



Electrochemical  
Reactions upfront  
MS – EC/MS

**Proteomics &  
Protein Chemistry**

S-S bond reduction  
HDX  
Peptide bond cleavage  
Na<sup>+</sup>, K<sup>+</sup> removal  
Drug-protein binding

**Lipidomics & Fatty Acids**

Cholesterol  
Oxysterol  
FAME Biodiesel

**Drug Metabolism**

Mimicking CYP 450  
Phase I & II  
Biotransformation

**Synthesis (mg)**

Metabolites & Degradants

**Pharmaceutical Stability**

Purposeful degradation  
API testing  
Antioxidants

**Environmental**

Degradation & persistence  
Transformation products  
Surface & drinking water

**Food & Beverages**

Oxidative stability  
Antioxidants

**Forensic Toxicology**

Designer drugs  
Illicit drugs

**Healthcare & Cosmetics**

Skin sensitizers

**Genomics**

DNA Damage  
Adduct formation  
Nucleic acid oxidation

## Fast Mimicking of Phase 1 and 2 Metabolism of Acetaminophen using the ROXY™ EC System

- **Acetaminophen, Paracetamol, Tylenol, Panadol, APAP**
- **Simulating CYP450 oxidative metabolism in <10 min.**
- **Controlled oxidation (phase 1) and detoxification (phase 2) reactions**
- **Fast and easy in use, complements HLM, RLM**

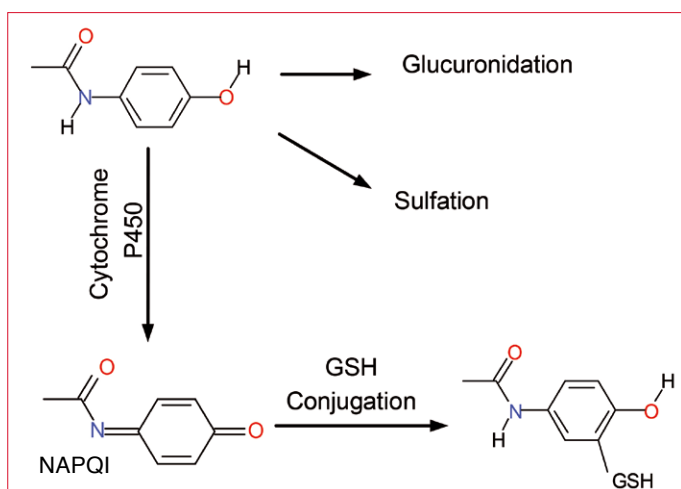
### Summary

Acetaminophen (paracetamol; APAP; IUPAC: N-(4-hydroxy phenyl)acetamide) was chosen as model drug to investigate oxidative metabolism using the ROXY EC System. Electrochemical conversion of the acetaminophen into reactive phase I metabolite – N-acetyl-p-benzoquinoneimine (NAPQI) and the NAPQI – GSH phase II conjugate was successfully achieved.

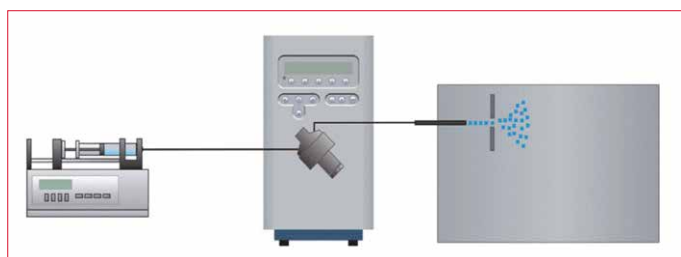
## Introduction

The knowledge of the metabolic pathways and the biotransformation of new drugs are crucial for elucidation of degradation routes of the new active compounds, especially in the area of possible toxicity. *In vitro* studies are based on incubating drug candidates with, e.g., liver cells (in microsomes activity of cytochrome P450 is high) and isolating and detecting the metabolic products. With the introduction of the ROXY™ EC system oxidative metabolism, which usually occurs in the liver cells by Cytochrome P450 oxidation, can be simulated successfully within seconds and detected by electrospray mass spectrometry (ESI-MS) [1-5].

Combining the ROXY EC System with MS creates a powerful platform for oxidative metabolite investigations and helps to overcome many of the laborious tasks by isolating the metabolites from *in vivo* studies, e.g., urine, plasma, etc., or *in vitro* studies, e.g., rat liver microsomes (RLM) or human liver microsomes (HLM).



**Figure 1:** Metabolic pathways of acetaminophen (APAP). 3 major pathways: Glucuronidation, Sulfation and enzymatic metabolism to NAPQI with direct conjugation to NAPQI-GSH.



**Figure 2:** ROXY™ EC System including a dual syringe infusion pump and the ReactorCell™ connected to electrospray MS.

## Acetaminophen Metabolism

Acetaminophen is a non-narcotic, analgesic and antipyretic drug, widely used as a pain relief medicine. Acetaminophen is metabolized primarily in the liver, into toxic and non-toxic products. Three metabolic pathways are known (see Figure 1). The non-toxic Glucuronidation which accounts for 45-55% and the Sulfation (sulfate conjugation) which accounts for 20-30%. N-hydroxylation and dehydration, then GSH conjugation, accounts for less than 15%. The hepatic cytochrome P450 enzyme system metabolizes acetaminophen, forming a minor yet significant alkylating metabolite known as NAPQI (N-acetyl-p-benzoquinoneimine). NAPQI is then irreversibly conjugated with the sulfhydryl groups of glutathione (GSH) [6].

All three pathways yield final products that are inactive, non-toxic, and excreted by the kidneys. In the third pathway, however, the intermediate product NAPQI is toxic. NAPQI is primarily responsible for the toxic effects of acetaminophen, causing acute hepatic necrosis. Production of NAPQI is primarily due to two isoenzymes of cytochrome P450: CYP2E1 and CYP3A4. At usual doses, NAPQI is quickly detoxified by conjugation with glutathione (phase II reactions).

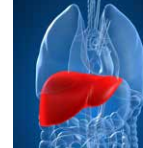
## Method

The ROXY™ EC System (Figure 2) for single compound screening includes the ROXY potentiostat equipped with a ReactorCell™, infusion pump and all necessary LC connections. The ROXY EC System is controlled by Antec Dialogue software. The ReactorCell equipped with a Glassy Carbon working electrode and a HyREF™ reference electrode was used for the generation of acetaminophen metabolite.

**Table 1**

Conditions	
EC	ROXY™ EC System
Cell	ReactorCell™ with GC WE and HyREF™
Flow rate	10 μL/min
Potential	0 – 1300 mV (100 mV steps)

The acetaminophen sample was delivered to the system with a syringe pump equipped with 1000 μL gas tight syringe. A MicrOTOF-Q (Bruker Daltonik, Germany) with Apollo II ion funnel electrospray source was used to record mass spectra. The relevant mass spectrometer parameters are listed in Table



2. The method was optimized on a 10 $\mu$ M paracetamol solution. Mass spectrometer calibration was performed using sodium formate clusters at the beginning of the measurements.

Table 2

MS settings	
Parameter	Value
Mass range	50 – 1000 m/z
Ion polarity	Positive
Capillary voltage	-4500 V
Nebulizer	0.4 Bar
Dry gas	4 L/min
Temperature	200 °C
Funnel 1 RF	200 Vpp
Funnel 2 RF	200 Vpp
ISCID energy	0 eV
Hexapole	100 Vpp
Ion energy	5 eV

### Oxidative metabolism – Phase I

A 10 $\mu$ M acetaminophen solution in 10mM ammonium formate (pH 7.4 adjusted with ammonium hydroxide solution) in 25% acetonitrile was pumped at a constant flow rate of 10  $\mu$ L/min through the ReactorCell using an infusion pump. The outlet of the reactor cell was connected directly (on-line) to the ESI-MS source. Working electrode potential was ramped from 0 – 1300 mV with incremental steps of 100 mV. After each change of the cell potential mass spectra were recorded. The total run time to record the MS voltammogram was approximately 10min. Instrumental set-up of ROXY EC System for oxidative metabolism phase I is shown in Figure 3.

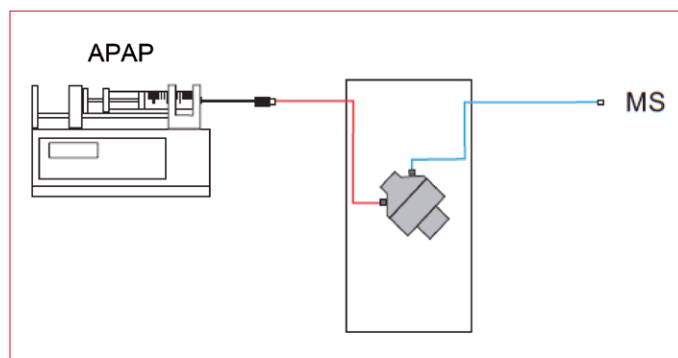


Figure 3: Instrumental set-up of ROXY EC System for oxidative metabolism phase I.

### Detoxification (GSH adduct formation) – Phase II

A 10 $\mu$ M acetaminophen solution in 10mM ammonium formate (pH 7.4 adjusted with ammonium hydroxide solution) with 25% acetonitrile was pumped with a constant flow of 10  $\mu$ L/min through the ReactorCell using an infusion pump. Adduct formation of acetaminophen and glutathione (GSH) was established using a 100 $\mu$ L reaction coil placed between the ReactorCell and the electrospray source and 50 $\mu$ M glutathione in mobile phase was added at the same flow rate via a T-piece into the coil. The reaction time at the specified flow rate is 5 min and the effluent from the reaction coil was injected directly into the ESI-MS. The instrumental set-up of the ROXY EC System for adduct formation (phase II) is shown in Figure 4.

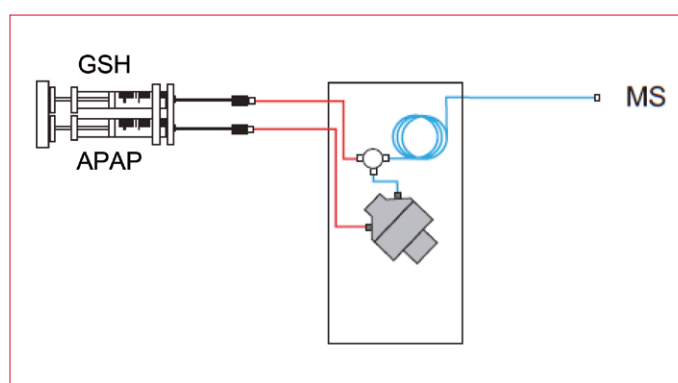


Figure 4: Instrumental set-up of ROXY EC System for adduct formation (Phase II reactions) by adding GSH via a T-piece after the ReactorCell. Mimicking the detoxification reaction of NAPQI by forming the NAPQI-GSH adduct.



# Fast Mimicking of Phase 1 and 2 Metabolism of Acetaminophen using the ROXY™ EC System

## Results

### Phase I

Table 3 consists of a list of compounds related to acetaminophen metabolism and their monoisotopic masses used for mass spectra interpretation. The mass voltammogram for acetaminophen (Figure 5) was recorded using an event table executed in Dialogue. In the Appendix 210.001A the background information is given about Dialogue and event table programming for automated recording of MS voltammograms.

Table 3

Compounds related to acetaminophen metabolism		
Name	Formula	Monoisotopic mass* [u]
Acetaminophen	C <sub>8</sub> H <sub>9</sub> NO <sub>2</sub>	151.063329
NAPQI	C <sub>8</sub> H <sub>7</sub> NO <sub>2</sub>	149.047678
GSH	C <sub>10</sub> H <sub>17</sub> N <sub>3</sub> O <sub>6</sub> S	307.083806
NAPQI-GSH	C <sub>18</sub> H <sub>24</sub> N <sub>4</sub> O <sub>8</sub> S	456.131484

\* In ESI ions are created by the loss or gain of a proton (Monoisotopic mass of proton: 1.00727646677 u).

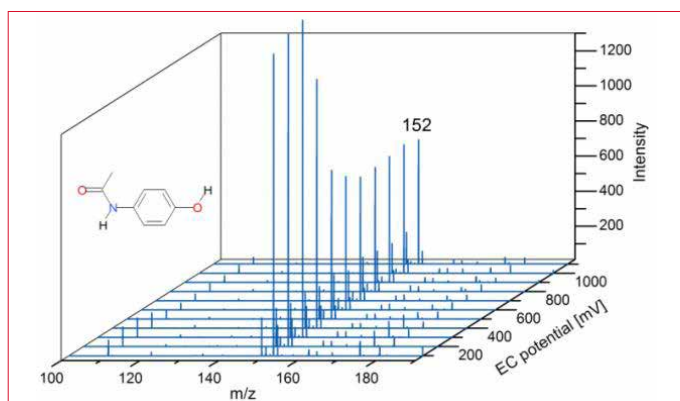


Figure 5: MS voltammogram of acetaminophen. Ion abundance versus m/z as a function of EC potential.

A significant drop in response is observed after the potential above 400 mV is applied. The drop of abundance is attributed to the oxidation of acetaminophen in the ReactorCell and the formation of reactive metabolite. The extracted ion chromatogram representing the mass-to-charge ratio (m/z) of 152 (+/- 0.2u), of protonated acetaminophen is shown in Figure 6.

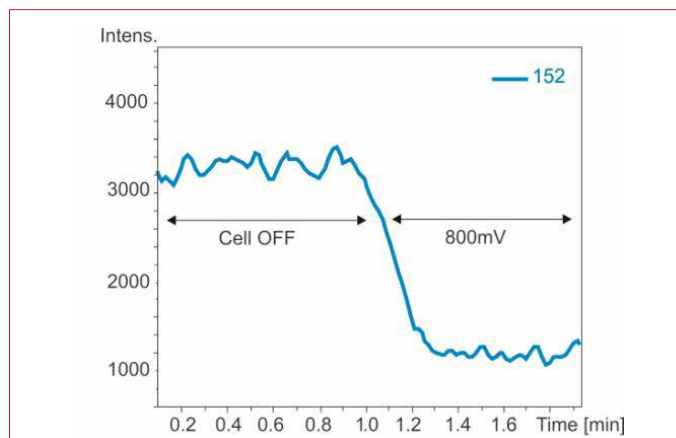


Figure 6: APAP abundance vs. EC potential. EC=800mV was applied to oxidize acetaminophen.

### Phase II

To confirm the presence of the conjugation product of acetaminophen reactive metabolite (NAPQI) and GSH, mass spectra were acquired with the ReactorCell off and at  $E_c = 800$  mV, when phase II instrumental set up was used. Figure 7 shows the spectra with the ReactorCell off (Fig. 7A) and on at 800 mV (Fig. 7B). Figure 8 shows zoom in of the mass spectrum from Figure 7 (the red circle). It is evident that the NAPQI – GSH conjugation product is only present in the spectrum recorded at 800 mV (Fig. 8B).

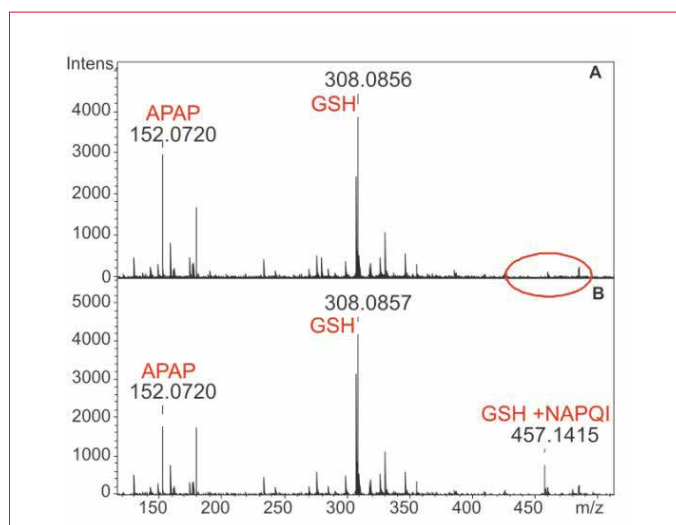
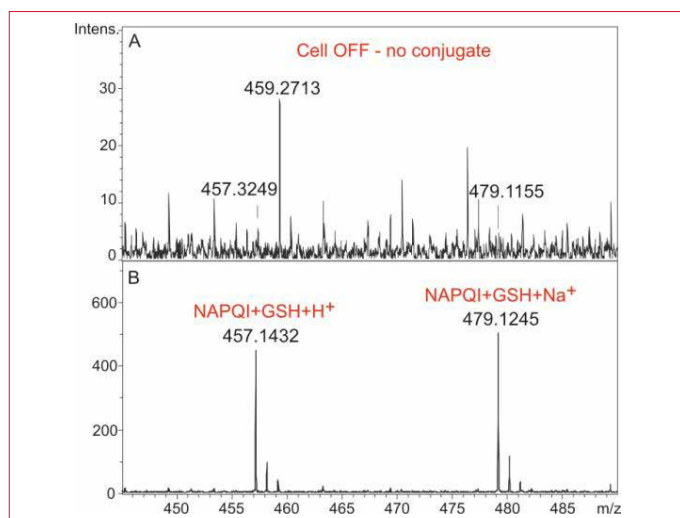
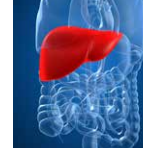


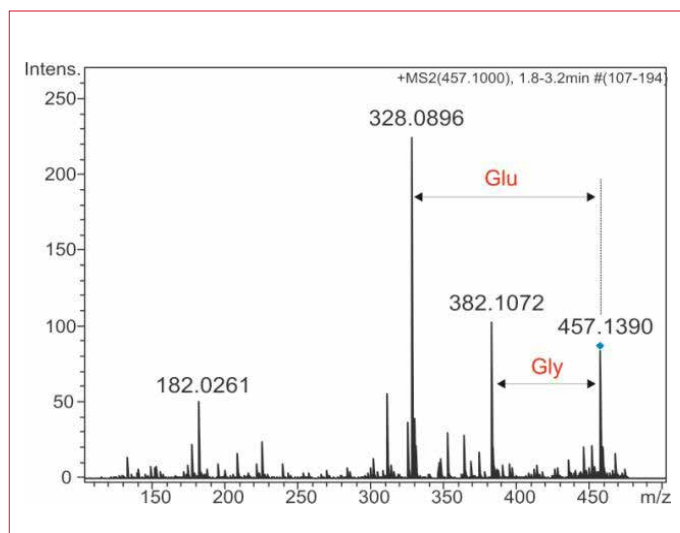
Figure 7: Result of conjugation of phase I metabolite of acetaminophen (APAP) and GSH. (A.) ReactorCell OFF, (B.) ReactorCell EC=800mV.



**Figure 8:** Zoom in of mass range from m/z of 445 to 490 (Red circle in the Figure 7). (A) ReactorCell OFF, (B) ReactorCell EC=800mV. Peak at m/z of 457.1432 corresponds to protonated ion of conjugation product. The peak of m/z of 479.1245 was identified as its Na<sup>+</sup> adduct.

To confirm that the peak at m/z of 457.1432 is originating from the NAPQI-GSH adduct, the fragmentation spectrum (Fig. 9) was acquired and the chemical formula of the adduct was calculated using Smart Formula (Bruker Daltonic software). The correct formula was found with relative error of 0.8 ppm.

The fragmentation pattern confirmed loss of Glycine and Glutamate, which are building block of glutathione (Glu-Cys-Gly).



**Figure 9:** Fragmentation spectrum of conjugation product.

## Conclusion

The on-line coupling of the ROXY™ EC System with MS (EC/MS) provides a versatile and user-friendly platform for fast screening of target compounds (drugs, pharmaceuticals, pollutants, etc.) for oxidative metabolism (phase 1 reactions), thereby mimicking the metabolic pathway of CYP450 reactions.

MS voltammograms can be recorded automatically to obtain a metabolic fingerprint of the compound of interest in less than 10 min.

In addition, rapid and easy studies of adduct formations can be performed simply by adding GSH after the ReactorCell (phase II reactions).



# Fast Mimicking of Phase 1 and 2 Metabolism of Acetaminophen using the ROXY™ EC System

## References

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6. Huber Ch., Bartha B., Harpaintner R., Schröder P., Metabolism of acetaminophen (paracetamol) in plants—two independent pathways result in the formation of a glutathione and a glucose conjugate, *Environ. Sci. Pollut. Res.*, 16 (2009) 206-213



**Figure 10:** ROXY™ EC System consisting of ROXY Potentiostat, dual syringe pump and ReactorCell.

## Ordering information

210.0070A	ROXY™ EC system, incl. dual syringe pump, ReactorCell, electrodes and LC connection kit for phase I and II reactions. All parts included for described Electrochemical (EC) application.
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