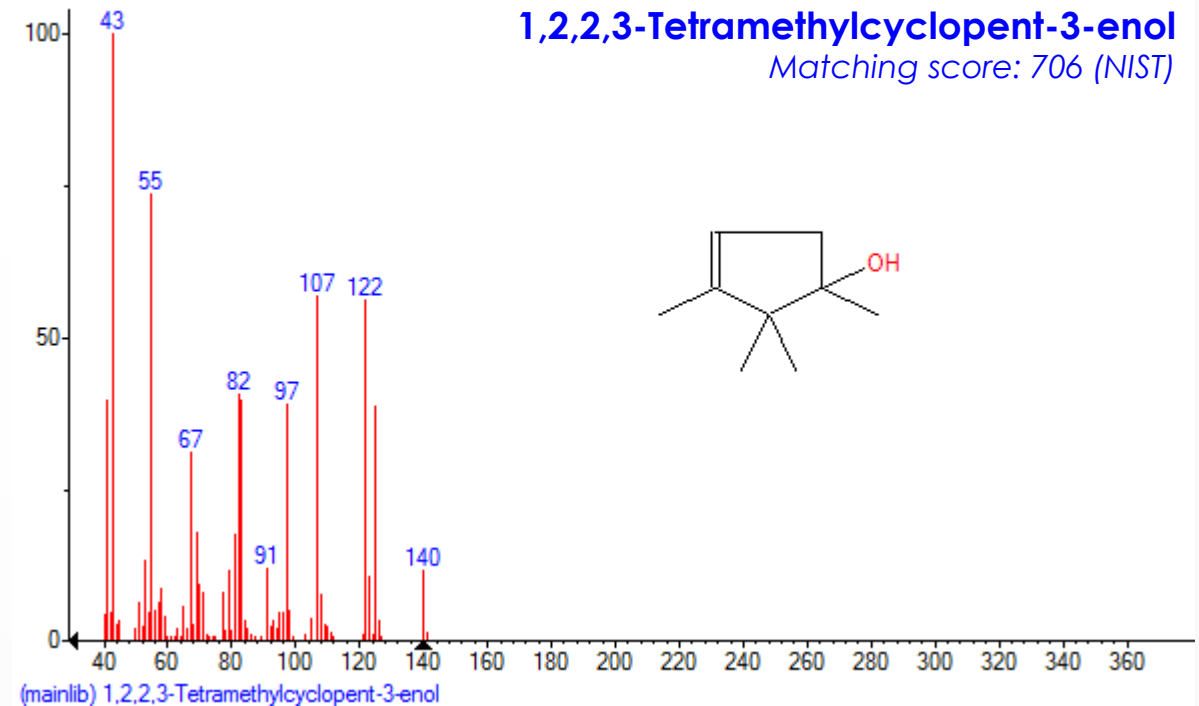
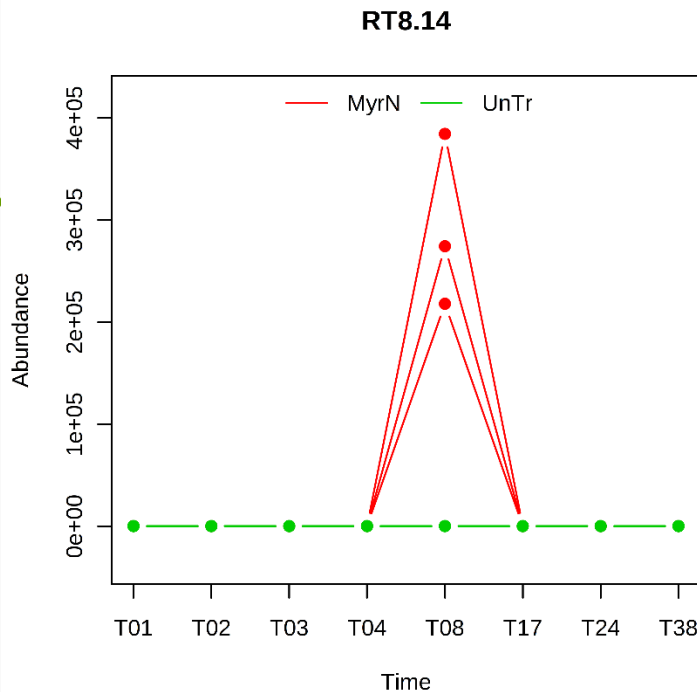


Appendix

"Fishing" of degradation by-products

Kinetics tracking by time evolution:

► Potential **degradation by-products**

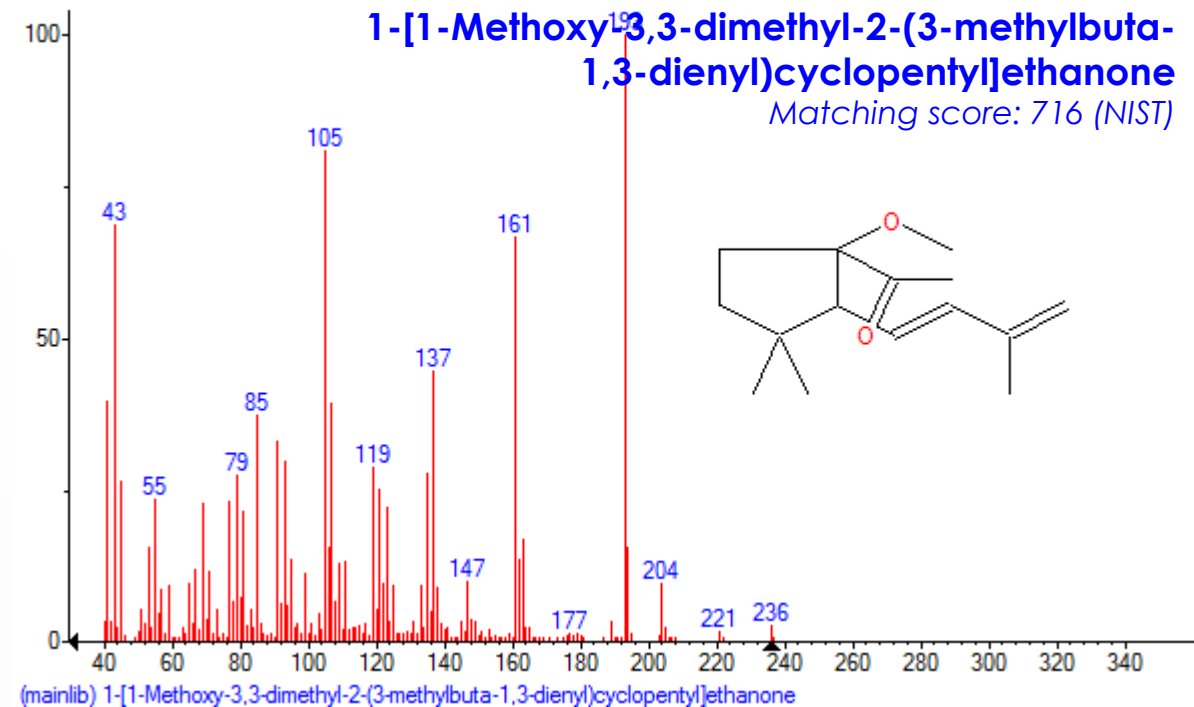
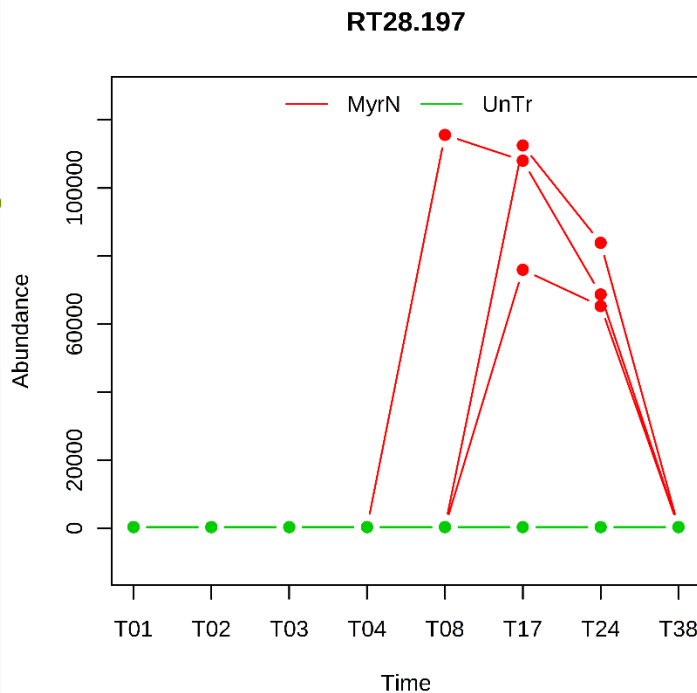


Appendix

"Fishing" of degradation by-products

Kinetics tracking by time evolution:

► Potential **degradation by-products**

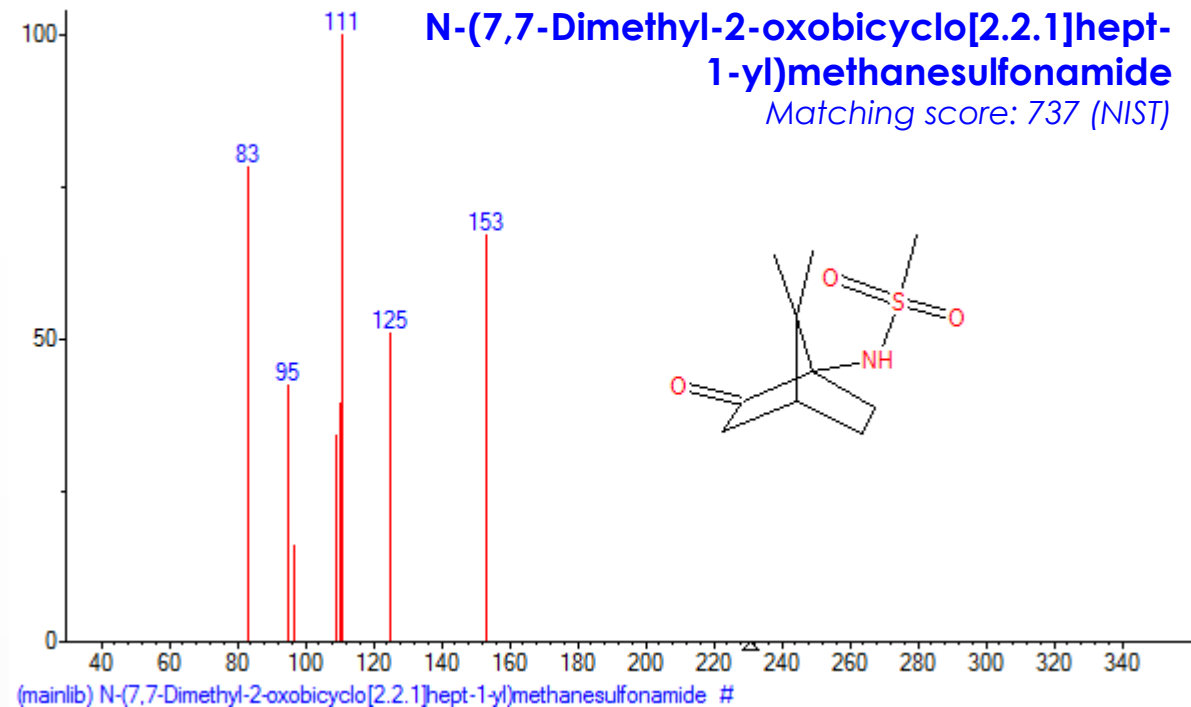
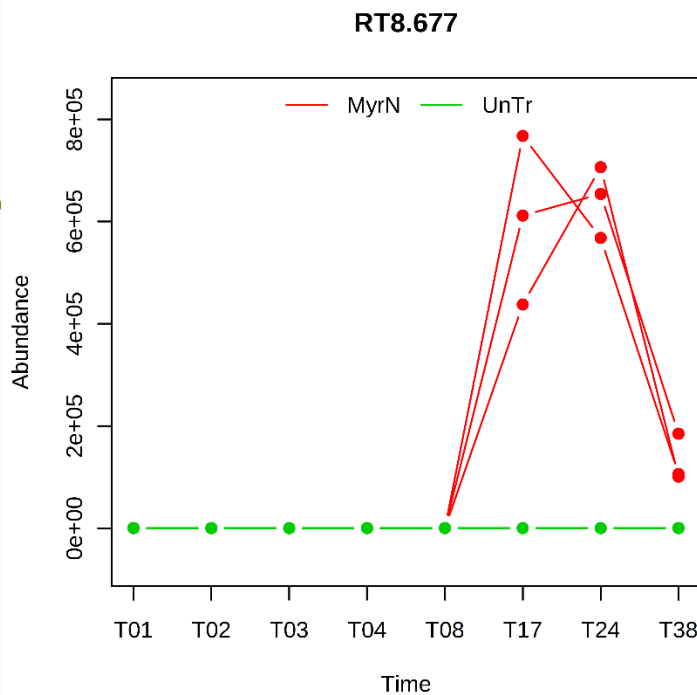


Appendix

"Fishing" of degradation by-products

Kinetics tracking by time evolution:

► Potential **degradation by-products**



Appendix

"Fishing" of degradation by-products

Kinetics tracking by time evolution:

➔ **Pesticide substances** or degradation **by-products?** Or **both?**

