

ANALYTICAL AND TOXICOLOGICAL INTEGRATION FOR GENOTOXICITY CHARACTERIZATION OF MOSH/MOAH

APPROACHES AND CHALLENGES

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International Collen-Francqui Chair, Classes of Excellence of Prof. Gary W. Miller

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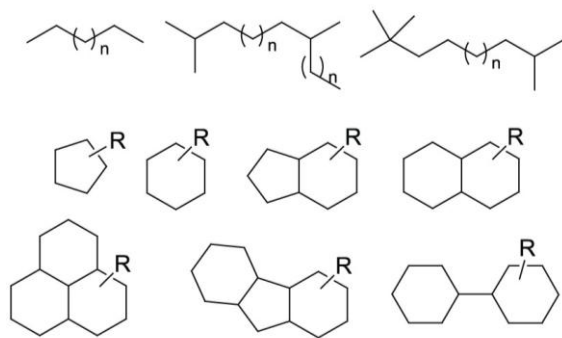
Context



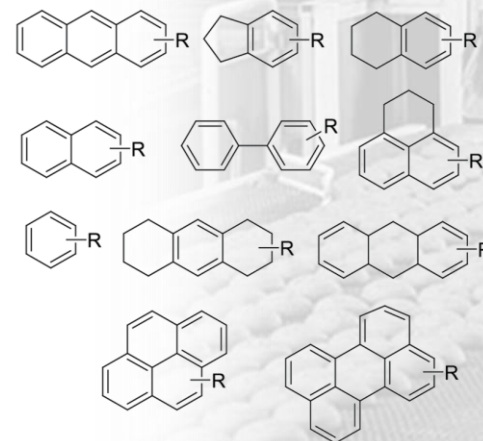
MOSH – mineral oil **saturated** hydrocarbons

MOAH – mineral oil **aromatic** hydrocarbons

- Ubiquitous, petroleum-derived **food contaminants**
- Enter the food chain through various pathways: contact with contaminated **food packaging, machinery lubricants**, or because of **poor storage practices**, ...



MOSH: Linear and branched alkanes
Branched cycloalkanes (C10-C50)



MOAH: Branched mono- or polyaromatic compounds (C10-C50)

Appear as complex mixtures of variable composition



Risk assessment



Risk assessments of mineral oil hydrocarbons in food (2012 & 2023)



MOSH

Generally considered **of no concern** at the
concentrations found today in food
Can **accumulate** in the human body.



MOAH

Considered of concern:
genotoxicity of **MOAH with ≥ 3 AR**
Absence of reliable toxicity data for **1-2 AR MOAH**



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Regulation (EU) 2023/915 limiting total MOAH levels in food
Entering into force in 2027



Gaps in the risk assessment



Since **MOAH** are considered as **potential genotoxicants**, the regulation is based on an **ALARA*** principle

In this case, the limits are defined by the **analytical LOQs for total MOAH (0.5 – 2 mg/kg)**

** As Low As Reasonably Achievable*

However, it has not been elucidated **which** are the **MOAH compounds** (or the **precise sub-fractions**) that are the genotoxic ones, although there are hypotheses.

Treating all MOAH as genotoxic may lead to unnecessarily strict regulations (penalizing for the food industry).

EFSA requests some clarification

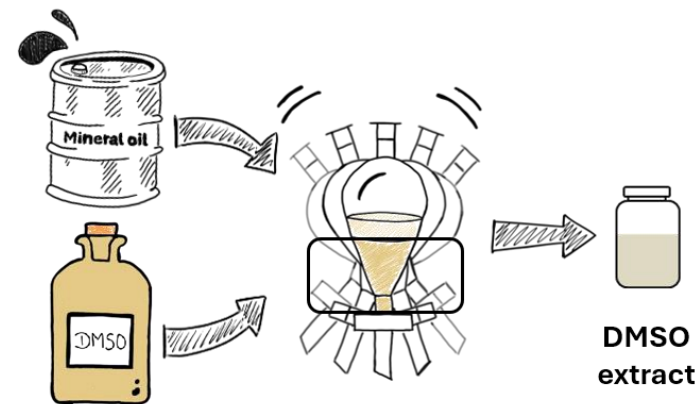


Challenges



Two elements that make toxicological evaluation challenging

- Mineral oils are highly **hydrophobic**
 - Direct application in conventional **aqueous-based** *in vitro* genotoxicity assays is usually not possible.
- Mineral oils are **complex mixtures** with variable composition
 - Testing each individual constituent is not realistic.





Goals of the presentation



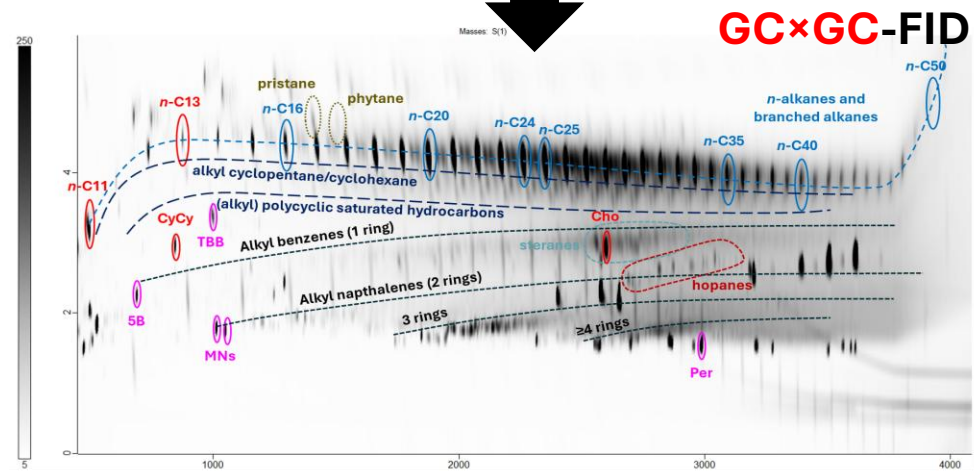
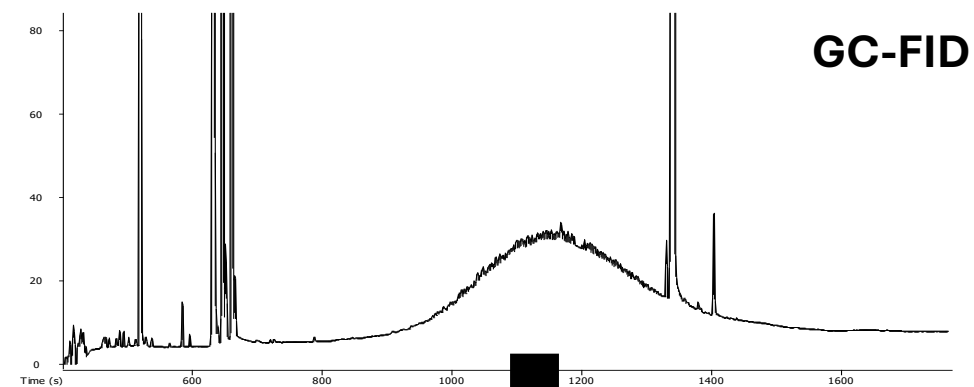
Illustrate how the combination of **GC×GC-FID/TOFMS** and *in vitro* **bioassays** can help **address knowledge gaps** identified by EFSA regarding **MOAH genotoxicity**.



Relevance of GC×GC-FID/TOFMS



- **GC×GC** generates **structured chromatograms**, allowing characterization of present HC sub-classes
 - Not possible with GC-MS alone due to the extensive coelution of isobaric species and the limited specificity of EI MS for HCs.
- **FID** allows for **accurate quantification** owing to the nearly **uniform response** of HCs across the range of compounds considered
- **TOFMS** coupled to GC×GC enables **post-targeted investigation** of owing to its wide m/z acquisition range



PART I

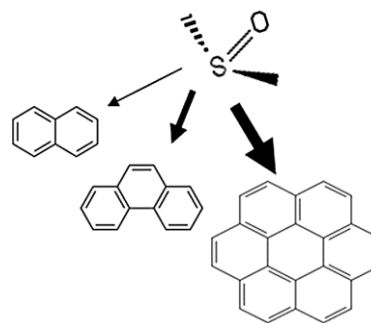
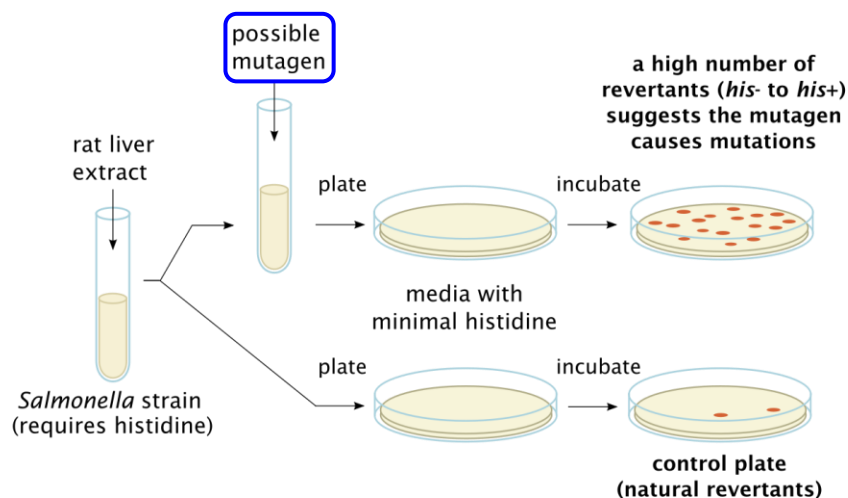
**Combination of GC×GC-FID/TOFMS characterization and
conventional *in vitro* bioassays for genotoxicity screening**



Mutagenicity screening in mineral oils

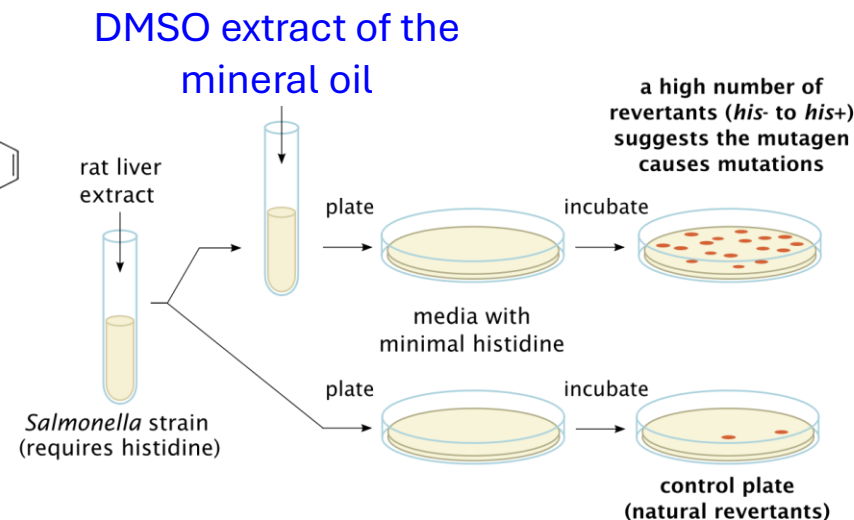


Principle of the Ames test



J.-C. Carrillo, et al. Reg Tox Ph 106 (2019) 316-333

Principle of the **Modified** Ames test



- DMSO selectively extracts only the most polar fraction of mineral oils (i.e., weakly alkylated PACs), which is considered toxicologically relevant.
- However, neither the composition of this fraction nor the compounds responsible for the observed genotoxicity have been elucidated.

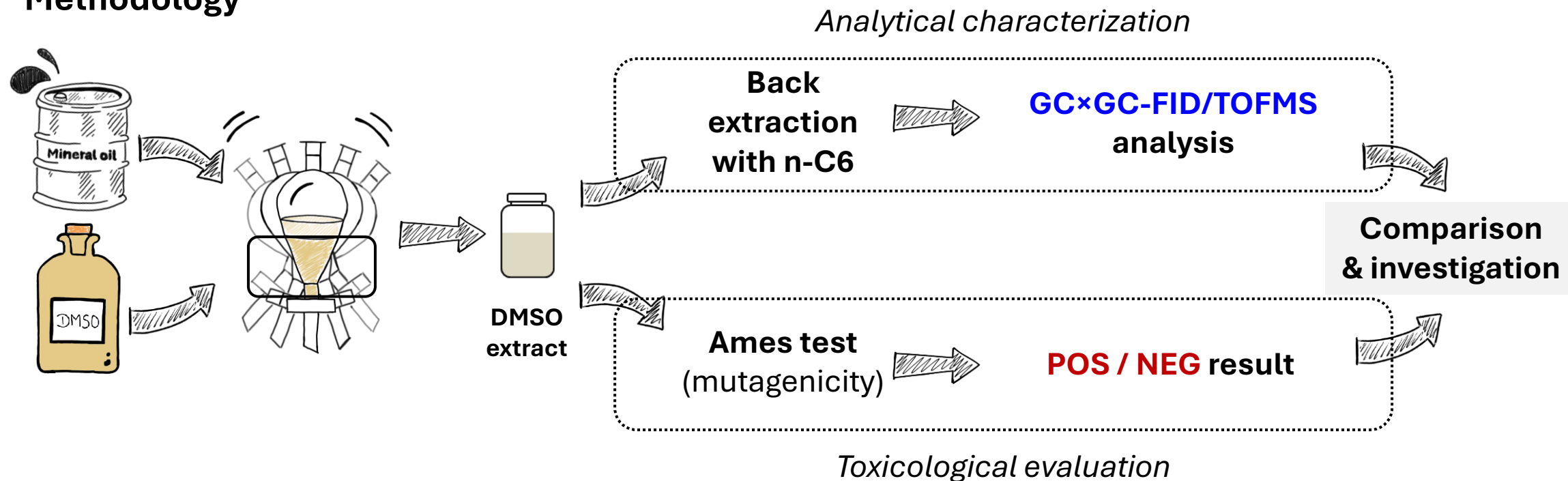


DMSO extract composition vs mutagenicity



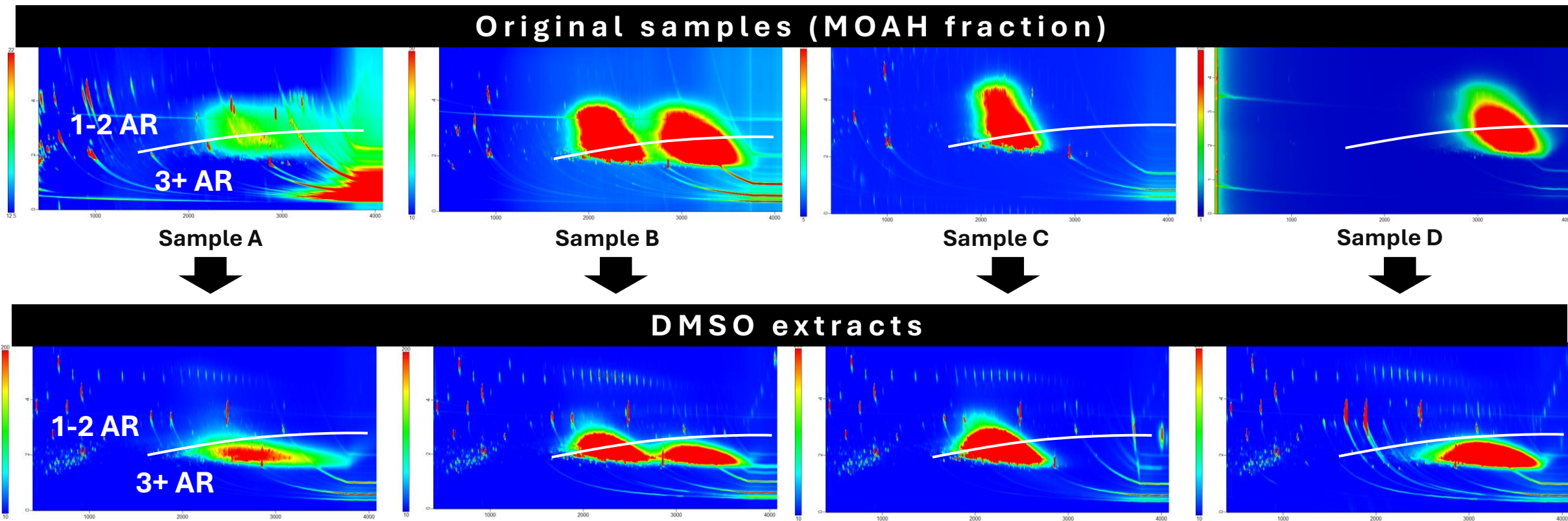
Project: characterize the DMSO extracts of various mineral oil samples (pure and formulations) and correlate it with the modified Ames test response

Methodology





DMSO extraction



→ Concentration of MOAH 3+ AR in the DMSO



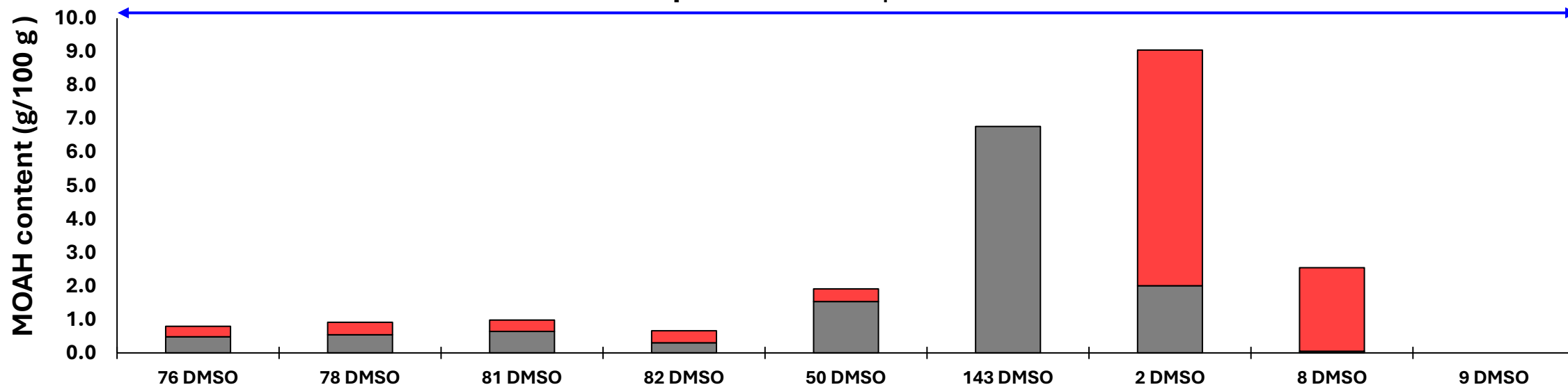
DMSO extract composition vs mutagenicity



■ Hexane - 1,2 Ring ■ Hexane - 3+ ■ DMSO - 1/2 Ring ■ DMSO - 3+

Characterization of the DMSO extracts of different MO samples in terms of MOAH 1-2 AR/3+ AR

→ sub-fraction quantification possible with GC×GC-FID



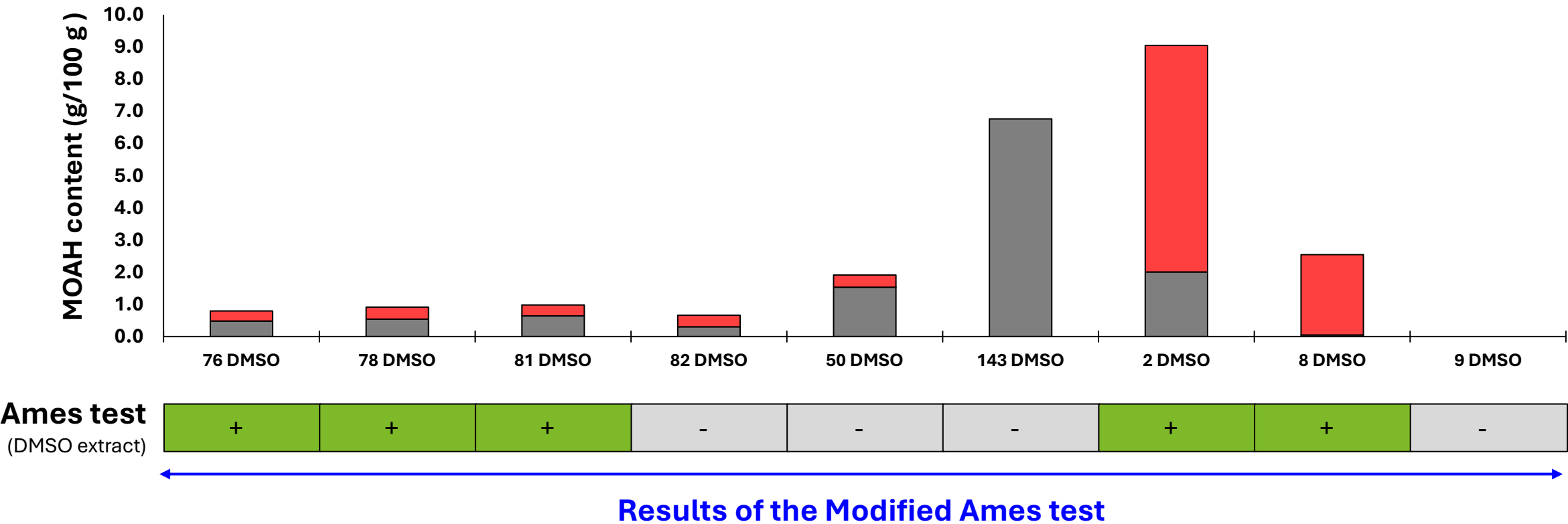


DMSO extract composition vs mutagenicity



Hexane - 1,2 Ring Hexane - 3+ DMSO - 1/2 Ring DMSO - 3+

Targeted correlation – step 1



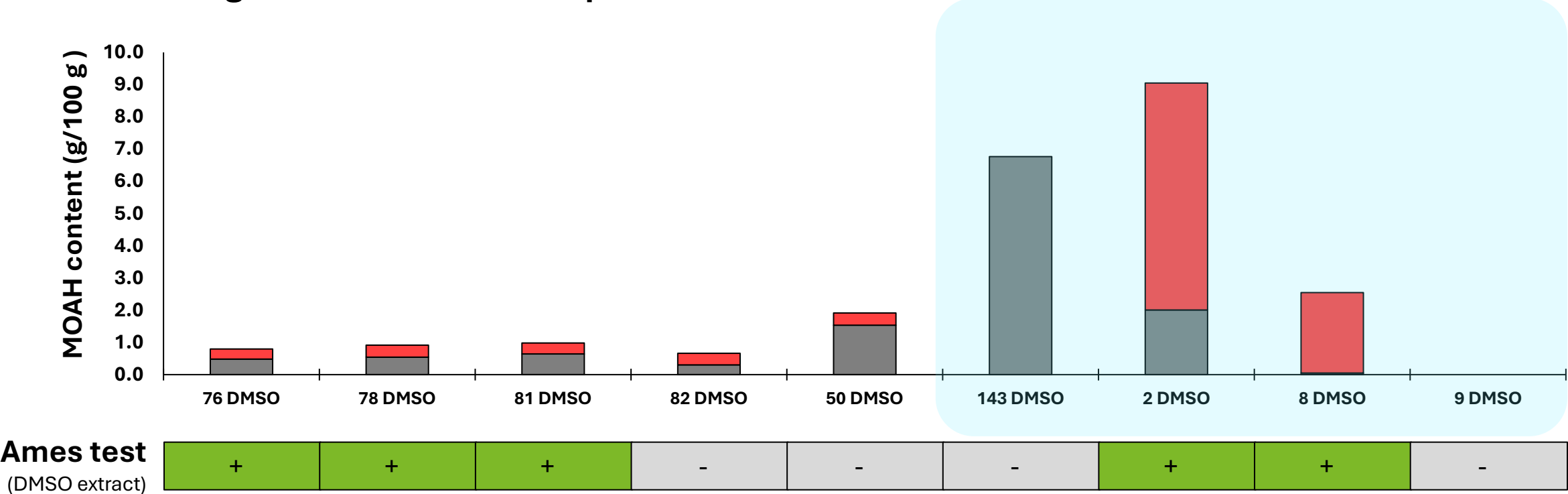


DMSO extract composition vs mutagenicity



■ Hexane - 1,2 Ring ■ Hexane - 3+ ■ DMSO - 1/2 Ring ■ DMSO - 3+

Targeted correlation – step 1



No MOAH or only MOAH 1-2 AR → **negative**

MOAH 1-2 & 3+ AR → **positive**

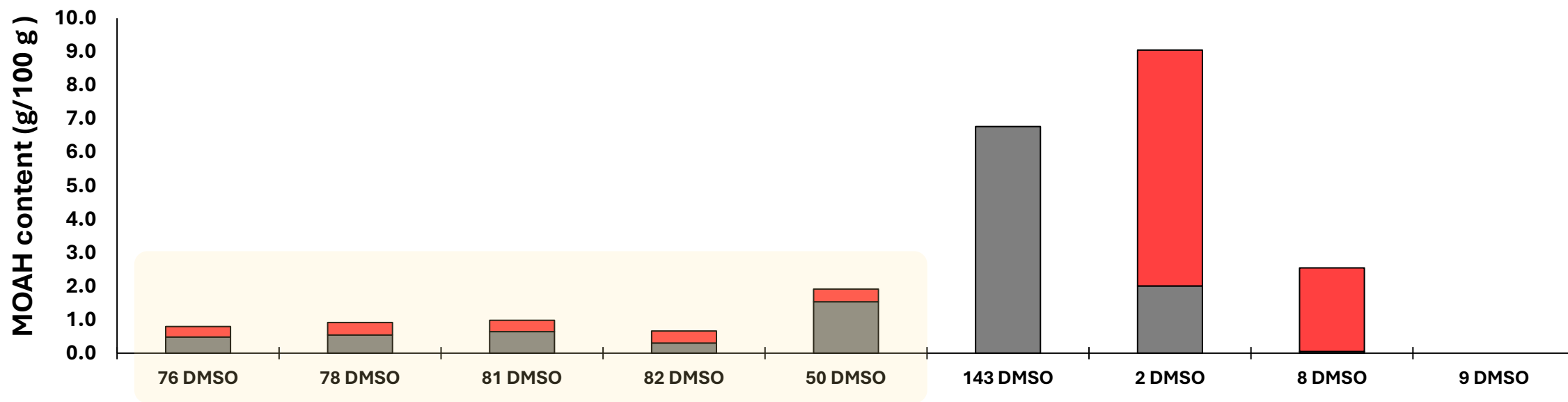


DMSO extract composition vs mutagenicity



■ Hexane - 1,2 Ring ■ Hexane - 3+ ■ DMSO - 1/2 Ring ■ DMSO - 3+

Targeted correlation – step 1



Ames test
(DMSO extract)

+	+	+	-	-	-	+	+	-
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MOAH 1-2 and 3+ AR, at lower concentrations
→ sometimes positive, sometimes negative,
despite similar 1-2/3+ AR content

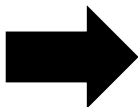
MOAH 1-2/3+ AR composition might not
explain alone positivity



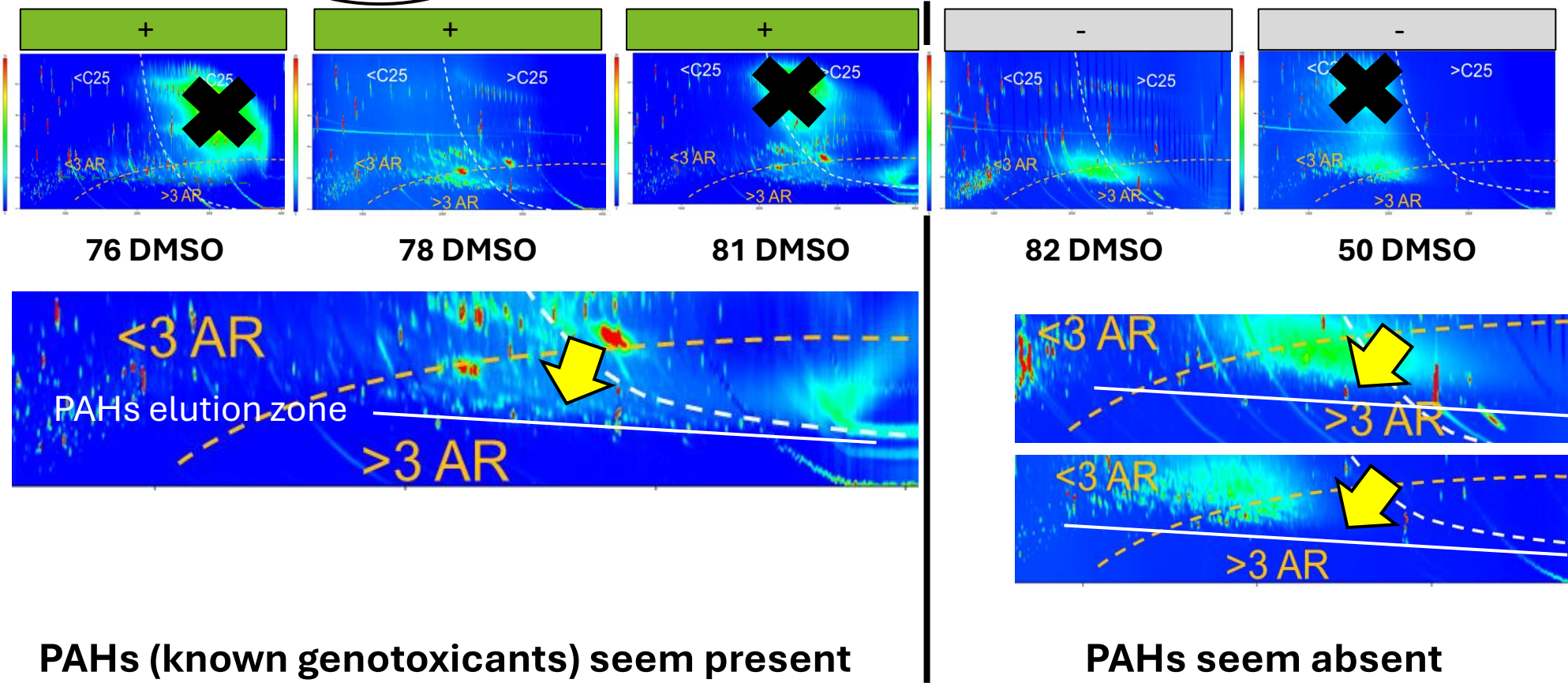
DMSO extract composition vs mutagenicity



What could explain positivity?



Investigation and comparison of GC×GC contour plots



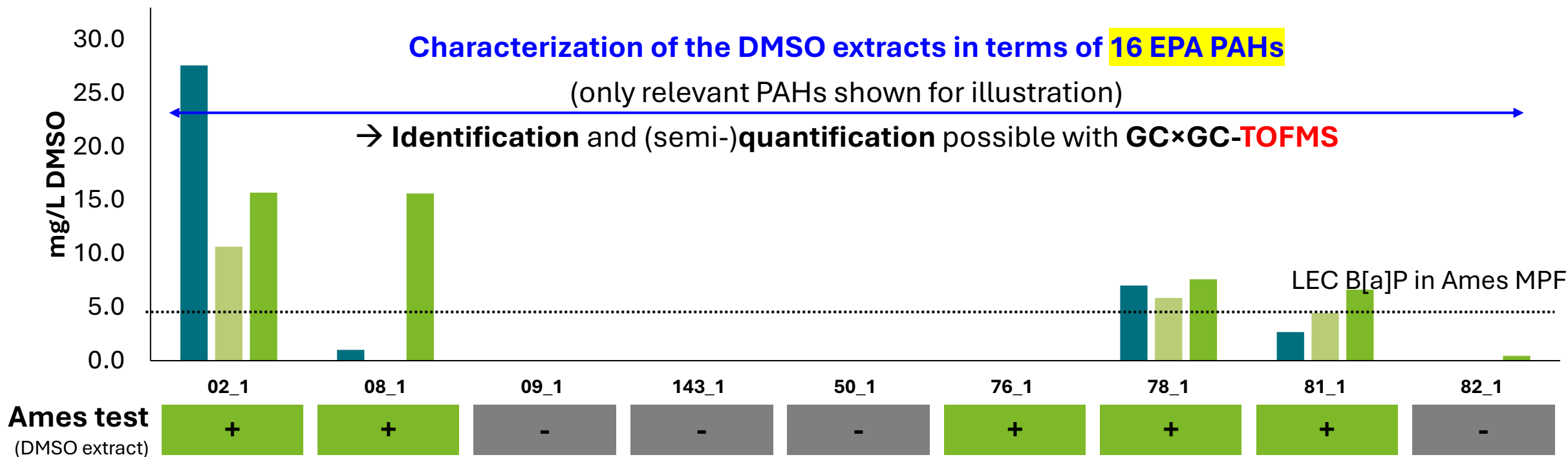


PAHs quantification



■ Chrysene ■ Benzo[k]fluoranthene + Benzo[f]fluoranthene ■ Benzo[a]pyrene

Targeted correlation – step 2



LEC: Lowest Effective Concentration = the lowest tested concentration of B[a]P that produces a statistically significant biological effect.



PAHs quantification

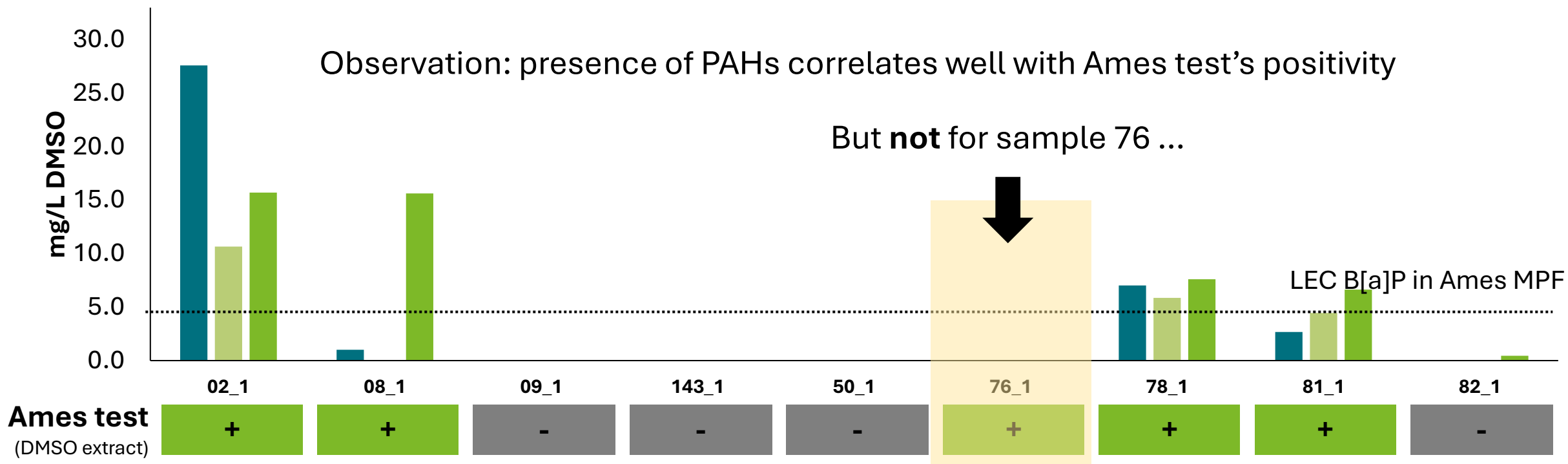


■ Chrysene ■ Benzo[k]fluoranthene + Benzo[f]fluoranthene ■ Benzo[a]pyrene

Targeted correlation – step 2

Observation: presence of PAHs correlates well with Ames test's positivity

But **not** for sample 76 ...



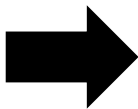
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DMSO extract composition vs mutagenicity

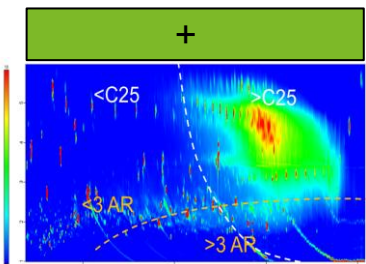


What could explain positivity?

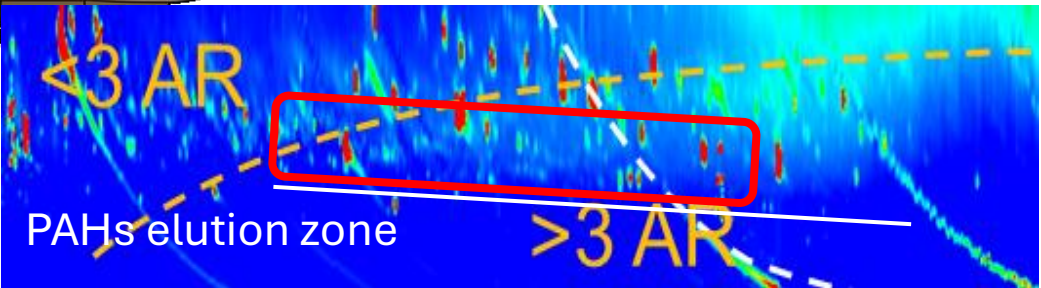


Investigation and comparison of GC×GC contour plots

NO PAHs

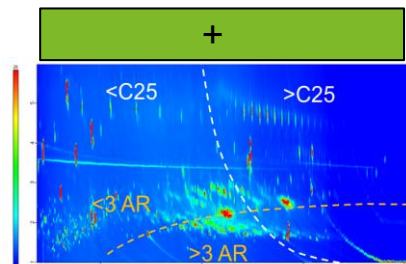


76 DMSO

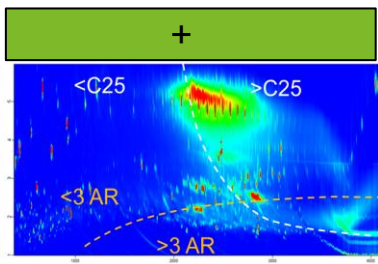


No PAHs but presence of **weakly alkylated PACs**

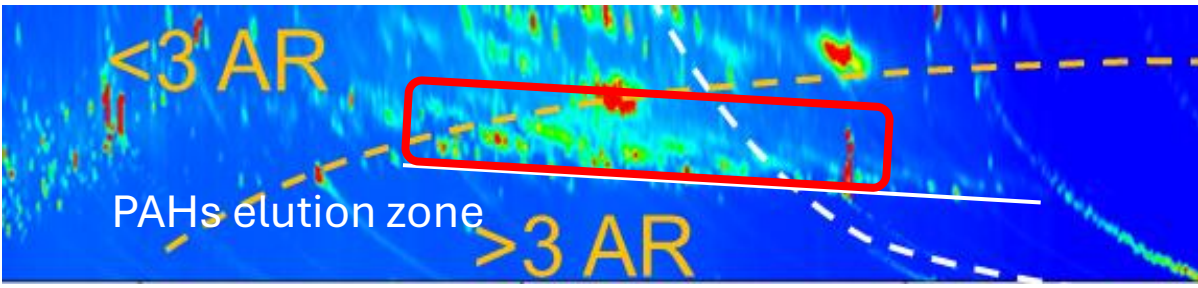
YES PAHs



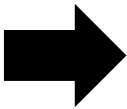
78 DMSO



81 DMSO



PAHs and presence of **weakly alkylated PACs**



Some **weakly alkylated PAHs** having bay-region like motives in their structure **can be bioactivated into mutagenic compounds** in a similar way to PAHs (Carillo et al., 2022)



Summary of the observations



- ✓ Not all “MOAH 3+” that are genotoxic, but rather co-extracted PAHs and weakly alkylated PACs

However, **this approach is not straightforward**, as identifying the compounds responsible for biological activity **requires access to numerous samples** with **complementary** and **sufficiently distinct compositions**.

PART II

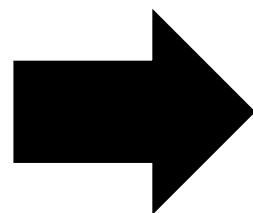
**Combination of GC×GC-FID/TOFMS characterization and
novel in vitro bioassays for genotoxicity screening**



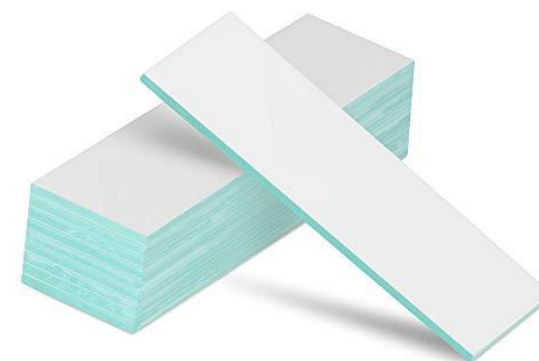
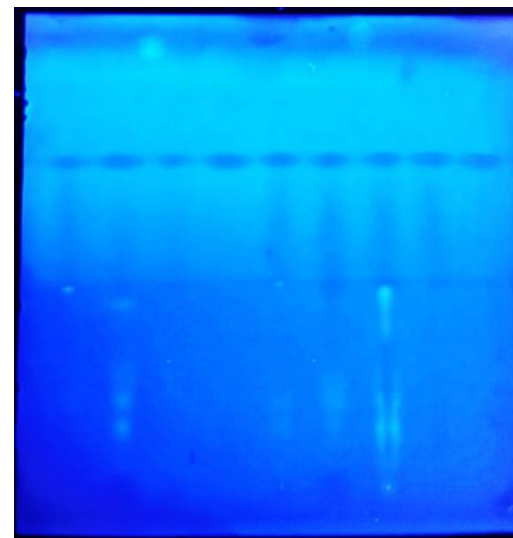
From liquid to solid



**Bioassay in aqueous-based
culture media**

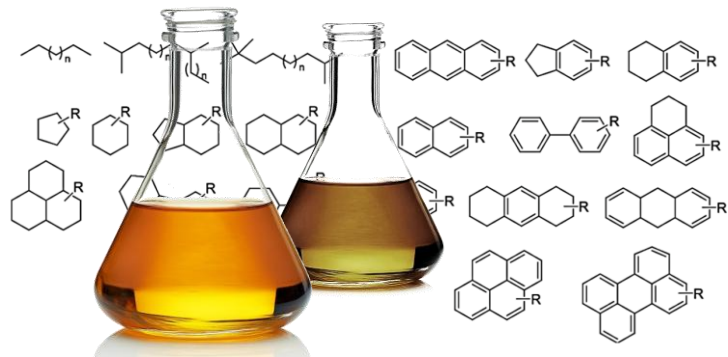


**Bioassay on a solid support
HPTLC-SOS-UmuC**



Principle of the HPTLC-SOS-UmuC method

Sample preparation (dilution in, e.g., *n*-C₆)



Sample plating (up to 15/plate)

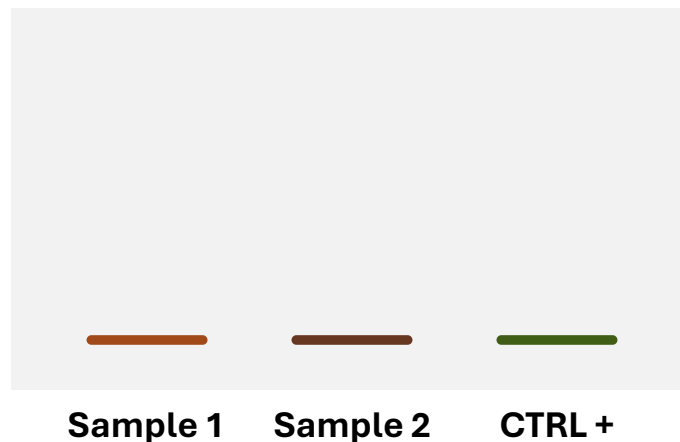
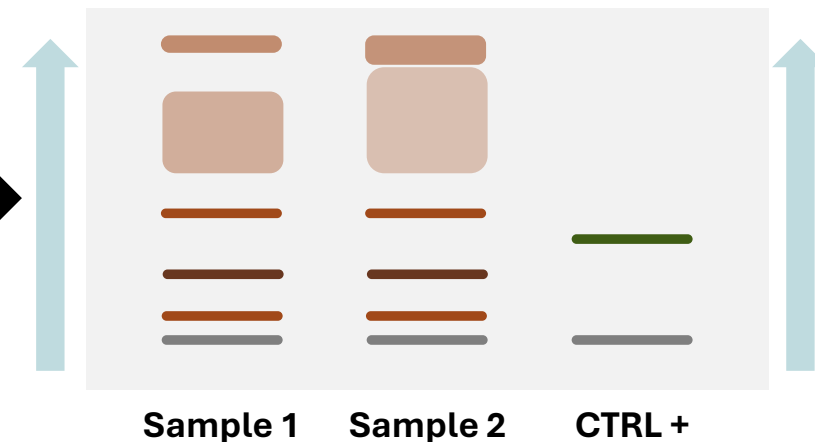
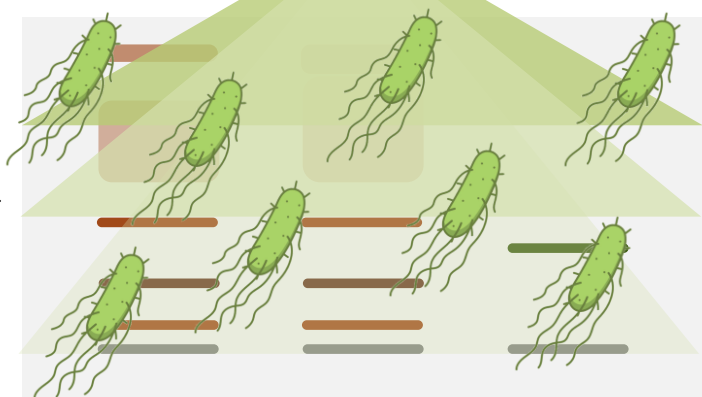


Plate elution and separation of compounds

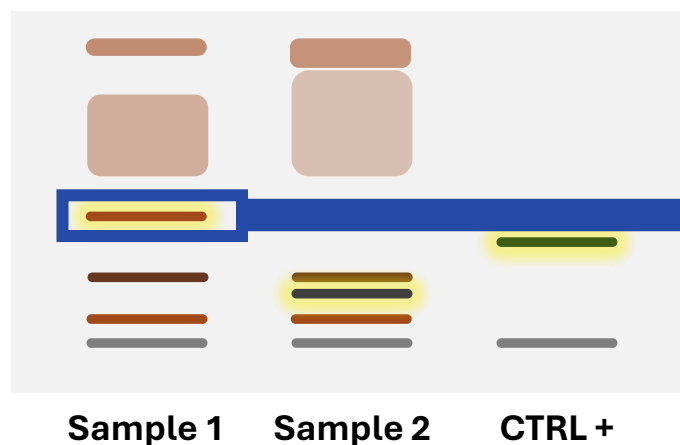


Spraying of *G.M. bacteria* + plate incubation



Sample 1 Sample 2 CTRL +

Genotoxicity screening result



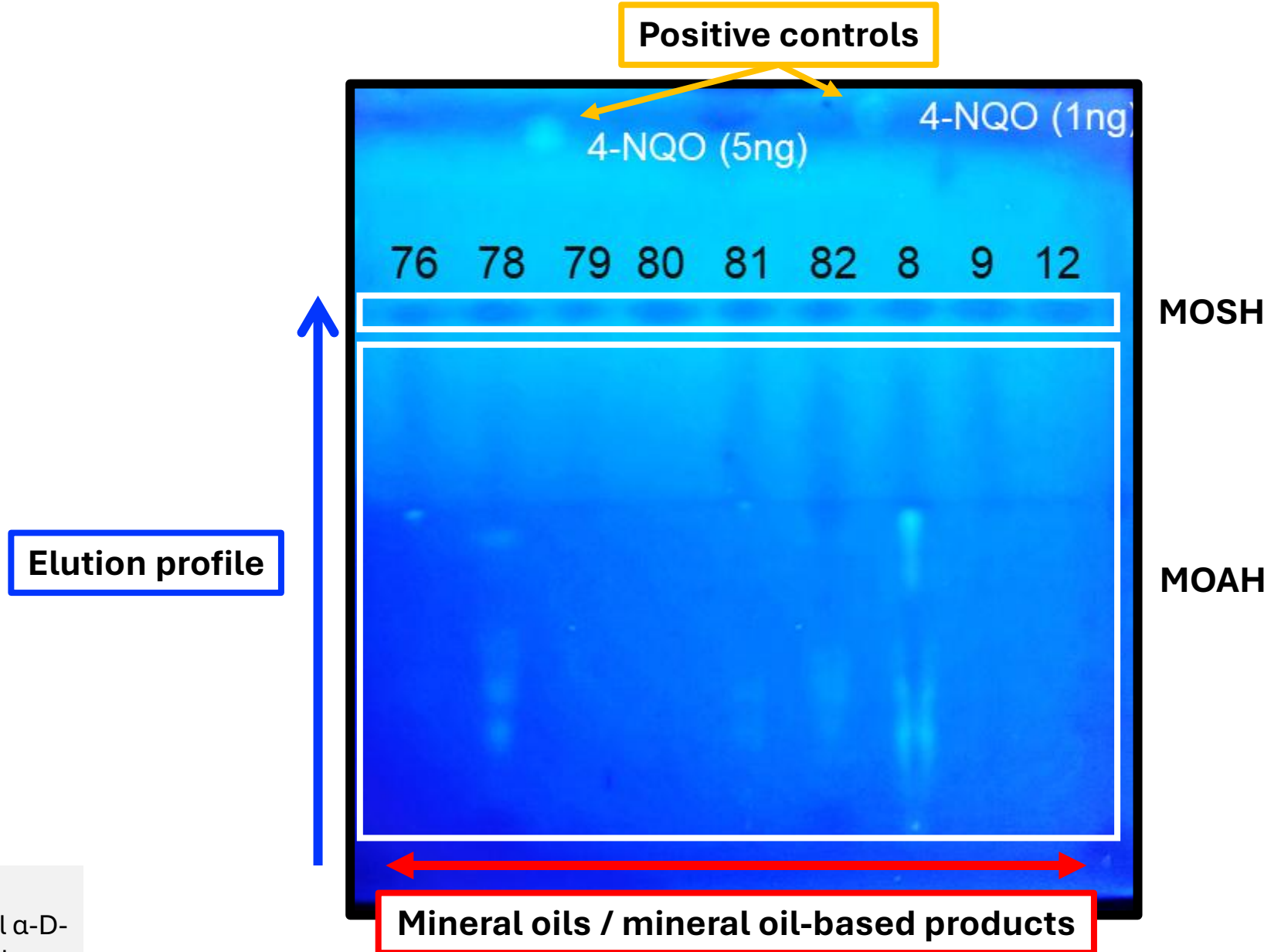
Sample 1 Sample 2 CTRL +

GC×GC

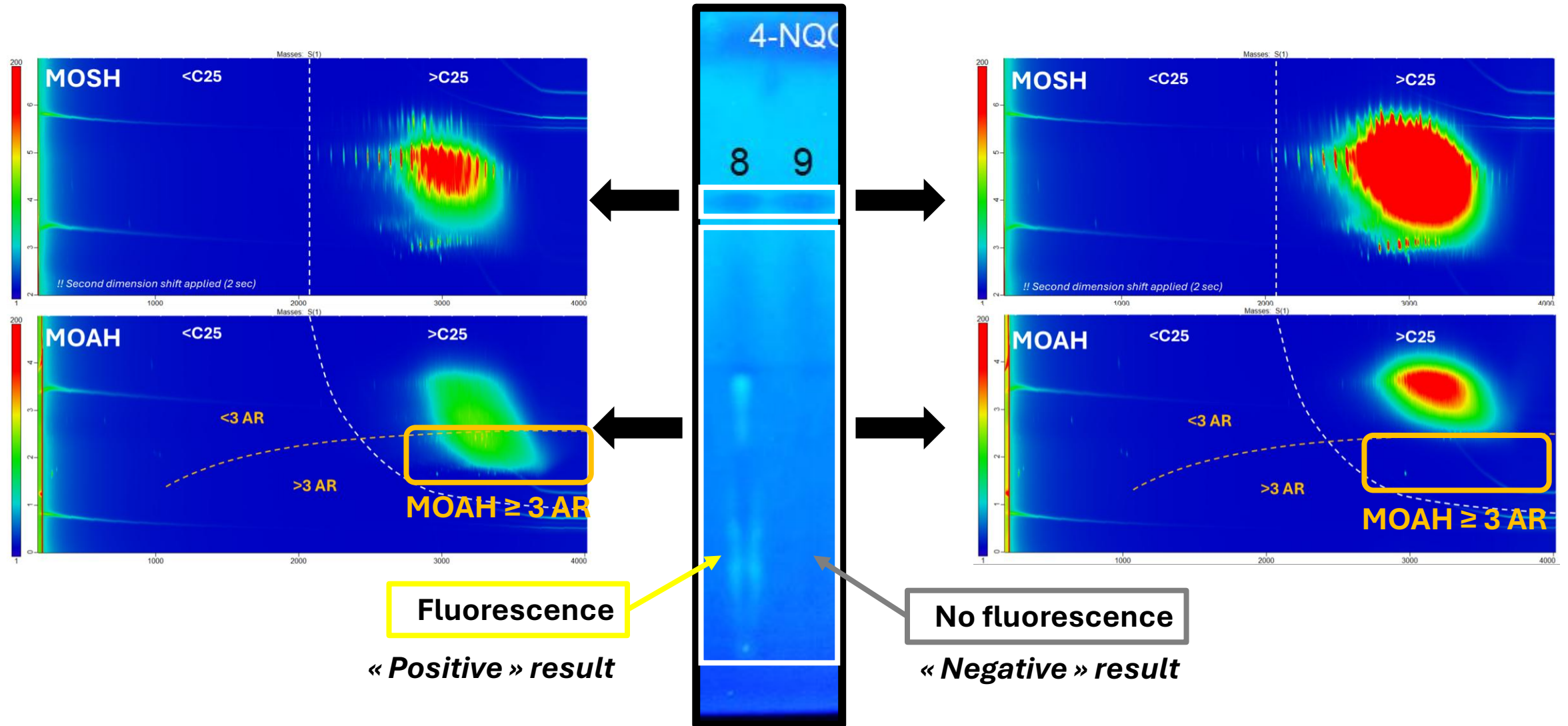
Fluorescent signal ↔ Genotoxicity

Application of the HPTLC-SOS-UmuC method to **mineral oils**

Application of the HPTLC-SOS-UmuC method to mineral oils

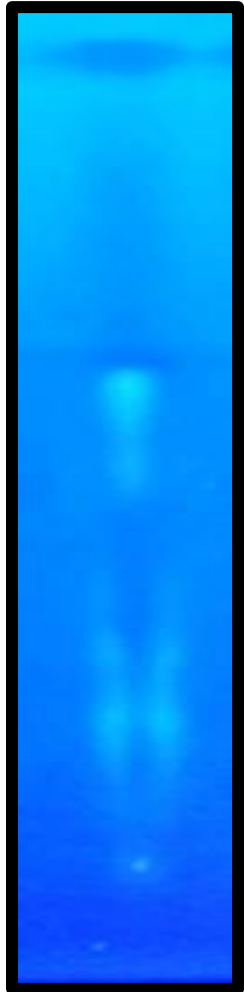


Application of the HPTLC-SOS-UmuC method to mineral oils

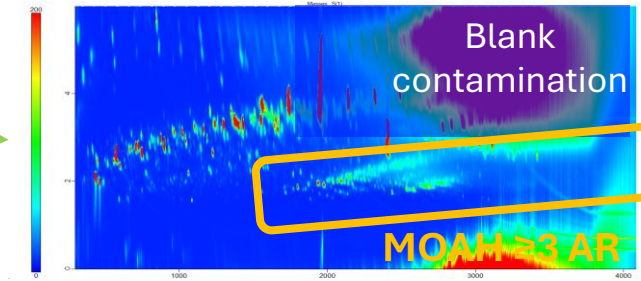
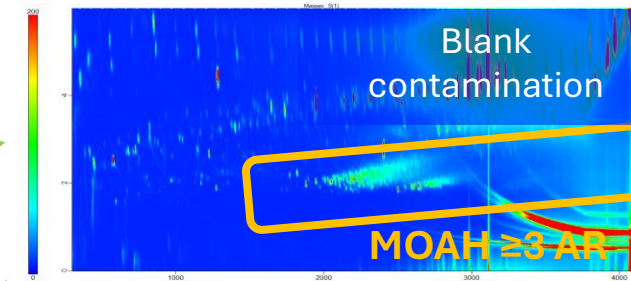
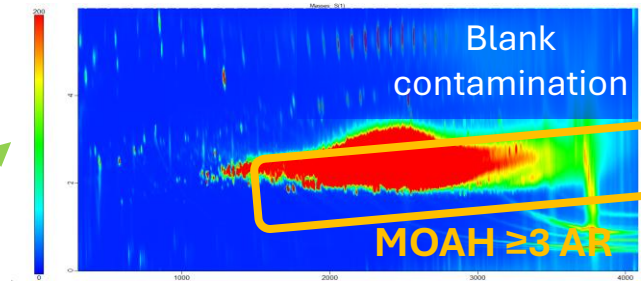
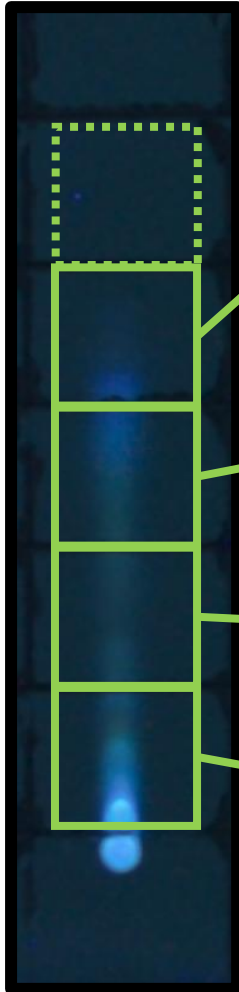


Application of the HPTLC-SOS-UmuC method to mineral oils

Sample 8
Derivatized



Other MO
Underderivatized



Scratch
Extract
Inject

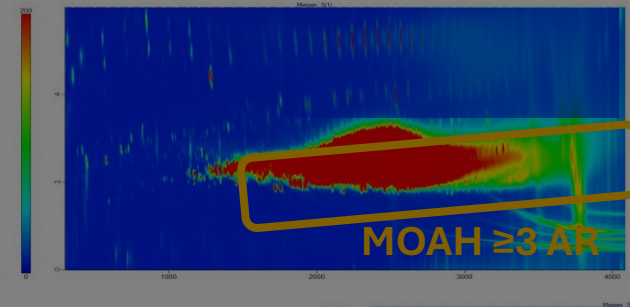
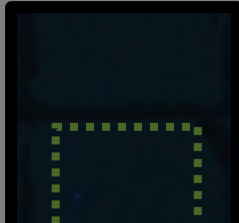
Note: the sample shown here is NOT sample 8. Another sample was used for illustration of the scratching – extraction – injection.

Application of the HPTLC-SOS-UmuC method to mineral oils

Sample 8
Derivatized

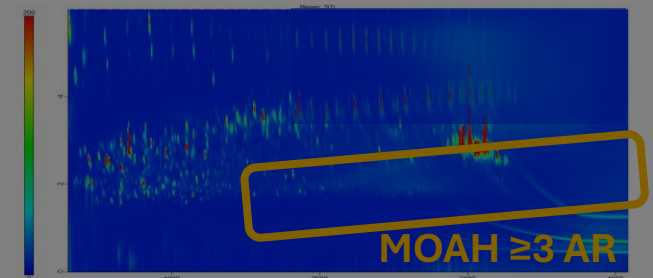
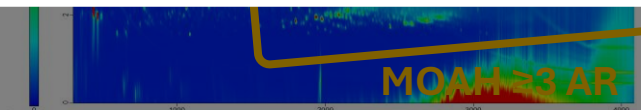
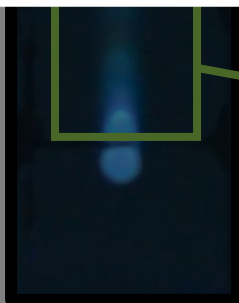
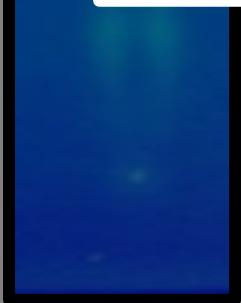


Other MO
Underivatized



Note: the sample shown here is NOT sample 8. Another sample was used for illustration of the scratching – extraction – injection.

- Fluorescence was observed in the elution region of **weakly alkylated PACs**.
- Consistent with the conclusions of the conventional approach.
- Provides **more direct evidence** of the compounds associated with genotoxicity and **enables testing of the whole complex mixture**

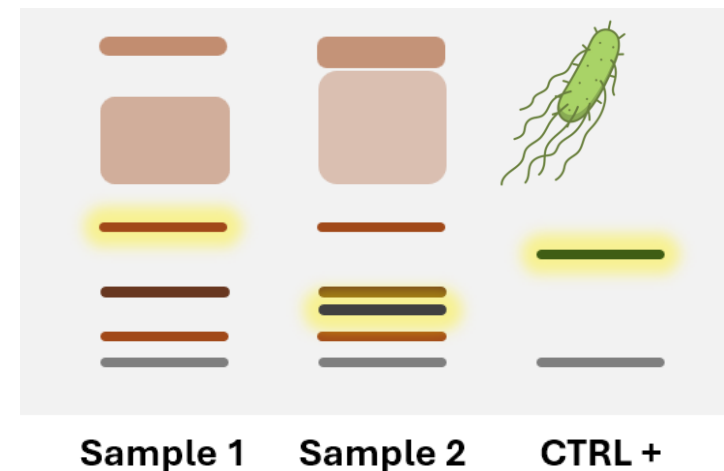
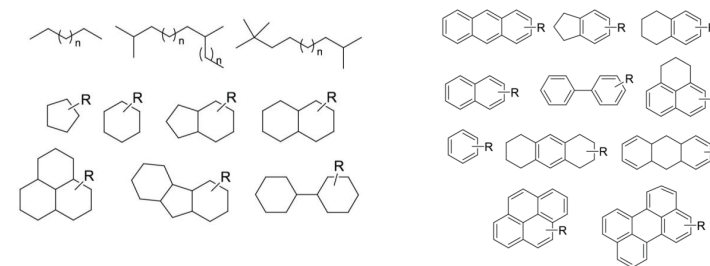
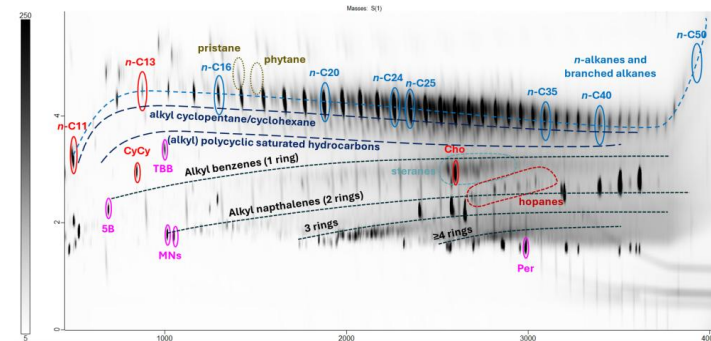




Conclusion and perspectives



- The toxicological evaluation of **hydrophobic complex mixtures** such as MOSH/MOAH remains **highly challenging**.
- **GC×GC-FID/TOFMS combined with bioassays** provides a powerful framework for investigating such mixtures.
- **Additional chromatographic separation** prior to bioassays (using **HPTLC-SOS-UmuC**) can further improve selectivity and facilitate interpretation.





Conclusion and perspectives



- The results presented here represent **only the beginning** of the journey. Further **investigations** and method **optimization** are ongoing.
- Nevertheless, these approaches have **the potential** to help resolve some of the **long-standing uncertainties** regarding the toxicological properties of MOSH/MOAH and support the **refinement of future regulatory thresholds**.

Team members

- **Prof. Giorgia Purcaro** – head of the Analytical Chemistry lab (Gembloux Agro-Bio Tech)
- **Prof. Andrea Hochegger (TU Graz)** – Visiting Professor; collaborators from Medical University of Graz and OFI
- **Damien Pierret** – PhD student
- **Sophie Vancraenenbroeck** – Head technician
- Damien Eggermont – Teaching assistant & PhD student
- Carlo Bellinghieri – PhD student
- Chaimae Chaibi – PhD student
- Mattia Grego – Master's student/intern
- Siebe Lievens – ACCESSS research manager



Partners



*Thank
you!*