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# In-vitro production of standards to identify biomarkers of exposure and effect of the emerging mycotoxin Versicolorin A

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## Introduction

The toxicity of bisfuranoid mycotoxins, like aflatoxin B1 (AFB1) or sterigmatocystin, is mostly determined by their biotransformation, detoxification, or bioactivation. It is well established that the in vivo biotransformation of AFB1 into a highly reactive molecule is the key step in the genotoxic process and therefore in carcinogenesis [1]. Hence, the presence of a bisfuran moiety in Versicolorin A (VerA), a precursor of AFB1 and co-detected with AFB1 in food, may also explain its genotoxicity. VerA is not commercially available and its metabolization has not been studied so far. Therefore, the purpose of this study was to establish the characterization of metabolites derived from synthesized VerA. To this aim, our strategy was based on the construction of a library of VerA metabolites using human in vitro S9 fraction incubations in the presence of appropriate cofactors coupled to UHPLC-ESI-HRMS [2]. The resulting chromatographic and mass-spectrometric data were used to identify VerA metabolites produced by intestinal cell lines as well as intestinal and liver tissues exposed *ex vivo*.

## Materials and methods

**VerA metabolites production with S9 fraction:**

- VerA (50uM) + human liver S9 fraction (mixed-gender) + cofactors (GSH, UDPGA, PAPS, NADP, G6P and G6Pdehydrogenase)
- 3 hours of exposure at 37 °C

**In-vitro model:**

- VerA (10 uM) + intestinal porcine epithelial cell (IPEC1)
- 24 hours of exposure at 39 °C

**Ex-vivo model:**

- VerA (50uM) + porcine jejunum explants / liver slices
- 3 hours of exposure at 39 °C

**LC-MS analyses:**

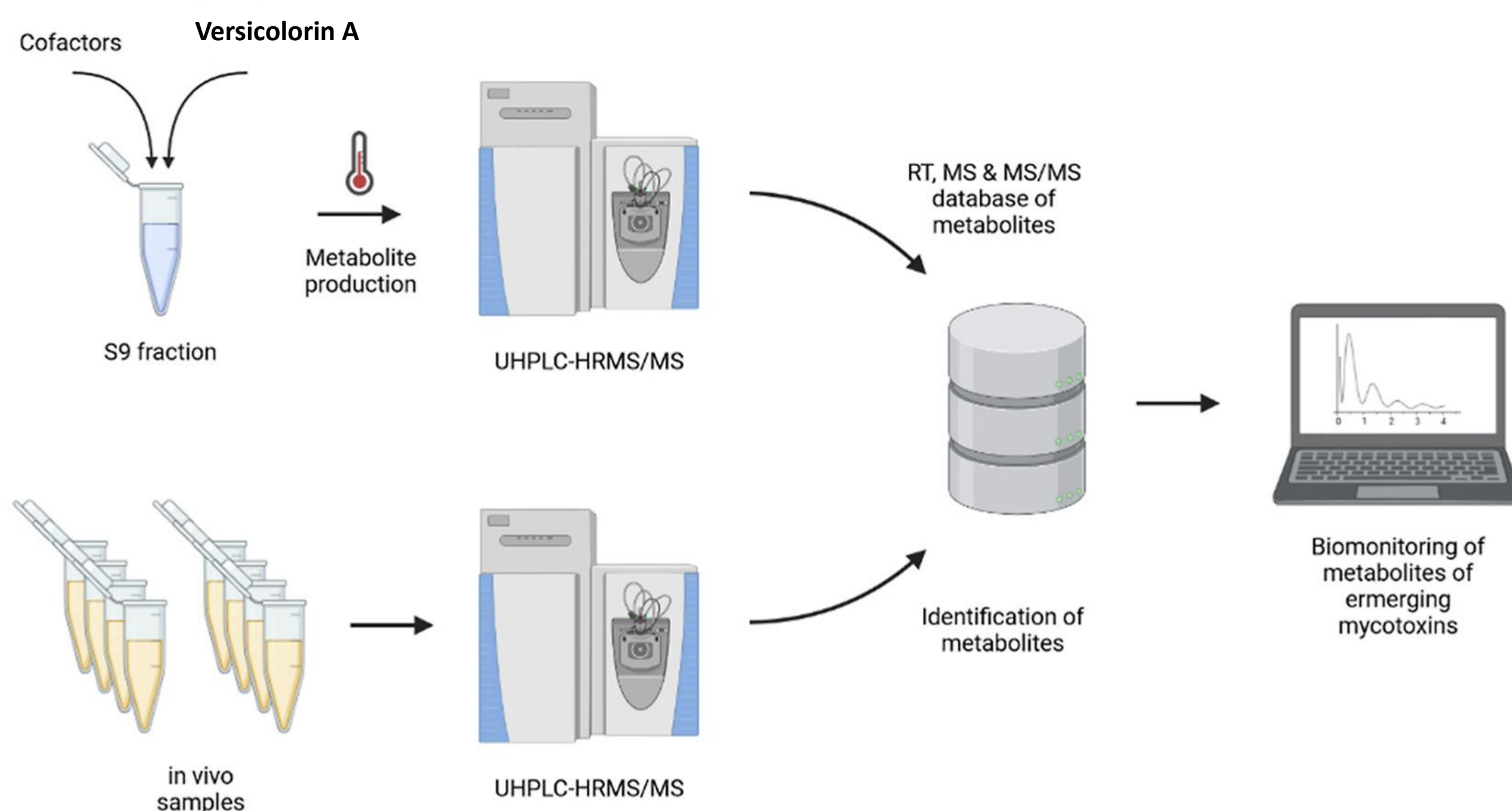
- U3000 UHPLC (Thermo), Hypersil Gold C18 column (100 x 2.1 mm, 1.9 μm) Vinj=10μL, A:B H<sub>2</sub>O/CH<sub>3</sub>OH/CH<sub>3</sub>CO<sub>2</sub>H at 0.3mL/min, 40°C, 0 to 100% of B in 30min
- LTO-Orbitrap XL (Thermo), HESI pos. & neg.

**Identification of metabolites:**

- Screening of suspected compounds (bibliography, expertise)
- Screening of *in-silico* metabolites (MetaSense®)
- HRMS<sup>n</sup> LTO-Orbitrap XL (Thermo), fragment ions interpretation (expertise, CFM-ID)

## Results and discussion

### Workflow of metabolites identification in complex samples using *in vitro* production of standards by S9 fraction:



### Construction of a library of VerA metabolites using the *in vitro* S9 fraction:

Compound	Measured m/z	Error (ppm)	Molecular formula	Retention time (min)	Identification level	Observed product ions m/z (relative abundance)
VerA	337.0359	-2.1	C <sub>18</sub> H <sub>10</sub> O <sub>7</sub>	23.2	1	309.1 (100), 308.1 (55), 293.1 (14), 337.1 (12), 320.0 (5), 265 (5), 252 (4), 280 (3)
M + O	353.0304	-2.8	C <sub>18</sub> H <sub>10</sub> O <sub>8</sub>	18.9	3	309.1 (100), 335.2 (97), 325.1 (93), 281.1 (23), 310.1 (17), 265.1 (11), 297.1 (10), 307.1 (5), 308.0 (5), 311.1 (3), 324.2 (3), 353.0 (2)
M + H <sub>2</sub> O	355.0465	1.7	C <sub>18</sub> H <sub>12</sub> O <sub>8</sub>	18.4	3	337.1 (100), 309.1 (60), 310.1 (24), 327.1 (21), 355.2 (14), 299.1 (13), 325.1 (9), 311.1 (6), 293.2 (5), 284.0 (5), 297.2 (4), 270.1 (3)
M + GlcAc	513.0677	2.7	C <sub>24</sub> H <sub>18</sub> O <sub>13</sub>	19.1	3	337.1 (100), 175.0 (2), 51.3.1 (2)
M + O + GlcAc	529.0619	-2.1	C <sub>24</sub> H <sub>18</sub> O <sub>14</sub>	14.2	3	353.1 (100), 529.1 (2)
M + SO <sub>3</sub>	416.9910	-2.9	C <sub>18</sub> H <sub>10</sub> O <sub>10</sub> S	22.0	3	337.1 (100), 417.0 (2)
M + O + SO <sub>3</sub>	432.9859	-2.8	C <sub>18</sub> H <sub>10</sub> O <sub>11</sub> S	14.9	3	353.1 (100), 433.0 (2)

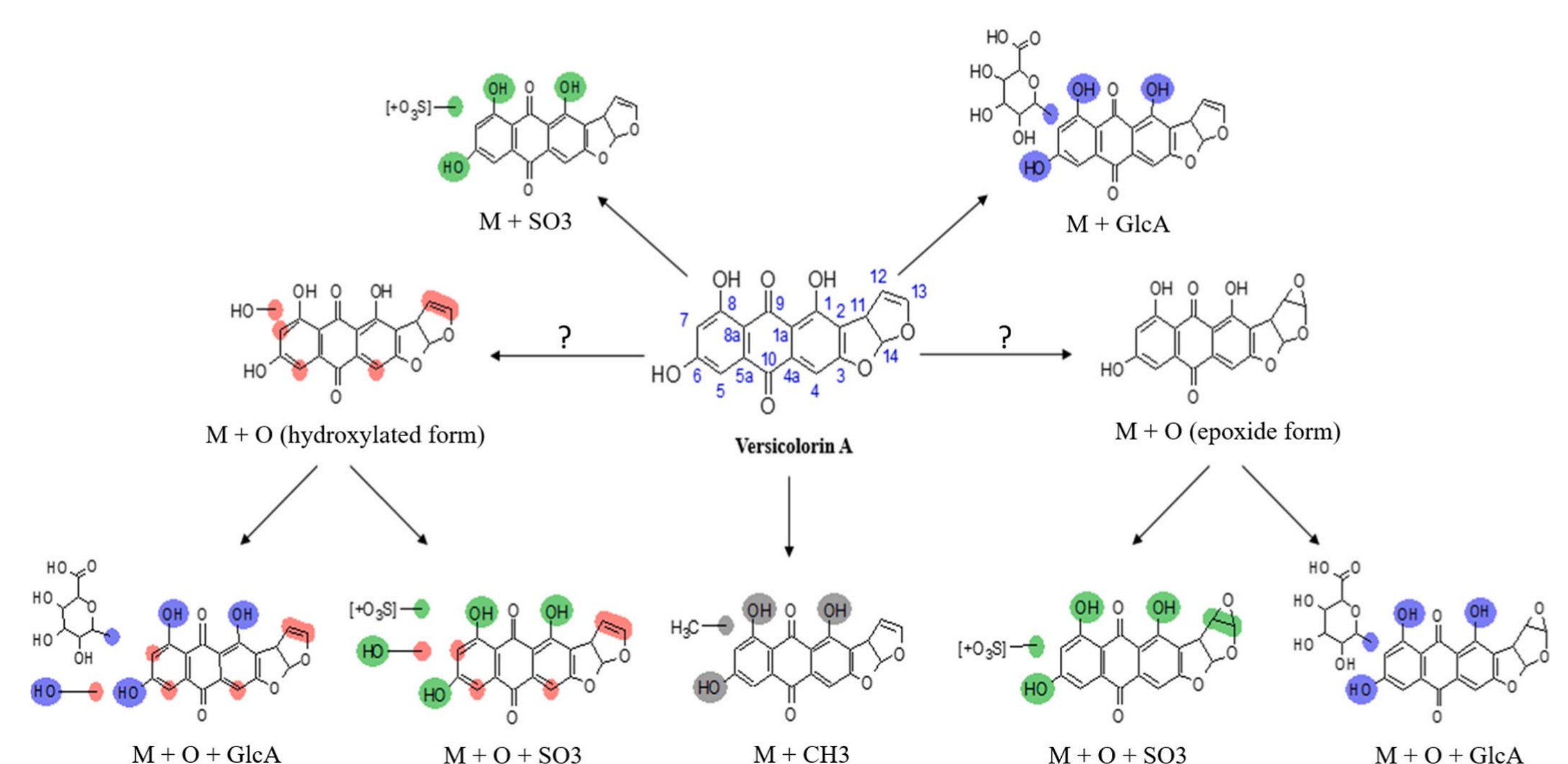
Six compounds were detected including phase I and phase II biotransformation metabolites along with intact VerA.

### VerA metabolites identified in intestinal cells, intestinal and hepatic tissues:

Compound	In vitro (intestinal cells)	Ex vivo (intestinal explants)	Ex vivo (liver explants)
VerA	Yes	Yes	Yes
M + O	Yes	No	Yes
M + H <sub>2</sub> O	No	No	No
M + GlcAc	No	Yes	Yes
M + O + GlcAc	No	No	Yes
M + SO <sub>3</sub>	Yes	No	No
M + O + SO <sub>3</sub>	No	No	No
M + CH <sub>2</sub>	No	No	Yes

- Two additional metabolites (M + O and M + SO<sub>3</sub>) were identified in cells exposed for 24 h and 48 h.
- Four compounds were identified from tissue samples, all characterized metabolites based on data acquired from human liver S9 incubations, except for one metabolite (M + CH<sub>2</sub>)
- Along with the intact toxin, the phase I metabolite M + O compatible with an hydroxylated, but also with an epoxide form of VerA was detected.

### Structures of identified VerA metabolites:



- Red positions correspond to hydroxylation (-OH), green to conjugation with sulfate (-SO<sub>3</sub>), blue to conjugation with glucuronic acid (-C<sub>6</sub>H<sub>7</sub>O<sub>6</sub>), grey to methylation (-CH<sub>3</sub>).
- Conjugation with glucuronic ac. (-C<sub>6</sub>H<sub>7</sub>O<sub>6</sub>) or sulfate (-SO<sub>3</sub>) occurs on hydroxyl functions (-OH)
- Question marks indicate the uncertain biotransformation of VerA into an epoxide and/or hydroxylated form.

## Conclusions

Using an *in vitro* production of standards, we were able to characterize metabolites representative of the exposure to VerA [2]. Moreover, one metabolite (M+O) suggests the possible bioactivation of VerA into an epoxide form, which might be responsible for the genotoxic and mutagenic effects. This result provide high-quality analytic evidence for further risk assessment of this harmful emerging mycotoxin. This detection of exposure biomarkers and effect biomarkers was possible only by the validation of identifications using S9 fraction incubations, which can be further applied to other emerging environmental and food contaminants for internal chemical exposomics studies.

## References

- Eaton D.L. et al., 2010, Hepato. Mycotox. Compr. Toxicol. 9, 527
- Al-Ayoubi C. et al., 2022, Food Chem. Toxicol., 167, 113272

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