

Electrospray Ionization and co-elution in Meta-metabolomics: a biomarker or a suppressed ion?

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Context of the study General objective

Development of a "universal" proxy to assess the environmental fate and impact of complex (bio)pesticides in environmental matrices by LC-HRMS and HS-SPME-GC-MS-based Metabolomics





Context of the study Biopesticides

Biopesticides (Biological Control Agents):

- Proposed as alternative solution instead of synthetic/chemical pesticides (European Directives, OECD Recommendations, French National Plan "Ecophyto")
- Supposed "less harmful" on nature and human health
- Complex biological/chemical nature (several unknown components, few studies on their "side-effects")





Context of the study Bacillus thuringiensis israelensis

A bio-insecticide: Bacillus thuringiensis israelensis (Bti) A commercialized anti-mosquito bio-insecticide Applied in lagoons and stagnant water swamps Active ingredient: Spores of Bacillus thuringiensis var. israelensis Complex molecular and metabolic nature (Proteins, crystals, metabolites, formulates)



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Context of the study Classical guidelines limitations

Complex molecular and metabolic nature:

> Half-life approach: not applicable (non-molecular active ingredient)

Degradation by-products: undiscoverable by the half-life approach

Methodological limitations

Development of a new approach



Environmental Metabolic Footprinting (EMF):

Alternative concept for pesticide risk evaluation

- Universal proxy to assess the environmental fate and impact of complex (bio)pesticides
- Untargeted meta-metabolomics-based approach



(Patil et al. 2016, Salvia et al. 2017)





Meta-metabolomics:

Studying a **holistic biological system** without attributing its **pool of metabolites** to a defined community. (Sans-Piché 2011)

e.g. pesticide-spiked environmental matrix (our case: sediments)



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Context of the study Environmental Metabolic Footprinting (EMF)



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Context of the study Environmental Metabolic Footprinting (EMF)







Comparison of Meta-metabolomes of Control and Spiked Samples









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Experiments Sample Preparation

10x field dose application: **5.5 μL/microcosm** (Field dose: 2.5 L/ha)

- Biopesticide: Bacillus thuringiensis israelensis (Bti)
- Environmental matrix: Sediments (15 g: 50% Dry sediment / 50% Water)
- Incubation in microcosms at 22 °C in dark
- Two groups of treatment: Control vs. Spiked sediment
 - ✓ 10 time points for kinetics tracking: day 0, 2, 4, 8, 15, 30, 45, 60, 90, 150
 - 5 replicates per group of treatment

Total number of samples: $2 \times 5 \times 10 = 100$ samples



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Data Analysis



Experiments Extraction

QUECHERS extraction (Lehotay 2006) Steps:

- 15 mL of Acetonitrile addition
- Vortex (15 sec)
- Salts addition (1 g NaCl + 4 g MgSO₄)
- Vortex (40 sec)
- Centrifugation (10 min, 20 °C, 4500 RPM)
- Vacuum vaporization of supernatant
- Dissolution in 1,5 mL of Methanol (9x enrichment)
- Filtration on 0.22 µm PTFE filters



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Data Analysis

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Experiments LC-HRMS Analysis

Liquid Chromatography:

✓ Thermo Fisher Vanquish UHPLC⁺ Focused system

- Phenomenex Kinetex® Polar C18 column (100 x 2.1 mm, 2.6 µm, 100 Å)
- ✓ 26 min/run Elution solvents:
 - Phase A: H₂O + 0.1 % Formic Acid
 - Phase B: ACN + 0.1 % Formic Acid
- ✓ Injection volume: **5 µL**
- ✓ Flow rate: 0.5 mL/min
- ✓ Column temperature: 30 °C



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Data Analysis

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Experiments LC-HRMS Analysis

Mass Spectrometry:

- ✓ Bruker maXis **QToF** HRMS
- Resolving power: 40'000 FWHM (at m/z 1000)
 - Positive mode Electrospray (ESI+):
 - Capillary voltage: 3.5 kV
 - Nebulizer pressure: 43.5 psi
 - Dry gas: 10 L/min
 - Dry Temperature: 200 °C
- ✓ FullMS: m/z 80-1600
- ✓ Scan rate: 1.25 Hz



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Sample preparation





At the "Day 15" of the kinetics tracking:

Principal Components Analysis (PCA)

Cross-Validation by OPLS-DA







Markers of discrimination discovery by Score plot of OPLS-DA



S-Plot









Molecular feature M274T611: a potential "Biomarker"

Manually treated data (by Compass DataAnalysis)





Molecular feature M274T611: a potential "Biomarker"

Manually treated data:



Peak area





p-Value: 0.02857 *

Significant difference











Mass Spectra... Oups!





Ion Suppression:

A decrease of an ion signal in Mass Spectrometry due to a lack of ionization efficiency.

Known mechanisms:

- Ionization competition with a co-eluted molecule in the liquid phase
- ✓ Decrease of evaporation efficiency in ESI source due to less-volatile (macro)molecules
- Co-precipitation of the metabolite with macromolecules (e.g. Peptides)
- Neutralization of the metabolite by a co-eluted molecule in the gas phase



(Antignac et al. 2005)



Conclusion and perspectives

Next step:

Validation of the ion suppression hypothesis by series of dilutions

Conclusion:

- Referring to raw data is essential to validate statistical results in metabolomics
- The awareness about Mass Spectrometry fundamentals assures reliable interpretations and conclusions



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- Risk of eliminating the feature from spiked samples data
- Statistically, the feature will be more significant (figure below)









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Appendix

Biomarker:

A factor associated with the **well-being** of living organisms that is used as a measure of **environmental change** and or **influence**. (MeSH, NCBI, **2018**)



Glutathione oxidized Sulfur stress biomarker for plants



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