Investigation of the Zearalenone Metabolism: Electrochemical vs. *in vitro* and *in silico* Approaches

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Objective

In this poster the use of Electrochemistry–MS (EC-MS) as a complementary technique to *in vitro* and *in silico* approaches is investigated to predict and mimic the oxidative metabolism the mycotoxins zearalenone.

- Can EC-MS facilitates the detection of instable metabolites (missed with *in vivo* and *in vitro* techniques)?
- Is EC-MS complementary to *in vivo, in vitro,* and *in silico* techniques?
- Can EC-MS as a purely instrumental approach provide rapid insight into the metabolic pathway?

1. Zearalenone Metabolism: Why and How?

Mycotoxins are toxic metabolites produced by mold fungi, which commonly contaminate cereal crops. One of the most dangerous mycotoxins is Zearalenone (ZEA), which shows strong estrogenic activity. The metabolites of ZEA that are produced in the liver of mammals also play a major role in the overall toxic properties of ZEA. Therefore, it is important to investigate the metabolic pathway of ZEA.

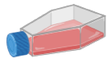
The oxidative metabolism usually covers a combination from *in vitro, in silico* and *in vitro* assays which use hepatic cells, cell microsomes, laboratory animals, theoretical methods and/or MS/MS for structure elucidation. The addition of Electrochemistry (as a purely instrumental approach) expands the possibility to detect metabolites, including short lived and/or reactive intermediates, in a quick and simple manner.

2. Materials and Methods

2.1. Investigation of ZEA Metabolism by Eucaryotic Cells (*in vitro*)

Human liver cancer cell line HepG2 (European Collection of Authenticated Cell Cultures) were cultured as monolayers:

- Medium: Eagle's Minimum Essential Medium (EMEM, Sigma Aldrich) supplemented with 10% (v/v) heat-inactivated Fetal Bovine Serum, 100 U/mL penicillin and 100 µg/mL streptomycin.
- Conditions: plastic tissue culture bottles with cells (37°C, humidified atmosphere of 5% CO_2) were passaged by 0.25% Trypsin/EDTA every 3-4 days
- Treatment: cells were seeded in bottles (25 cm^2) in density 10^5 cells/mL, and after 24 h the cells were exposed to ZEA, and the supernatant was sampled at time 0, 24 h and 48 h.
- Analysis of ZEA and metabolites: an HPLC-ESI-MS/MS 8050 system (Shimadzu, Tokyo, Japan) was used.



2.2. Investigation of ZEA Metabolism by Theoretical Calculation (*in silico*)

The optimization of the ground-state geometry was carried out using the B3LYP functional. The 6–311 ++ G (d,p) basis set was used for all the quantum chemical calculations. All the electronic structure calculations were performed using the Gaussian 2009 program (Frisch et al., 2009).

2.3. Investigation of ZEA Metabolism by EC-MS

For the EC-MS experiment the ROXY™ system, equipped with a ReactorCell™ (Antec Scientific, The Netherlands) and a boron-doped diamond working electrode, directly connected to a 6460 TripleQuad MS with an ESI interface (Agilent, Germany) was used.

Generation of Phase I metabolites by on-line EC-MS:

- Sample: 5 µM ZEA solution in 10 mM ammonium formate (pH 7.2 ± 0.3) and acetonitrile
- Flow rate: 10 µL/min
- Potential: from 0 to 3000 mV (at 10 mV/sec) or selected constant voltages
- Reaction temperature: 36.7 °C

Generation of Phase II metabolites (conjugation)

- Simply by adding 25 µM glucuronic acid after the ReactorCell via a T-piece to conjugate the phase I metabolites.

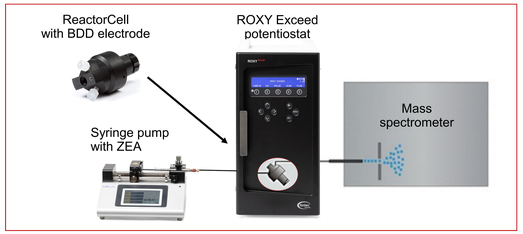


Figure 1: *Schematics instrumental set up for generation of phase I and II metabolites.*

For molecular structure elucidation of the detected metabolites, in-source fragmentation in the ESI interface was used. Settings of the tandem mass spectrometry are listed in [2].

3. Results

3.1. ZEA Metabolism by Eucaryotic Cells

Both *α*-ZOL and *ß*-ZOL became detectable after 24 h of exposure to ZEA. The dominant metabolite of ZEA as produced *in vitro* was found to be *ß*-ZOL.

Table 2: *Metabolization of ZEA, α-ZOL and ß-ZOL in HepG2 cells line.*

Time [h]	Concentration µg/mL		
	ZEA	α-ZOL	β-ZOL
0	0.1424	–	–
24	0.0622	0.0118	0.3881
48	0.0397	0.0025	0.2547

3.2. Phase I and II ZEA metabolism by EC

Extracted ion currents of ZEA and its metabolites are shown in Figure 3. With increasing working potential, the intensity of ZEA decreases, and the intensity of the proposed ZEA metabolites of phase I increase.

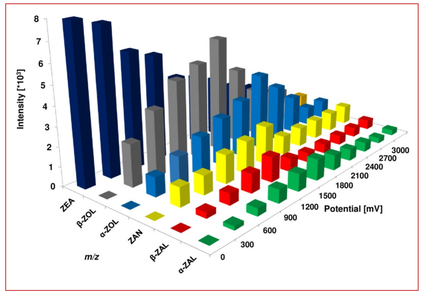


Figure 3: *MS Voltammogram of ZEA and its major oxidation products (metabolites) generated by EC. Peaks represent the proposed metabolites of phase I.*

In Figure 4 the mass spectra of phase I and phase II metabolites are shown, and the 14-*O*-Gluc/16-*O*-Gluc of α-ZOL and β-ZOL were detected too.

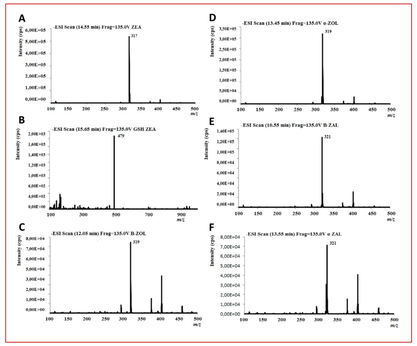


Figure 4: *MS spectra of ZEA (A) and its potential phase I and II metabolites generated at constant voltages:*
GSH ZEA (B) at 1800 mV
ß-ZOL (C) at 1500 mV
α-ZOL (D) at 1800 mV
ß-ZAL (E) at 1200 mV
α-ZAL (F) at 1500 mV

3.3. Theoretical Calculation of ZEA Metabolites

Based on the calculations we found two lowest energy geometries for α-ZOL and β-ZOL (Rogowska *et al.*, 2022). Hence, we can conclude that each metabolite can be created in reaction, but in non-equal amounts. The results obtained from the calculation were in agreement with the results obtained by EC-MS.

3.4. Proposed Metabolic Pathway of ZEA

The potential metabolites from ZEA and the proposed transformational pathway based on the presented results are shown in Figure 5

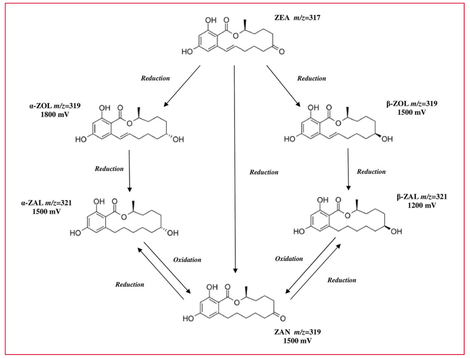


Figure 5: *Proposed transformational pathway of ZEA*

Conclusions

The oxidative metabolism of ZEA was investigated with *in vitro* eucaryotic liver cell incubations, direct infusion EC-MS analysis, and *in silico* theoretical calculation. Based on the results we propose a ZEA metabolic pathway in mammals and we draw the following conclusions about the use of EC-MS:

- EC-MS made it possible to detect 14-*O*-Gluc and 16-*O*-Gluc in addition to α and β metabolites, whereas eukaryotic cells and the theoretical study only showed the ZEA metabolization to α and β forms.
- The purely instrumental EC-MS is an interesting alternative approach to investigate oxidative reactions that occur in the human body. It can quickly generate the main oxidative reaction products. EC-MS provides a valuable addition to *in vivo* and *in vitro* experiments.
- EC is a very simple and rapid technique (minutes) to generate phase I and II metabolites.

References

[1] Zloch *et al.* "Use of *Lactobacillus paracasei* strain for zearalenone binding and metabolization." Toxicon 181 (2020) 9-18

[2] Rogowska *et al.* "Investigation of the mechanism of zearalenone metabolization in different systems: Electrochemical and theoretical approaches." Toxicon 210 (2022) 19-24