Application Note
Screening on Redox Reactions

Electrochemical Reactions upfront MS – EC/MS

Proteomics & Protein Chemistry
S-S bond reduction
HDX
Peptide bond cleavage
Na+, K+ 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

Automated Screening on REDOX Reactions using the ROXY™ EC/LC System

- **Simulating REDOX reactions, e.g., oxidative stress, oxidative metabolism, biotransformation, degradation, ROS, etc.**
- **Automated screening of multiple samples (96 or 384 well plate)**
- **Automated phase I (REDOX) and phase II (adduct formation) reactions**
- **Most versatile and powerful platform for REDOX studies**

Summary

A novel and flexible EC/LC/MS approach is demonstrated for automated screening of multiple samples based on the integration of an EC cell into the autosampler flow path of the ROXY EC/LC system [10, Patented].

Introduction

In vitro oxidation of drugs in electrochemical (EC) reactors has been proposed as a valuable screening tool in the investigation of potential oxidative metabolites [1-11]. During the last three decades, extensive research has been conducted in this field showing that it is possible to simulate typical phase I reactions by using EC coupled to mass spectrometry (MS), even though the enzymatic mechanism differs from the electrochemical oxidation pathway.

Extending the EC/MS set-up by integrating liquid chromatography (LC) provides additional information regarding the polarity of the metabolites and the formation of isomeric products.

The benefits of EC/(LC)/MS in metabolism studies has already been shown for a variety of well known pharmaceuticals such as paracetamol, diclofenac, tetrazepam, amodiaquine, naltrexone, statin drugs and many more.

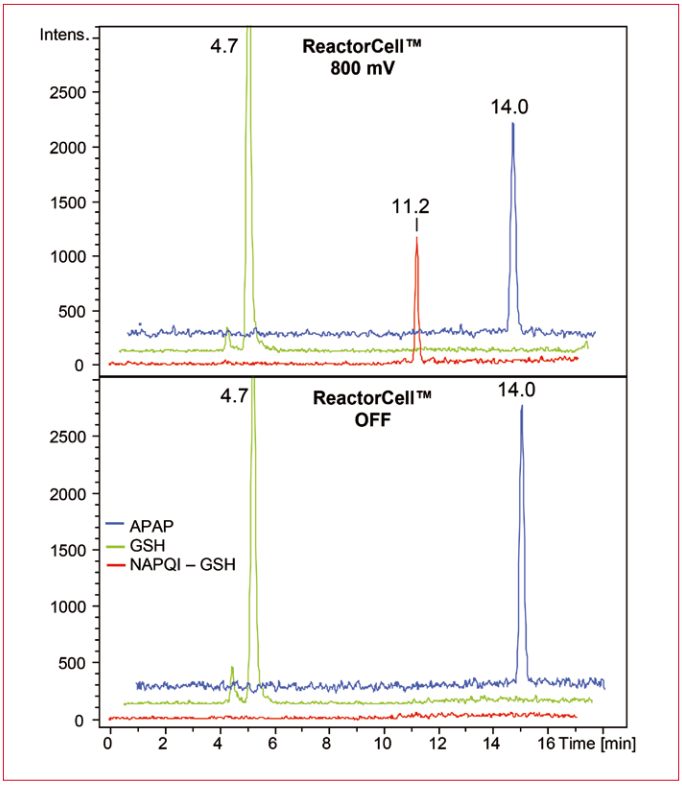

Figure 1: Automated generation of phase I and II metabolites of acetaminophen (APAP) using the ROXY™ EC/LC system: with Reactor Cell™ ON (top) immediate formation of the NAPQI—GSH conjugate (phase II reaction) is observed (red).

Mimicking of oxidative metabolism

The knowledge of the metabolic pathways and the biotransformation of new drugs is crucial for elucidation of degradation routes of new active compounds and assessing the toxicity of formed metabolites. Traditional research in the field of oxidative metabolism involves time-consuming *in-vivo* or *in-vitro* methods. A new fast alternative for the classical method is the application of electrochemistry in conjunction with MS, a purely instrumental technique, for the simulation of oxidative metabolism.

Current EC/LC/MS approaches are either based on the generation of metabolites (1) online using an electrochemical cell integrated in the LC flow path or (2) offline with the EC cell connected to a sampling valve [1-9].

In the first approach, the LC separation conditions such as flow rate, mobile phase composition and pH may have a significant effect on the generation of metabolites via EC. Moreover coulometric EC cells are often used. These cells are sensitive to adsorption onto the electrode surface, which affect the reproducibility.

In the second approach, a (syringe) pump is used to deliver sample into the EC cell and fill a loop of an injection valve with oxidized product(s). Although such a configuration has the advantage that the oxidation conditions are decoupled from the LC conditions, it does not allow the automated handling (oxidation, separation and MS analysis) of multiple samples.

In the ROXY EC/LC system, a ReactorCell™ (amperometric thinlayer cell) is placed between the injection capillary and the injection valve of an AS110 autosampler which allows fully automated oxidation, conjugation (i.e., adduct formation), separation and online MS analysis of multiple samples. A major advantage of this configuration is that the decoupling from the LC flow path allows that samples can be oxidized or reduced under optimal conditions (flow rate, mobile phase conditions and pH) which may differ from the LC conditions required for the separation making the ROXY EC/LC a powerful and versatile platform for automated metabolic screening. The capabilities of the system are demonstrated below using acetaminophen (APAP, paracetamol) as a model compound.

Model compound

Acetaminophen is a non-narcotic, analgesic and antipyretic drug, widely used as a pain relief medicine. Acetaminophen is metabolized in the liver by enzyme cytochrome P 450 to a highly reactive metabolite – N-acetyl-p-benzoquinoneimine (NAPQI), which can cause acute hepatic necrosis if not followed by conjugation with glutathione (GSH) (figure 2). Automated phase II reactions are demonstrated on the ROXY EC/LC system using the conjugation reaction of GSH with NAPQI.

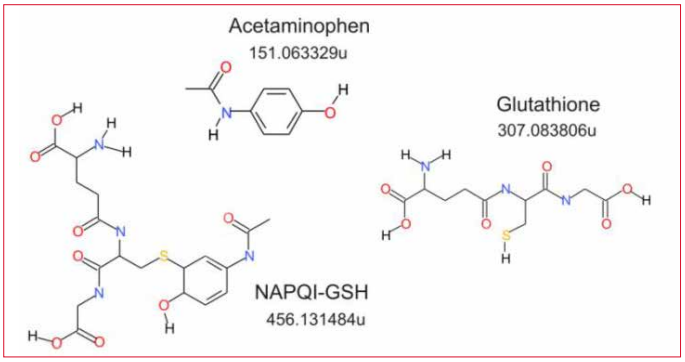

Figure 3: Structures and monoisotopic masses of acetaminophen, glutathione and conjugate of the reactive metabolite of acetaminophen (NAPQI-GSH).

Method

The ROXY™ EC/LC System (figure 3) for automated screening (p/n 210.0080C) includes the ROXY potentiostat equipped with a ReactorCell™, an AS110 autosampler, two LC 110S HPLC pumps and all necessary LC connections for user-friendly installation and use with a MS (Table 1). The pumps are configured to work in high-pressure gradient mode and the final mobile phase composition is achieved by mixing phase A and B in a 250µL binary tee mixer.

The ROXY EC/LC System is controlled by Clarity chromatography software (DataApex). The ReactorCell with Glassy Carbon working electrode and HyREF™ reference electrode was used for the generation of acetaminophen metabolites.

Table 1

Configuration of the ROXY EC/LC system

1	AS110 autosampler, cool, micro, 6-PV
2	Reactor Cell with Pt, GC, Au, MD WE, and HyREF
3	LC 110S HPLC pump (2x)
4	OR 110 organizer rack, dual channel
5	ROXY potentiostat DCC
6	Clarity chromatography software, including LC, AS modules

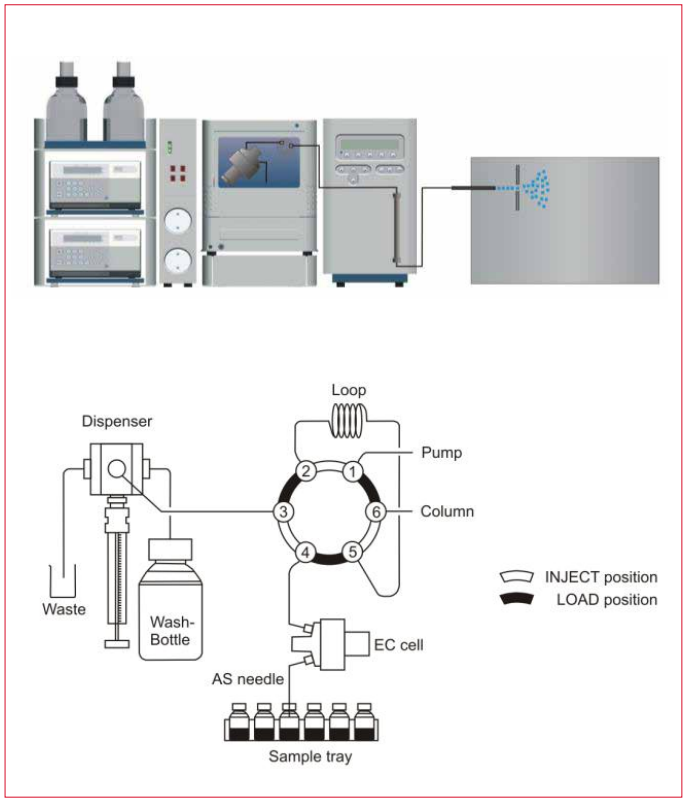

Figure 3: Top: ROXY™ EC/LC System including ReactorCell™ integrated in the AS110 autosampler flow path. Bottom: Detailed lay-out of autosampler flow path with 6-port injection valve and ReactorCell™

The automated electrochemical conversion of samples (Phase I), and addition of reagents for follow-up reactions (Phase II) are controlled by means of user-defined injection programs (UDP) of the AS110 autosampler (See appendix 210.002A). The ReactorCell was integrated in the auto sampler flow path as shown in figure 3 (Bottom) and the volume of the buffer tubing, speed of autosampler syringe was optimized to facilitate optimal conditions for efficient electrochemical conversion. A 25 µL syringe was installed to be able to run at the lowest possible aspiration flow rate (lowest speed is 3µL/min).

Table 2

Gradient composition

Time[min.]	A [%] (Buffer)	B [%] (Methanol)
Initial	90.0	10.0
2.00	90.0	10.0
3.00	50.0	50.0
15.00	50.0	50.0
16.00	90.0	10.0

The potential applied to the working electrode (WE) of the ReactorCell was controlled by the ROXY potentiostat and can be programmed within the ROXY control module in the Clarity chromatography software. The optimal potential used for acetaminophen oxidation was determined based on a recorded mass voltammogram shown in figure 4 (for details see Application note 210.001).

The use of UDP's in combination with the unique ROXY LC/EC hardware offers a fully automated and flexible solution for metabolic screening of multiple samples and is ideally suited for sample screening on REDOX reactions (phase 1) including follow-up reactions (phase 2).

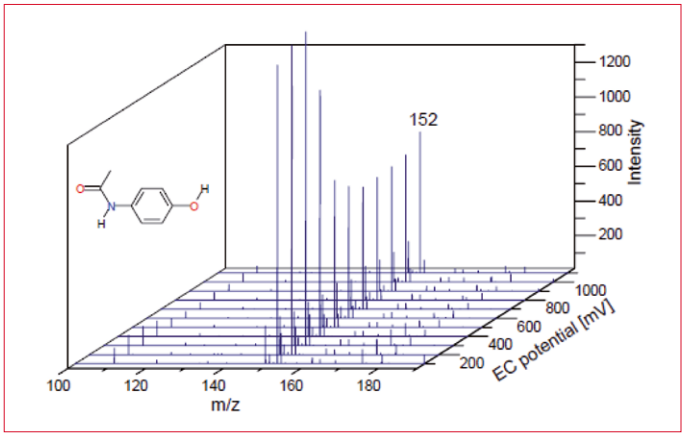

Figure 4: Mass voltammogram of acetaminophen.

Table 3

Conditions

System	ROXY™ EC/LC System (p/n 210.0080C)
Cell	ReactorCell™ with GC WE and HyREF™
Flow Rate	300 µL/min
Column	BetaSil Phenyl, 250x3mm; 3µm
Injection	10µL
Mobile phase (MP)	A. 20 mM ammonium acetate pH 6.9 B. 50% methanol
Potential	Off or 800 mV
Standard Phase I	10 µM acetaminophen in MP A
Standards Phase II	1. 10 µM acetaminophen in MP A 2. 50 µM GSH* in MP A (25 µL of standard 1 mixed with 50 µL of reagent 2; see paragraph Phase II for details)

*GSH should be freshly prepared to avoid spontaneous oxidation to glutathione disulfide (GSSG).

The EC and LC conditions are listed in table 2. Separation was achieved by gradient elution over a BetaSil Phenyl column. The gradient was adapted from Lohmann et al. [1], as described in table 3. Total analysis time was 17 minutes.

Mass Spectrometry

A MicrOTOF-Q (Bruker Daltonik, Germany) with an Apollo II ion funnel electrospray source was used to record mass spectra and MS data were analyzed by Compass software. The relevant mass spectrometer parameters are listed in the Table 4. The method was optimized on use of a 10 µM paracetamol (APAP) solution. Mass spectrometer calibration was performed using sodium formate clusters at the beginning of the measurements.

Table 4

Bruker MicrOTOF-Q MS settings

Parameter	Value
Mass range	50 – 1000 m/z
Ion polarity	Positive
Capillary voltage	-4500 V
Nebulizer	1.6 Bar
Dry gas	8 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

Results

Table 5 consists of a list of compounds related to the metabolism of acetaminophen, their empirical formulas, their monoisotopic masses and the mass-to-charge (m/z) ratio used for mass spectra interpretation.

Table 5

Compounds related to acetaminophen metabolism

Name	Formula	m/z [Th]
Acetaminophen	$C_8H_9NO_2$	152.070605
NAPQI	$C_8H_7NO_2$	150.054954
GSH	$C_{10}H_{17}N_3O_6S$	308.091082
NAPQI-GSH	$C_{18}H_{24}N_4O_8S$	457.138760

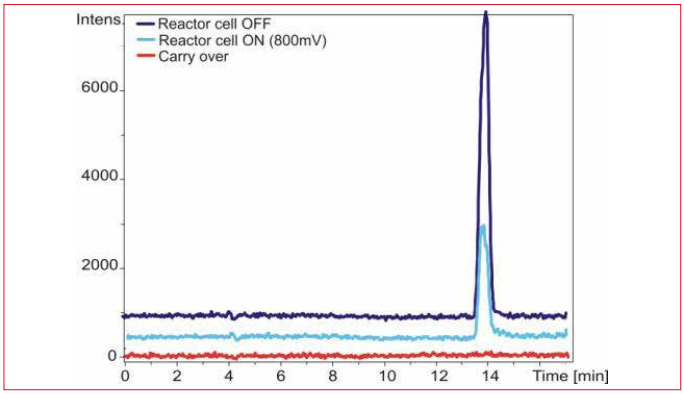

Figure 5: Extracted ion chromatograms (EIC) of APAP (m/z= 152 Th), eluted in 14 min. 65% conversion of the APAP is observed with ReactorCell™ ON (800mV; light blue). Dark blue trace corresponds to control measurements with ReactorCell™ OFF. Red trace is a mobile phase injection

Phase I

To evaluate phase I metabolism of APAP using the ROXY EC/LC system three experiments were performed:

1.) Oxidative conversion

In this experiment, a potential of 800mV was applied to the working electrode (Glassy Carbon) of the ReactorCell to generate metabolites.

2.) Control measurement

During the control measurement, no potential was applied to the ReactorCell. This was done to measure the area of the parent ion signal when no electrochemical conversion takes place.

3.) Carry-over check

Injection of mobile phase under the same conditions after the first two experiments were run to check carry-over of the system.

All experiments were executed automatically by means of a sample sequence with methods containing specific settings and UDP's for each. The results are shown in figure 5. Note that the reactive NAPQI metabolite of APAP cannot be detected by ESI-MS directly and the formation of metabolite has to be judged based on the attenuation of the parent ion signal. When a potential of 800 mV was applied to the ReactorCell, a 65% attenuation of the parent ion of acetaminophen was observed (figure 5) indicating the formation of metabolite. The mobile phase injected after the control measurement does not show any significant acetaminophen carry-over in the system.

Phase II

To evaluate phase II metabolism with APAP & GSH using the ROXY EC/LC system, three experiments were performed:

1.) Conjugation reaction

In this experiment, a potential of 800mV was applied to the working electrode (Glassy Carbon) to generate metabolites. The acetaminophen was oxidized in the ReactorCell and then 25µL of acetaminophen was mixed in a destination vial containing 50µL of GSH. The loop was subsequently filled with NAPQI-GSH conjugate and injected in the column. The GSH reagent does not undergo oxidation in this protocol. See figure 6 for a simplified schematic representation of the Phase II injection routine.

2.) Control measurement

An identical experiment was performed as described above for the conjugation reaction, with the difference that during the control measurement no potential (Cell off) was applied to the ReactorCell. Due to the fact that in such case no electrochemical conversion takes place it is expected that no NAPQI-GSH conjugate is formed.

3.) Carry-over check

Injection of mobile phase under the same conditions directly after run 1 and 2 to check carry-over of the system.

The mass chromatograms of the Phase II experiments are shown in figure 1. The conjugation product, corresponding to a m/z ratio of 457 is present only when a potential was applied and the acetaminophen sample was electrochemically oxidized. In the control experiment, no NAPQI-GSH is found in the mass chromatogram as expected.

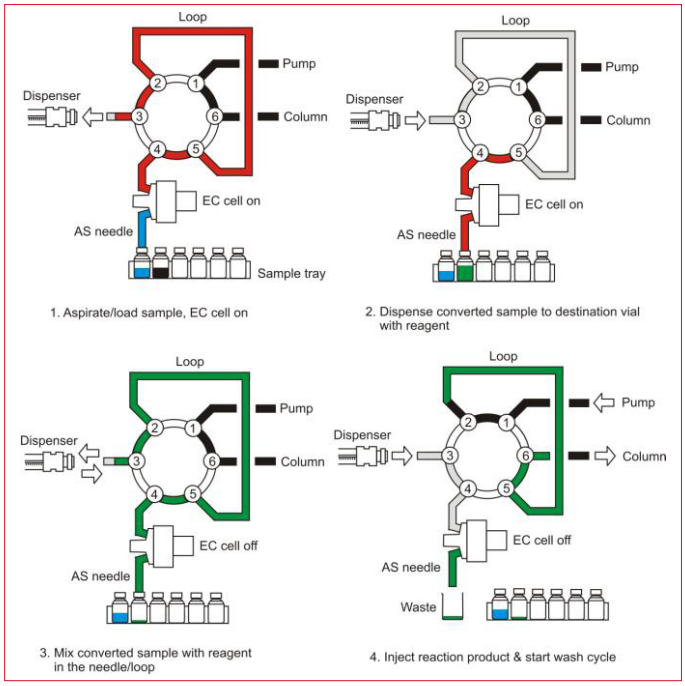

Figure 6: Simplified schematic representation of the automated Phase II injection routine. Blue:sample,red: oxidized sample, black: reagent and green: oxidized sample mixed with reagent.

In addition to the mass chromatograms (figure 1) the mass spectra are presented (figure 7) to confirm the presence of the conjugation product of acetaminophen reactive metabolite (NAPQI) and GSH. The protonated ion of NAPQI-GSH conjugate (m/z = 457.1432 Th) as well as its sodium adduct (m/z = 479.1245 Th) were identified based on high resolution measurement (figure 7B). When the ReactorCell was OFF none of these peaks were formed (Figure 7B).

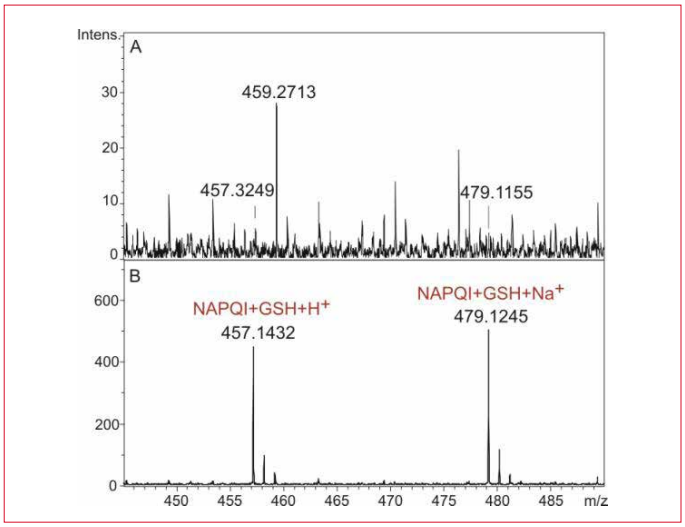

Figure 7: Result of conjugation of phase I metabolite of acetaminophen (NAPQI) and GSH. (A.) ReactorCell OFF, (B.) Reactor Cell EC = 800mV.

To confirm that the peak at m/z of 457 is originating from the NAPQI-GSH adduct, the fragmentation spectrum (figure 8) was acquired and the chemical formula 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 blocks of glutathione (Glu-Cys-Gly).

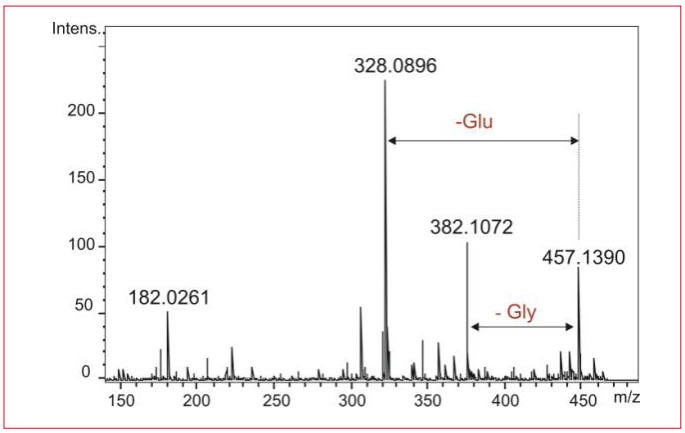

Figure 8: Fragmentation spectrum (MS/MS) of conjugation product.

An injection of mobile phase (A) was executed to evaluate carry-over in the system (figure 9) after the phase II injection method was applied with APAP and GSH. No carry-over in the system was observed.

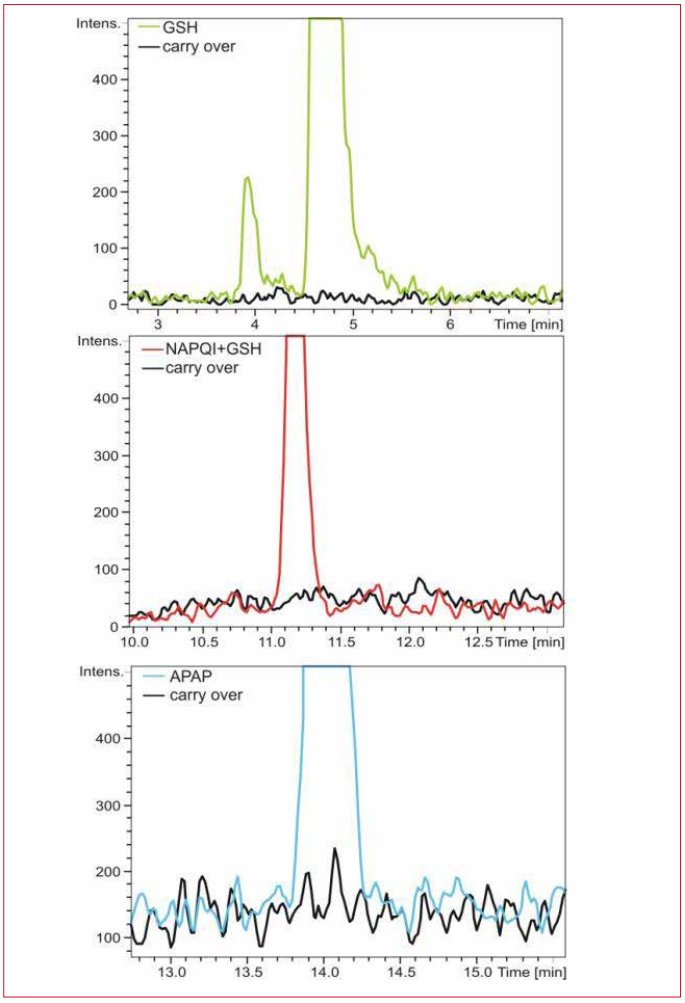

Figure 9: Carry-over experiment: Black traces in all panels correspond to EIC of 152 (acetaminophen); 457(conjugation product) and 308 (GSH), respectively, for injection of mobile phase A. No peaks were detected when mobile phase was injected.

Conclusion

The ROXY™ EC/LC system provides a powerful and versatile platform capable of automated screening on REDOX reactions of large series of samples (96 vials, 96 and 384 well plates) under different conditions (type of electrolyte, pH, organic modifier etc.). The combination of user-defined injection programs (UDP's) with the unique ROXY LC/EC hardware gives full flexibility and control over the automated electrochemical conversion and allows studying of both phase I reactions (oxidations or reductions) and subsequent phase II reactions (adduct formation). Another important key feature is the 'decoupling' of the EC conversion from the chromatographic separation which enables flexible optimization of the separation and MS detection without compromising on the EC conditions.

References

1. W.Lohmann, U. Karst, A. Baumann, Electrochemistry and LC–MS for Metabolite Generation and Identification: Tools, Technologies and Trends, LC-GC, 23 (2010) 1-7.
2. Lohmann W., Karst U., Simulation of the detoxification of acetaminophen using on-line electrochemistry/liquid chromatography/mass spectrometry, Anal. Bioanal. Chem. 386 (2006) 1701–1708.
3. Lohmann W., Hayen H., Karst U., Covalent Protein Modification by Reactive Drug Metabolites Using Online Electrochemistry/Liquid Chromatography/Mass Spectrometry, Anal. Chem. 80 (2008) 9714–9719.
4. Permentier H. P., Bruins A. P., Bischoff R., Electrochemistry-Mass Spectrometry in Drug Metabolism and Protein Research, Mini-Rev. Med. Chem. 8 (2008) 46-56.
5. Jurva U., Washroom H. V., Weidolf L., Bruins A.P., Comparison between electrochemistry/mass spectrometry and cytochrome P450 catalyzed oxidation reactions, Rapid Commun. Mass Spectrom. 17 (2003) 800–810.
6. W. Lohmann, B. Meermann, I. Moller, A. Scheffer, U. Karst, Quantification of Electrochemically Generated Iodine-Containing Metabolites Using Inductively Coupled Plasma Mass Spectrometry, Anal. Chem. 80 (2008) 9769–9775.
7. Huber Ch., Bartha B. et. al., 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.
8. Baumann A., Lohmann W., Schubert B., Oberacher H., Karst U., Metabolic studies of tetrazepam based on electrochemical simulation in comparison to in vivo and in vitro methods, J. Chromatogr. A, 1216 (2009) 3192–3198.
9. Lohmann W., Hayen H., Karst U., Covalent Protein Modification by Reactive Drug Metabolites Using Online Electrochemistry/Liquid Chromatography/Mass Spectrometry, Anal. Chem. 80 (2008) 9714–9719.
10. Brouwer, H. J., Chervet, J. P., Kraj, A., Analytical apparatus comprising an electrochemical flow cell and a structural elucidation spectrometer, Eur. Patent EP2572188.
11. Sandra Jahn, Anne Baumann, Jörg Roscher, Katharina Hense, Raniero Zazzeroni, Uwe Karst, Investigation of the biotransformation pathway of verapamil using electrochemistry/liquid chromatography/mass spectrometry – A comparative study with liver cell microsomes, J. Chromatogr A, 1218 (2011) 9210-9220.

Figure 10: ROXY™ EC/LC/System consiting of (from left to right): binary high pressure gradient pumps, degassing unit, Autosampler AS110 with integrated ReactorCell, cooled sample tray, working electrodes, ROXY Potentiostat and Clarity software.

Ordering information

210.0080C	ROXY™ EC system consisting of: binary high pressure gradient pumps, degassing unit, Autosampler AS110 with integrated ReactorCell, cooled sample tray, working electrodes, ROXY Potentiostat and Clarity software. All parts included for described Electrochemical (EC) application.

Antec Scientific (USA)
info@AntecScientific.com
www.AntecScientific.com
T 888 572 0012

Antec Scientific (worldwide)
info@AntecScientific.com
www.AntecScientific.com
T +31 71 5813333