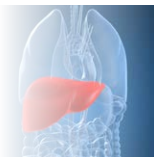
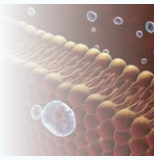
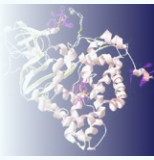
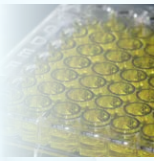
Antec® Scientific




Applications Notebook

ROXY™ EC System for Electrochemical Reactions with MS Detection - EC/MS

Design: MGO-studio, Maarssen, NL
Illustrations: Antec Scientific

For research purpose only. The information shown in this communication is solely to demonstrate the applicability of the ALEXYS system. The actual performance may be affected by factors beyond Antec Scientific's control. Specifications mentioned in this notebook are subject to change without further notice.

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Scientific research is one of the most exciting and rewarding of occupations.

Frederick Sanger

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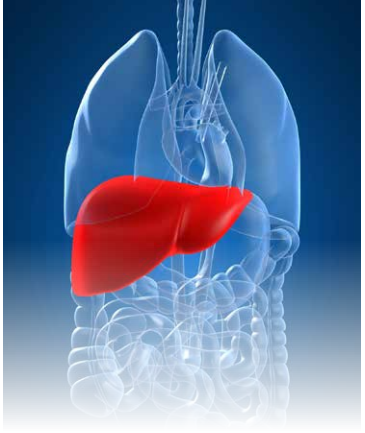

Electrochemical Reactions upfront MS – EC/MS

Proteomics & Protein Chemistry
S-S bond reduction
HDX
Peptide bond cleavage
Na+, K+ removal
Drug-protein binding

Lipidomics & Fatty Acids
Cholesterol
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Metabolites & Degradants

Pharmaceutical Stability
Purposeful degradation
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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.

ROXY Application Note # 210_001_09

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

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 form *in vivo* studies, e.g., urine, plasma, etc., or *in vitro* studies, e.g., rat liver microsomes (RLM) or human liver micorosomes (HLM).

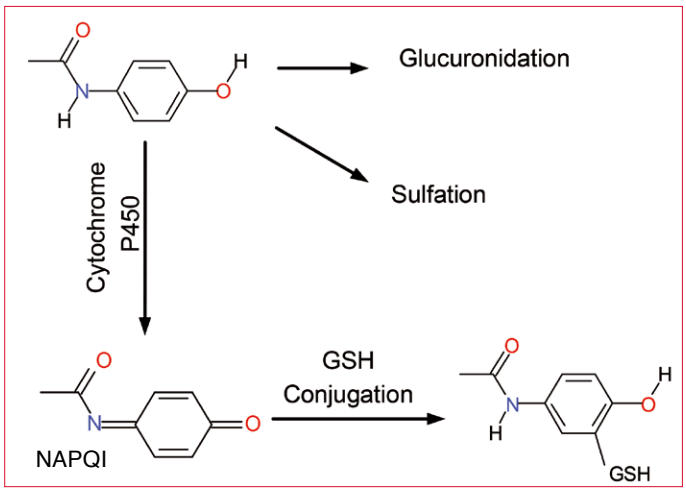

Figure 1: Metabolic pathways of acetaminophen (APAP). 3 major pathways: Glucuronidation, Sulfation and enzymatic metabolism to NAPQI with direct conjugation to NAPQI-GSH.

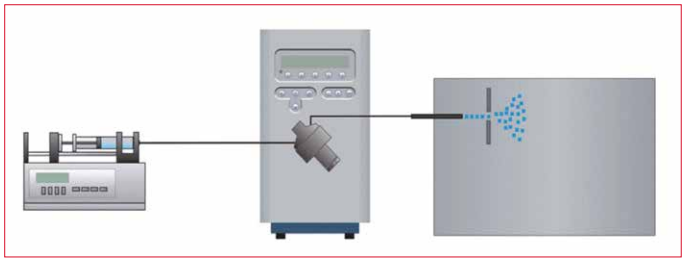

Figure 2: ROXY™ EC System including a dual syringe infusion pump and the ReactorCell™ connected to electrospray MS.

Acetamionphen 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	10µL/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 10µM 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 10µM 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 µL/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.

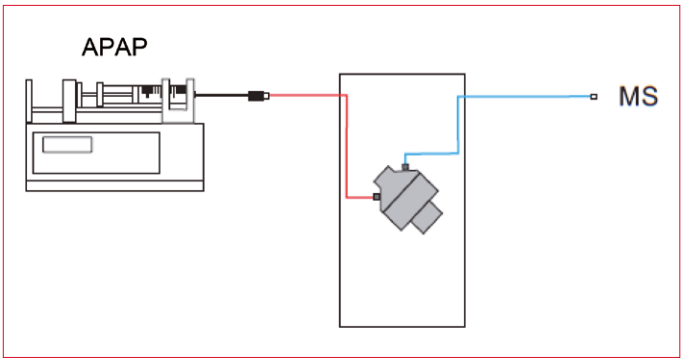

Figure 3: Instrumental set-up of ROXY EC System for oxidative metabolism phase I.

Detoxification (GSH adduct formation) – Phase II

A 10µM 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 µL/min through the ReactorCell using an infusion pump. Adduct formation of acetaminophen and glutathione (GSH) was established using a 100µL reaction coil placed between the ReactorCell and the electrospray source and 50µM 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.

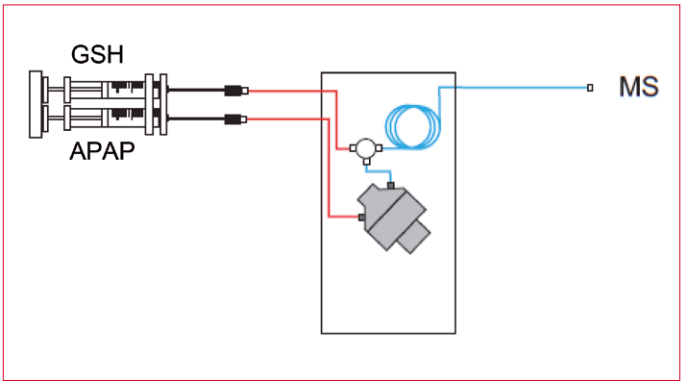

Figure 4: Instrumental set-up of ROXY EC System for adduct formation (Phase II reactions) by adding GSH via a T-piece after the ReactorCell. Mimicking the detoxification reaction of NAPQI by forming the NAPQI-GSH adduct.

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	C8H9NO2	151.063329
NAPQI	C8H7NO2	149.047678
GSH	C10H17N3O6S	307.083806
NAPQI-GSH	C18H24N4O8S	456.131484

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

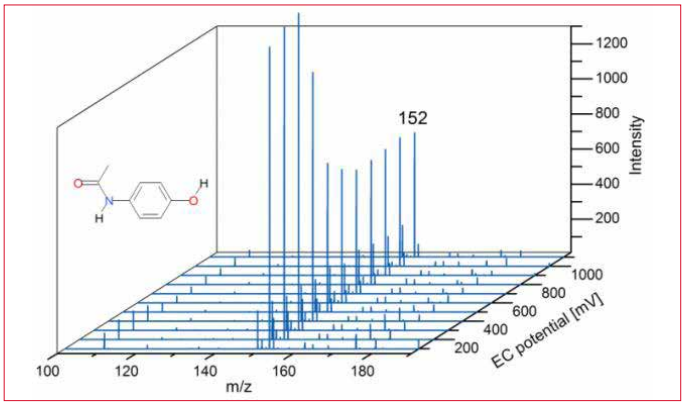

Figure 5: MS voltammogram of acetaminophen. Ion abundance versus m/z as a function of EC potential.

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.

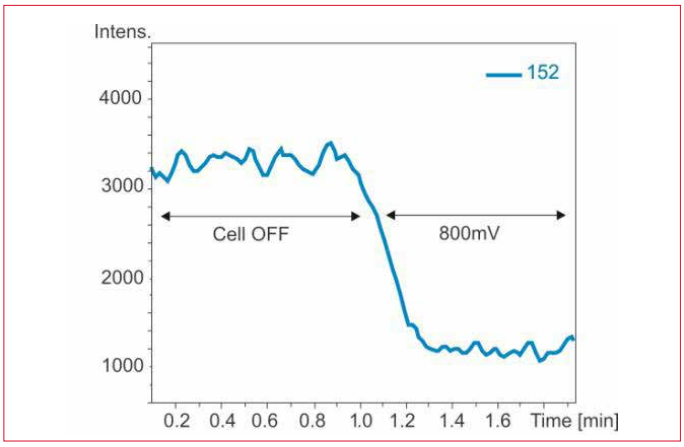

Figure 6: APAP abundance vs. EC potential. EC=800mV was applied to oxidize acetaminophen.

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 Ec = 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).

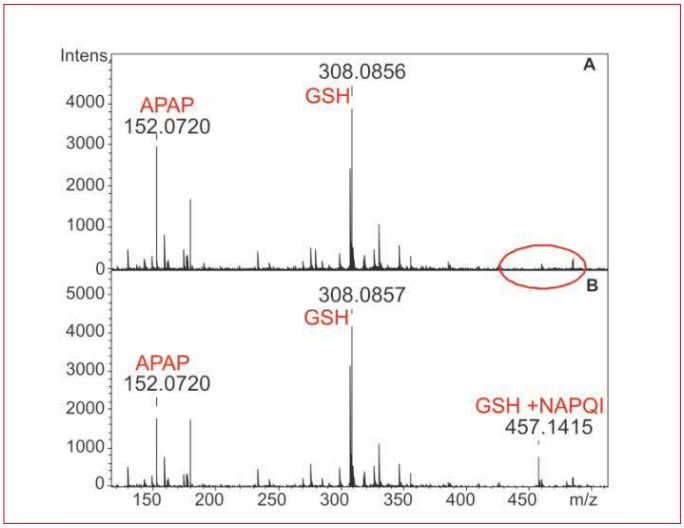

Figure 7: Result of conjugation of phase I metabolite of acetaminophen (APAP) and GSH. (A.) ReactorCell OFF, (B.) ReactorCell EC=800mV.

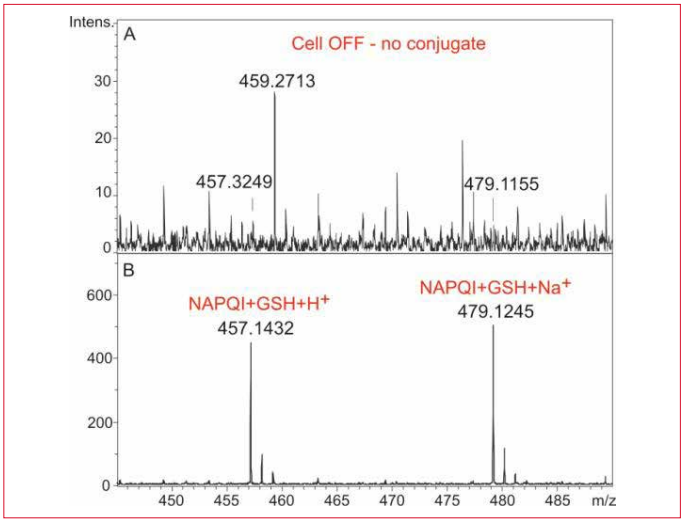

Figure 8: Zoom in of mass range from m/z of 445 to 490 (Red circle in the Figure 7). (A) ReactorCell OFF, (B) ReactorCell EC=800mV. Peak at m/z of 457.1432 corresponds to protonated ion of conjugation product. The peak of m/z of 479.1245 was identified as its Na+ adduct.

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).

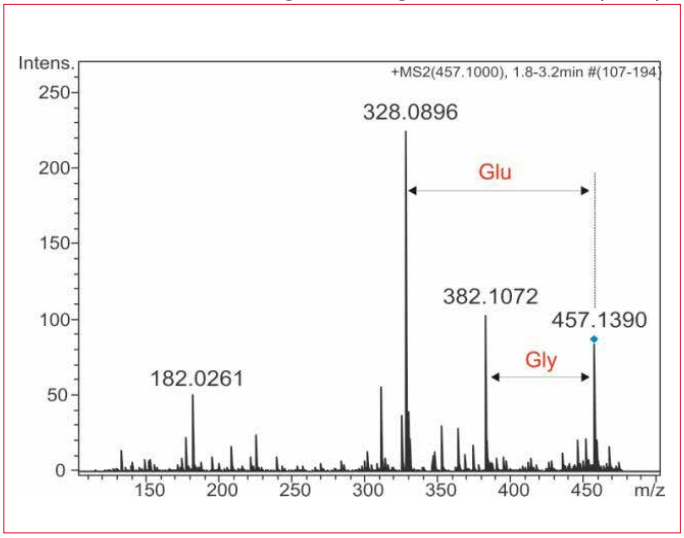

Figure 9: Fragmentation spectrum of conjugation product.

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).

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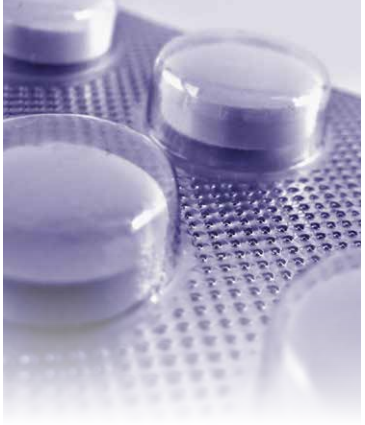
5. W. Lohmann, B. Meermann, I. Moller, A. Scheffer, U. Karst, Quantification of Electrochemically Generated Iodine-Containing Metabolites Using Inductively Coupled Plasma Mass Spectrometry, Anal. Chem., 80 (2008) 9769-9775
6. Huber Ch., Bartha B., Harpaintner R., Schröder P., Metabolism of acetaminophen (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.

Application Note
Drug Metabolism



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

Oxidative Metabolism of Amodiaquine using the ROXY™ EC System

- **Amodiaquine, Camoquin, Flavoquine**
- **Fast mimicking and predicting drug metabolism < 10 min.**
- **Oxidative metabolism (phase I) and adduct formation (phase II)**
- **Ideal for system performance evaluation (reference system)**

Introduction

Amodiaquine (AQ) is an antimalarial agent which is used against Plasmodium falciparum, a protozoan parasite which can cause cerebral malaria. Though the drug was withdrawn from the market because of its hepatotoxicity, it is still widely applied for the treatment of Malaria in Africa. Amodiaquine is metabolized to reactive electrophilic metabolites, which are difficult to detect since they are shortlived, and the metabolites can undergo further reactions resulting in stable products.

Amodiaquine (trade names: Camoquin, Flavoquine; IUPAC: 4-[(7-chloroquinolin-4-yl)amino]-2-(diethylaminomethyl)phenol) was chosen as a model drug to investigate the nature of the oxidative metabolism using the ROXY EC System.

Electrochemical conversion of the amodiaquine into reactive phase I metabolites and their GSH conjugates were successfully achieved.

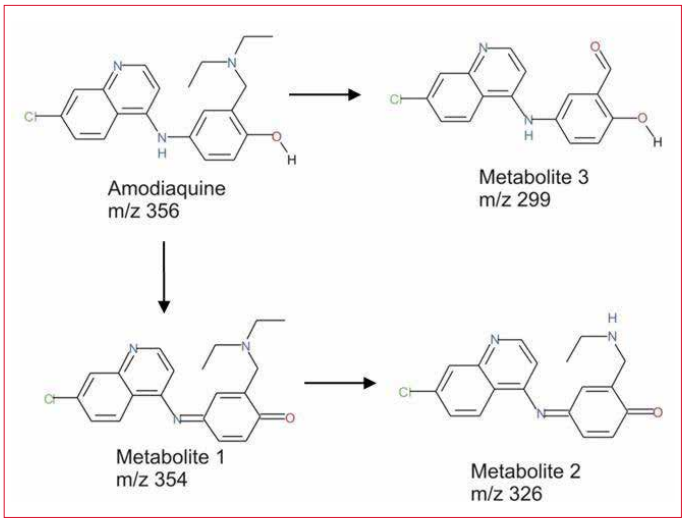


Figure 1: Metabolic pathway of amodiaquine with the 3 most abundant metabolites.

Table 1

Amodiaquine and its (selected) metabolites

Name	Formula	Monoisotopic mass [u]
Amiodaquine (AQ)	$C_{20}H_{22}ClN_3O$	355.14514
1 (quinoneimine)	$C_{20}H_{20}ClN_3O$	353.12949
2 (desethyl; quinoneimine)	$C_{18}H_{16}ClN_3O$	325.09819
3 (bis desethyl; aldehyde)	$C_{16}H_{11}ClN_2O_2$	298.05091

Method

The ROXY EC System (Figure 2) for compound screening (p/n 210.0070A) 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.

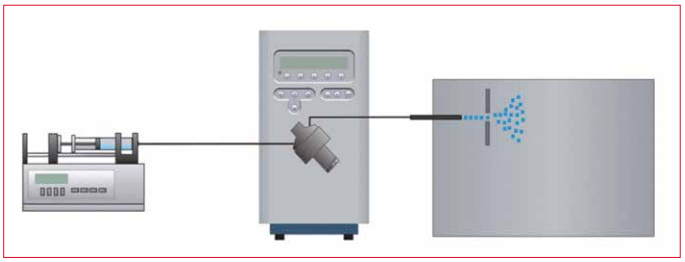


Figure 2: Instrumental set-up of ROXY EC System for oxidative metabolism phase I.

The ReactorCell equipped with Glassy Carbon working electrode and HyREF™ reference electrode was used for the generation of amodiaquine metabolites.

Table 2

Conditions

EC	ROXY™ EC System (p/n 210.0070)
Cell	ReactorCell™ with GC WE and HyREF™
Flow rate	10 µL/min
Potential	0 – 1500 mV (scan mode)
Mobile phase	20 mM ammonium formate (pH 7.4 adjusted with ammonium hydroxide) with 50% acetonitrile

The amodiaquine sample was delivered to the system with a syringe pump equipped with a 1000 µL gas tight syringe. 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 Table 3. The method was optimized on a 10µM amodiaquine solution. Mass spectrometer calibration was performed using sodium formate clusters at the beginning of the measurements.

Table 2

Conditions

Name	Formula
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
ISCID energy 100 Vpp	0 eV
Hexapole	100 Vpp
Ion energy	5 eV

Oxidative metabolism – Phase I

A 10µM amodiaquine solution in 20mM ammonium formate (pH 7.4 adjusted with ammonium hydroxide) with 50% acetonitrile was pumped at a constant flow rate of 10 µL/min through the ReactorCell using an infusion pump. The outlet of the reactor cell was connected directly (online) to the ESI-MS source. The scan mode was used to register the MS Voltammogram with the working electrode potential ramped from 0 – 1500 mV at a scan rate of 10 mV/s in the half cycle. The mass spectra for each change of the cell potential were recorded continuously and saved in one file. The total run time to record the mass voltammogram was approximately 2.5 min. Instrumental set-up of ROXY EC System for oxidative metabolism phase I is shown in Figure 3.

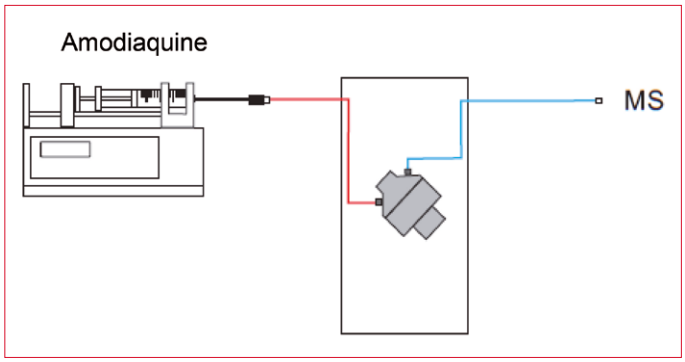


Figure 3: Instrumental set-up of ROXY EC System for oxidative metabolism phase I.

Adduct formation – Phase II

A 10µM amodiaquine solution in 20mM ammonium formate (pH 7.4 adjusted with ammonium hydroxide solution) with 50% acetonitrile was pumped with a constant flow of 10 µL/min through the ReactorCell using an infusion pump. Adduct formation of amodiaquine metabolites and glutathione (GSH) was established using a 100µL reaction coil placed between the ReactorCell and the electrospray source. 100µM glutathione in mobile phase was added at the same flow rate via a T-piece into the coil and the reaction time at the specified flow rate was 5 min. 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 reactions) is shown in Figure 4. The DC potentials of 400mV and 1200mV were applied to form conjugates with Metabolite 1, and Metabolites 2 and 3 (Fig. 1), respectively.

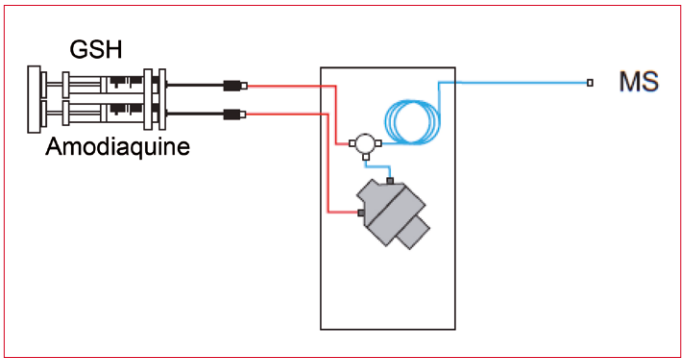


Figure 4: Instrumental set-up of ROXY EC System generating the oxidative metabolites in the ReactorCell (phase I) and subsequnet addition of glutathione via a T-piece for GSH-adduct formation (phase II).

Results

Phase I

Table 1 provides a list of compounds related to amodiaquine metabolism and their monoisotopic masses used for mass spectra interpretation. The 3-D MS Voltammogram shown for amodiaquine (Fig. 5) is a graphical representation of oxidative pattern of the analyte. The data for the MS Voltammogram were recorded using a scan mode with a potential range between 0 and 1500mV, scanned at a 10mV/s rate in the half cycle (Fig. 6).

The background information about MS Voltammogram acquisition using Dialogue are given in the "Dialoque for ROXY user guide" (P/N 210.7017) and in the application note 210_001A "Event Programming for Automated Recording of MS Voltammograms" for details, see our web.

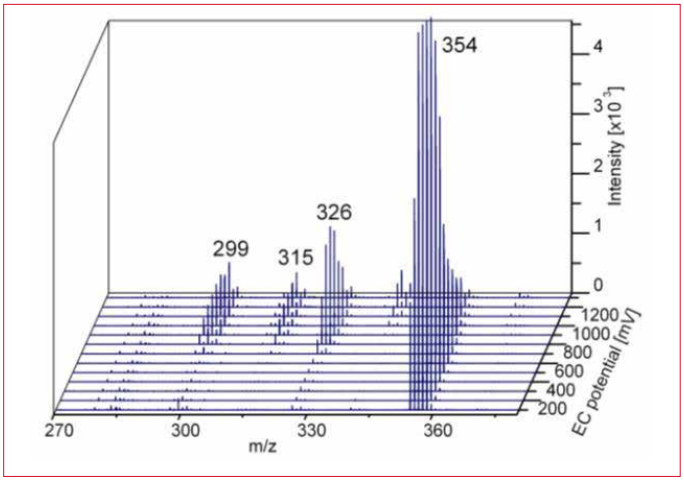


Figure 5: Mass voltammogram of Amodiaquine. Ion abundance versus m/z as a function of EC potential.

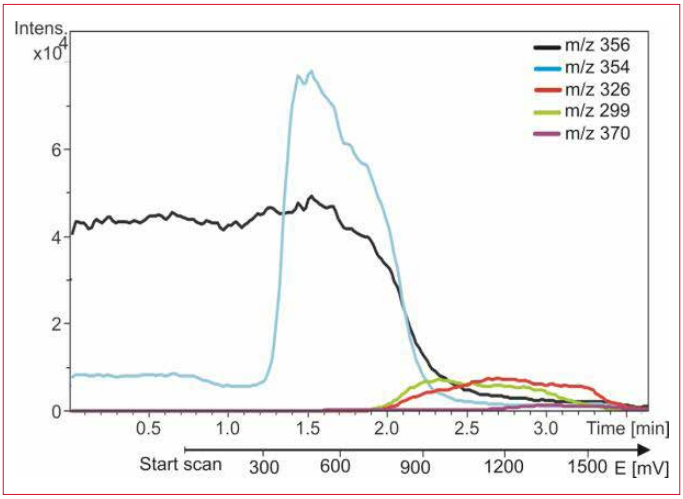


Figure 6: Amodiaquine abundance vs. EC potential. The 2-D MS Voltammogram was acquired using scan mode.

The extracted ion chromatograms for the mass-to-charge ratio (m/z) of amodiaquine (m/z of 356) and its metabolites (m/z of 354; 326; 299 and 370) are shown in Figure 6 as a 2-D MS Voltammogram. Based on the 2-D MS Voltammogram (Fig. 6), the optimum potential for the formation of the particular metabolites was estimated as 400mV for amodiaquine dehydrogenation (metabolite 1), and 1200mV for formation of metabolites 2, 3 and 4.

Furthermore if the potential is higher than 1400mV, hydroxylation of Amodiaquine (m/z of 370) was observed. Fig. 7 shows the mass spectra corresponding to ReactorCell OFF (control measurement) with applied voltages of 400mV and 1200mV.

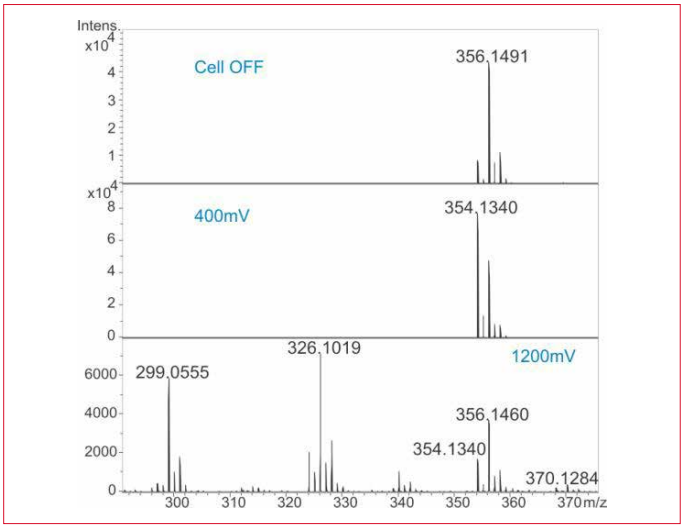


Figure 7: Mass spectra of phase I metabolites of Amodiaquine.

Phase II

To confirm the presence of the conjugation products of Amodiaquine metabolites and GSH, mass spectra were acquired with the ReactorCell off and at Ec = 400 mV and 1200 mV. EIC traces of Amodiaquine metabolites (1 and 2) are presented in Fig. 8. Mass spectra obtained with different potentials and a control experiment with ReactorCell OFF are shown in Fig. 9.

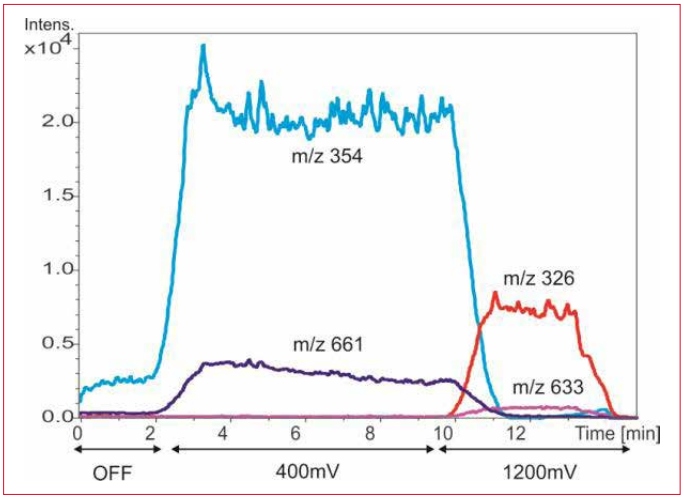


Figure 8: Result of conjugation of phase I metabolites of Amodiaquine with GSH. Example of EICs of Metabolite 1 (m/z 354) and its conjugate (m/z 661) and Metabolite 2 (m/z 326) and its conjugate (m/z 633)

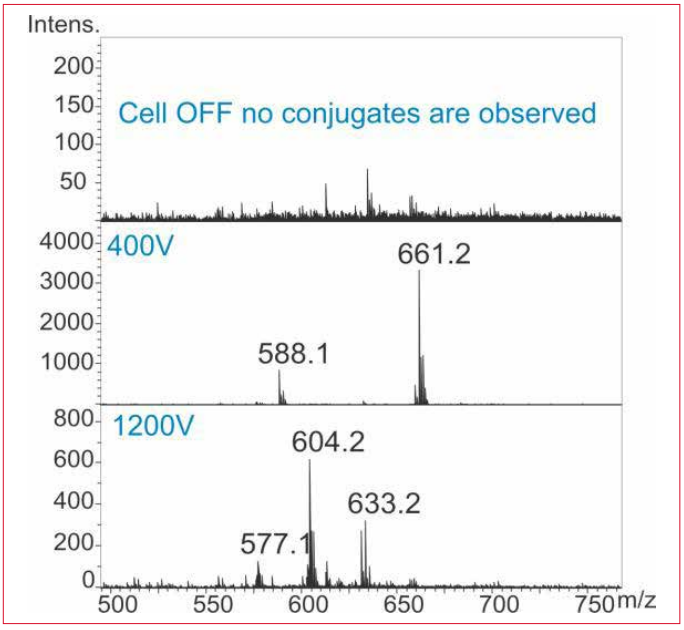


Figure 9: Mass Spectra of GSH-Metabolite adducts formed at 400 and 1200 mV with m/z 661.2 and 663.2, respectively. The spectrum with ReactorCell OFF confirms that the conjugates are formed only if potential is applied.

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).

References

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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.

Application Note
Electrochemical Synthesis

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

SynthesisCell™ – Efficient Synthesis of Metabolites/ Degradants

- **Rapid and cost-efficient synthesis of mg quantities**
- **Superior than traditional wet chemistry/microsomal techniques**
- **Various large surface-area working electrodes**
- **Proven track record in Big Pharma**

Summary

A fast and efficient method for electrosynthesis of metabolites, degradants and reference materials is presented. Using the SynthesisCell oxidation and reduction products can be produced in milligram quantities in a short period of time [1-7]. The Oxidation of 3-methoxy 4-hydroxyphenylglycol (MOPEG) Lidocaine and two drug compounds from Big Pharma (Pfizer and Novartis) are used to demonstrate the electrochemical synthesis of their major metabolites. Almost complete conversion of 0.1 mmol/L MOPEG (1.4 mg) was achieved in 10 min. For Lidocaine 5 µmol/L (ca. 94 µg) was converted by almost 80% in 15 min into the relevant oxidation products. For the drug compound Cipargamin (Novartis) a key secondary metabolite M16 could be synthesized for the fist time and for Fesoterodine (Pfizer) two degradants (oxidation products) could be synthesized with almost 100% yield.

ROXY Application Note # 210_005_03

SynthesisCell™ – Efficient Synthesis of Metabolites and Reference Materials

Introduction

In most areas of drug discovery & development, including environmental degradation of drugs/xenobiotics, there is a severe need for reference materials. The same need exists for most bio-degradation and bio-transformation reactions, which lead to small amounts of REDOX products. In addition, scale-up to mg quantities of these REDOX products are required for comprehensive structural identification by MS, NMR and subsequent toxicology studies.

Conventional methods for synthesis include classical organic synthesis, microsomal incubation or porphyrin-catalyzed chemical oxidation. However, these methods are usually time consuming, cumbersome and not always successful. Electrochemical synthesis is a purely instrumental technique often capable to synthesize such REDOX products in absence of biological matrix in a very short period of time (less than 1 hour).

Figure 1: ROXY™ Potentiostat with SynthesisCell™. The cell contains a Reticulated Glassy Carbon (RGC) working electrode (WE), a Pd/H2 reference electrode (HyREF), and a Pt auxiliary electrode (AUX).

Method

A ROXY™ Potentiostat with extended current range (up to 20 mA) was used with Dialogue Elite software (version 2.0.0.81). The SynthesisCell was equipped with a Reticulated Glassy Carbon (RGC) working electrode, a HyREF™ reference electrode and an auxiliary electrode without frit.

Table 1

Synthesis Conditions

EC	ROXY™ EC System
Cell	SynthesisCell™ with RGC WE, Pt coil AUX and HyREF™
Volume	80mL
Solution A	50 mmol/L acetic acid, pH 4.4, with 5% methanol
Sample	10 or 100µmol/L MOPEG in solution A
Potential	1000 mV
Range	10mA

The SynthesisCell was filled with 80 mL of 10 or 100 µmol/L MOPEG dissolved in solution A (see Table 1). A constant potential of 1V was applied to oxidize MOPEG. The progress of the synthesis was checked each 5 min by taking an aliquot of 100 µL of the SynthesisCell solution. The sample was diluted a factor 20 (10 µmol/L) or 200 (100 µmol/L) prior to HPLC/ECD analysis (see Table 2).

A porous frit can be used to prevent mixing of products that are formed at the working and auxiliary electrodes and was also compared in this study. The conversion is calculated by the % decrease in MOPEG peak area when switching on the cell.

Table 2

Detection Conditions

HPLC	LC 110; AS 110; DECADE II
Flow cell	VT03 flow cell with ISAAC and GC WE
Column	Antec HPLC Column for PQ
Detection potential	650 mV
Range	10mA

Table 3

Cleaning Conditions

Detection mode	scan
E1	- 200mV
E2	+1000mV
Scan rate	50 mV/s
Cycle	continuous
Time	30min

Results

Case Study 1 - MOPEG

Figure 2 depicts the progress of electrosynthesis for different experimental conditions using 100 and 10 µmol/L MOPEG and an AUX electrode with or without frit. No significant difference in conversion efficiency was observed for both AUX configurations. The complete oxidation of MOPEG was achieved in less than 30 minutes and near 100% conversion was reached in only 10 minutes. The current response was measured during the electrosynthesis using the Dialogue software (Figure 3). Evidently, only during first 15 minutes of oxidation the current response was significantly declining from 7.5 mA to approx. 0.5 mA. After 25 minutes of oxidation the current stabilized at about 130 µA. This observation corresponds to conversion efficiency (Fig. 2), which reached 100% after 15 min of electrosynthesis. Registering of the current response can give an insight in the electrosynthesis progress even without the control sample measurement.

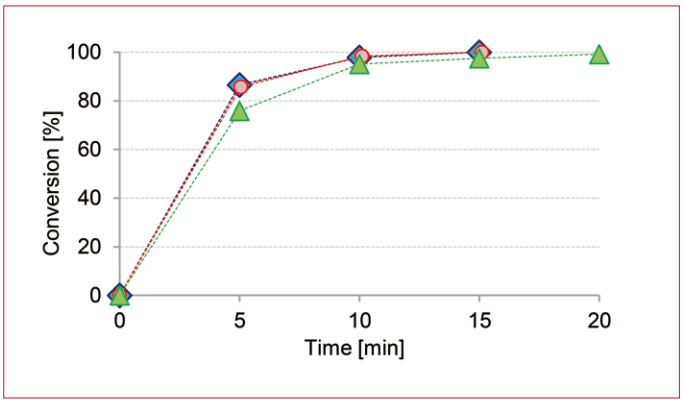

Figure 2: Oxidation of MOPEG. Green/Red: 10 µmol/L MOPEG. Blue: 100 µmol/L MOPEG. Green: using AUX with frit, the others are without frit.

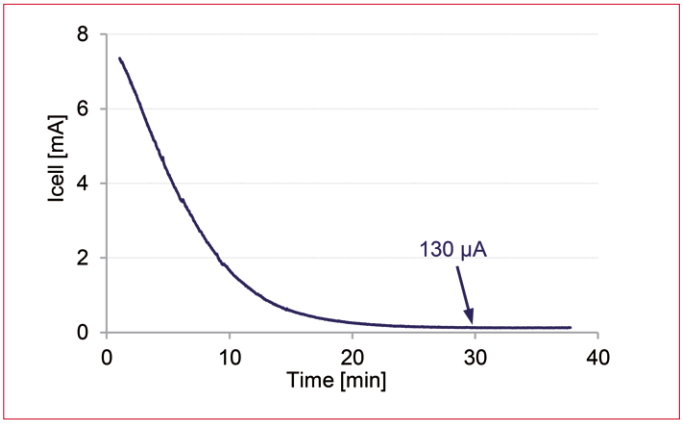

Figure 3: The current (I-cell) measured in the SynthesisCell during oxidation of 10 µmol/L MOPEG, using Dialogue.

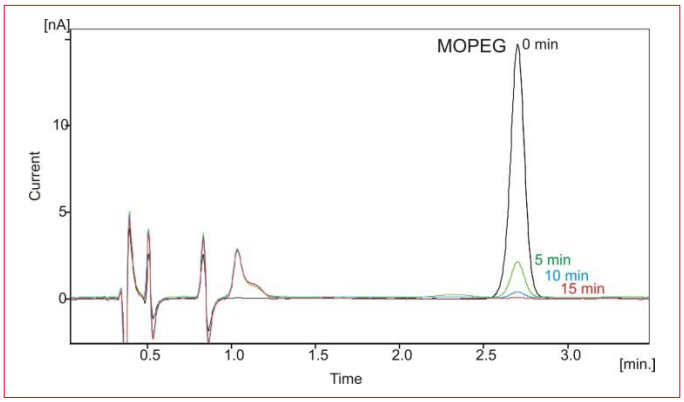

Figure 4: Oxidation of MOPEG. At time 0, 5 ,10 and 15 min. After 15 min almost 100% conversion.

Figure 4 shows the oxidation of MOPEG in the SynthesisCell. After 15 min almost full conversion of the the MOPEG was obtained.

Case Study 2 - Lidocaine

Lidocaine is a common local anesthetic and class-1b antiarrhythmic drug. Lidocaine is used topically to relieve itching, burning, and pain from skin inflammations, injected as a dental anesthetic, or as a local anesthetic for minor surgery. It is listed as essential medicine by WHO and applied in numerous healthcare products.

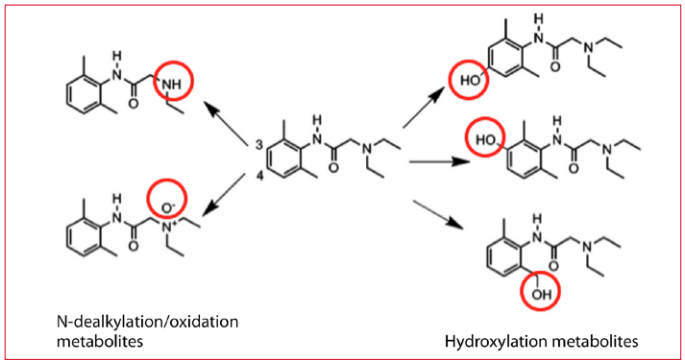

Figure 5: *In-vivo* metabolites of Lidocaine due to oxidative metabolism by Cytochrome P450. Metabolites result from N-dealkylation, N-oxidation, and aromatic and benzylic hydroxylation

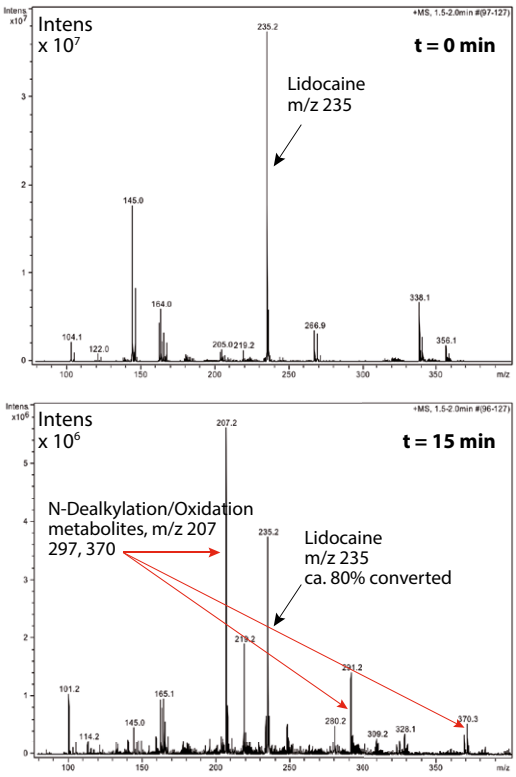

Figure 6: MS spectra of aliquots taken from the SynthesisCell at t=0 and t=15 minutes. Ca. 80 % of the Lidocaine was converted into 3 main reaction products (N-Dealkylation and N-Oxidation metabolites using the conditions listed in Table 4 and the Reticulated Glassy Carbon (RGC) electrode.

In Figure 6 the MS spectra are shown for aliquots taken at 0 and 15 minutes from the 80 mL SyntesisCell analyzed by direct infusion ESI/MS. At 0 minutes only Lidocaine is present. After 15 minutes of electrolysis ca. 80% of Lidocaine was converted into the oxidation products with m/z 207, 297 and 370, which correspond to the N-dealkylation and N-Oxide metabolites of Lidocaine. For the generation of larger amounts of hydroxylation metabolites, the use of Boron Doped Diamond (BBD) working electrode is required. Data not shown.

Table 4

Synthesis Conditions	
EC	ROXY™ EC System
Cell	SynthesisCell™ with RGC WE, perforated glass tube as AUX and HyREF™
Volume	80mL
Solution A	20 mM NH4Ac + 0.1M Acetic Acid in ACN:H2O (90:10)
Sample	5 µM Lidocaine*)
Potential	1500 mV, DC mode
Range	20mA

*) Up to 100 x higher concentrations are typically used. This low concentration was chosen for direct infusion MS of aliquots from the SynthesisCell without any sample preparation, i.e., filtration or dilution.

Examples from Pharma

Cipargamin (KAE609), Novartis

In case of Novartis, a key secondary metabolite M16 of the antimalarial drug Cipargamin (KAE609) was identified in all biological matrices at very low levels.

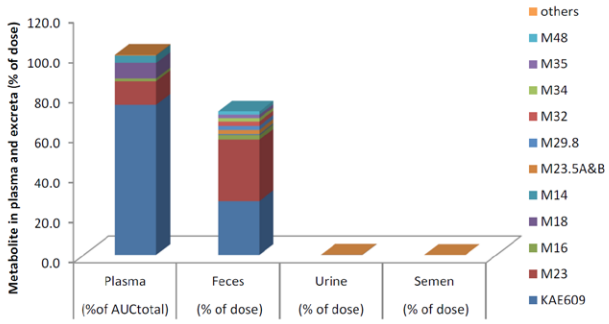

Figure 7: Overall metabolism of Cipargamin (KAE609)

All 19 recombinant human CYP enzymes were capable of catalyzing the hydroxylation of M23 to form M16 but with insufficient turn-over for structural characterization by NMR.

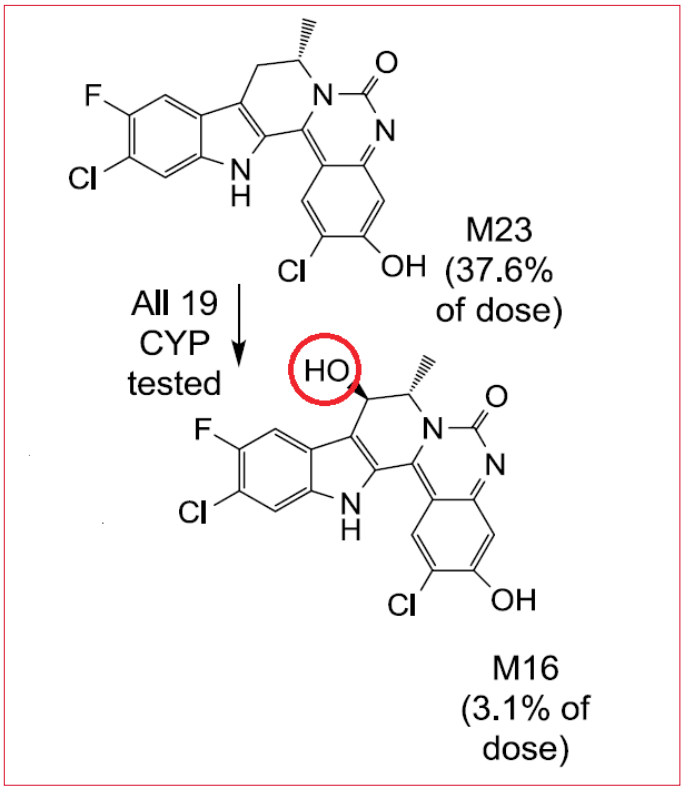

Figure 8: Excerpts of the metabolic pathway of Cipargamin. Hydroxylation of metabolite M23 to M16

As the proposed structure of M16 suggest benzylic oxidation, electrochemical synthesis was applied using the ROXY EC system equipped with SynthesisCell. A boron doped diamond electrode under acidic conditions gave the desired stereoselective product in 10% yield. For the first time ever, sufficient quantities of M16 could be synthesized, to allow full structural characterization by NMR, previously unable using traditional enzymatic techniques [5].

Fesoterodine, Pfizer

At Pfizer, electrochemical synthesis was used for the fast and convenient synthesis of pharmaceutical oxidation products (degradation products) of N-dealkylation reactions of Fesoterodine.

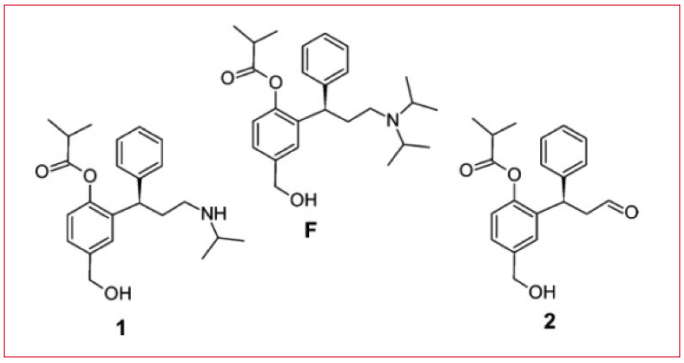

Figure 9: Chemical structure of Fesoterodine (F) and its two oxidative N-dealkylation products (degradants) 1 and 2.

A working potential of 950 mV was applied using the ROXY Potentiostat equipped with the SynthesisCell for the generation of the two oxidative N-dealkylation products (degradants). A glassy carbon working electrode (Reticulated Glassy Carbon – RGC) was used as the supporting electrolyte. The reaction was monitored over a 2 h period of time. Aliquots of the reacting solution were taken at given time points and analyzed using high-performance liquid chromatography with UV and mass detection. After turning on the cell voltage, a decrease in fesoterodine peak area was observed with concomitant formation of the two N-dealkylated oxidation products. These experimental conditions generated an almost complete conversion of fesoterodine into the two N-dealkylation products after 2 hrs of operation.

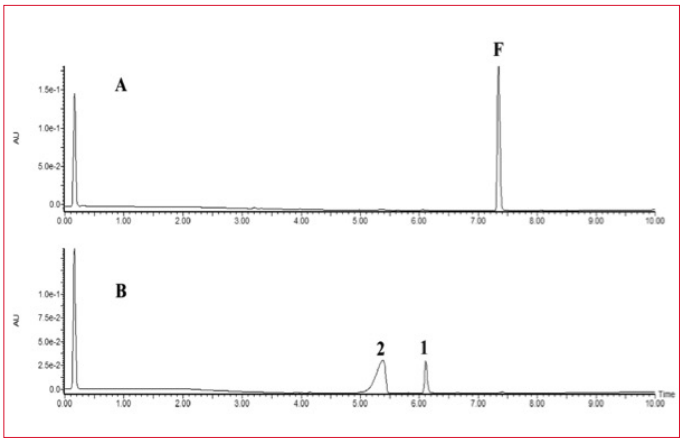

Figure 10: UV chromatograms at 224 nm. (A) 0.25 mg/mL fesoterodine fumarate solution in 50 mM aqueous ammonium acetate (no potential). The observed peak "F" corresponds to fesoterodine. (B) Reaction mixture after 2 hrs (a constant potential of 950 mV was applied to the cell) with the two oxidative N-dealkylation products (degradants) 1 and 2.

The two oxidation products were purified by reverse-phase preparative high-performance liquid chromatography and subsequent characterization by NMR.

Pfizer reported that the electrochemical procedure proved to be rapid, clean, and efficient compared to traditional synthetic methods and that it is particularly useful for generating milligram quantities of oxidative degradants [6].

References

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Ordering information

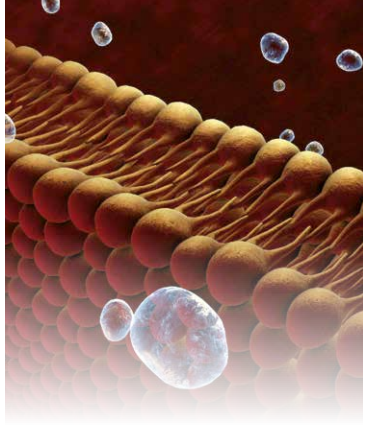
ROXY EC System	
210.0010A	ROXY Potentiostat, High Current
SynthesisCell	
206.0037	SynthesisCell, consisting of 80 mL reaction vessel with Teflon cap, WE (Reticulated Glassy Carbon), RE (HyREF) and AUX electrode, stir bar, all parts included for immediate use with high current ROXY Potentiostat
Optional	
206.0306	Magic Diamond (BDD) working electrode
206.0322	Platinum (Pt) working electrode

Conclusion

The electrosynthesis using the SynthesisCell is fast, efficient and cost-effective. Full conversion in less than 30 min has been demonstrated for MOPEG, using the large surface area Reticulated Glassy Carbon working electrode. With same type of electrode all major N-dealkylation and N-oxide metabolites of Lidocaine can be produced. Moreover, other types of working electrodes such as Magic Diamond (BDD) and Platinum (Pt) are available for increased selectivity such as aromatic and benzylic hydroxylation reactions on BDD. In the examples of Novartis and Pfizer the obtained oxidation products could be synthesized for the first time for characterization by NMR.


Science is organized knowledge. Wisdom is organized life.
Immanuel Kant

Application Note
Lipidomics / Cholesterol



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

Generation of Multiple Oxysterols by Oxidation in an Electrochemical Flow-Through Cell

- **Cholesterol oxidation**
- **Easy and fast generation of oxysterols**
- **Mimicking free radical and enzymatic oxidation**

Introduction

Electrochemical (EC) oxidation using EC flow-through cells becomes a popular technique for fast simulation of biological and technologically relevant redox reactions. Combined with mass spectrometry (MS), EC oxidation allows characterization of diverse oxidation products and intermediates formed during an oxidation process, and thus provides deeper understanding of free radical oxidation mechanism and indications for potential products generated in vivo (Jahn & Karst 2012, Faber et al. 2014). EC-MS, often in combination with liquid chromatography (LC), was successfully applied for simulation of oxidation processes in the environment (Hoffmann et al. 2010), elucidation of xenobiotics degradation (Chen et al. 2012), mimicking cytochrome P450 enzyme activities (Jurva et al. 2003), fast prediction of phase I and II drug metabolism and detoxification (Lohmann & Karst 2006, Baumann et al. 2009a), as well as for studying nucleic acid oxidation (Baumann et al. 2009b), disulfide bond arrangements of peptides/proteins (Zhang et al. 2011, 2012), and other protein post-translational modifications (Lohmann et al. 2008, Jahn et al. 2012).

Oxysterol species are formed in vivo by enzymatic and non-enzymatic oxidation of cholesterol. Oxysterols are intermediates in the biosynthesis of bile acids and steroid hormones, but also possess per se versatile bioactivities, such as controlling gene expression, affecting calcium-signaling and immune or inflammatory responses. Many functions of oxysterols are not fully understood and

others may not have been discovered yet, especially those of non-enzymatic origin. The limited number of commercially available standards challenges both analyses and functional studies.

Here we report the generation of numerous cholesterol oxidation products in short reaction times by using an amperometric flow-through cell (ROXY EC system, Antec, NL) and characterization of obtained species by normal phase thin layer chromatography (NP-TLC) and reverse phase (RP)-HPLC-MS.

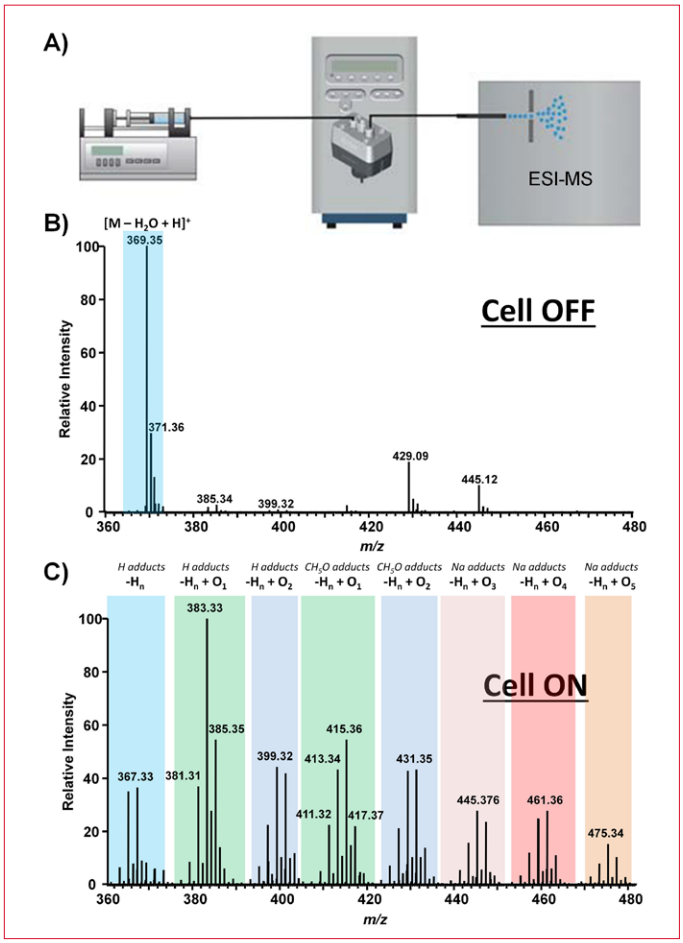


Figure 1: Schematical representation of the ROXY EC system (including the syringe pump and the ROXY potentiostat with the µ-PrepCell) coupled to ESI-MS (A) and the mass spectra aquired by the ESI-LTQ-Orbitrap XL mass spectrometer in positive ion mode for a 100 µmol/L cholesterol solution under the applied EC-cell OFF (B) vs. EC-cell ON (C) conditions. Protonated ion species, sodium- and methanol-adducts are shown.

Material & Methods

Cholesterol (100 µmol/L in 90 % MeOH containing 20 mmol/L ammonium formate) was introduced by syringe pump (50 µL/min) into the µ-PrepCell equipped with a boron doped diamond working electrode and a Pd/H2 reference electrode controlled by the ROXY potentiostat via the Dialogue Software (ROXY EC system, Antec, NL, Fig. 1A). The voltage-dependent oxidation-process was first monitored by coupling the system directly to the ESI-LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, Fig. 1B,C). Later, 2V were constantly applied, the output flow collected and stored at -20°C (with/without 0.05 % BHT) for further analyses by NP-TLC and RP-LC-MS. The electrochemically (EC) generated oxidized products of cholesterol were compared to a set of 19 commercially available oxysterols. The EC-oxidized solution (200 µL) and standards (2 µg) were separated on HPTLC Silica gel 60 F_{254} plates (15 cm x 10 cm, Merck KGaA, Darmstadt, Germany), developed with a mixture of ethylacetate and toluene (50:50, v/v) and dipped into primuline solution (0.02 % in acetone/water, 8:2, v/v) for visualization (Biorad GelDoc EZ Imager, UV Tray). The generated cholesterol oxidation products were additionally analyzed by LC-MS and LC-MS/MS analysis by multiple reaction monitoring (MRM) on a QTRAP 4000 (AB-Sciex) mass spectrometer coupled on-line to C18-column.

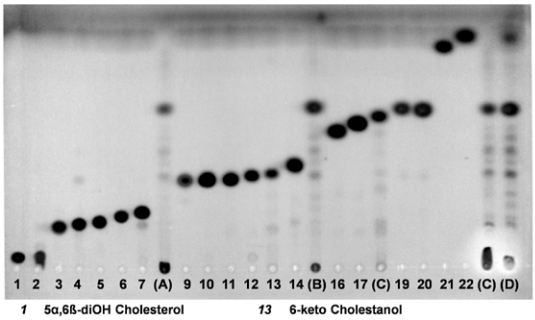


Figure 2: Alalysis of the compounds generated by EC-oxidation of cholesterol by NP-TLC. Lanes 1-22: commercially available oxysterol standards (2µg). A-D: EC-oxidized cholesterol mixture equivalent to 2 pmol of initial cholesterol)

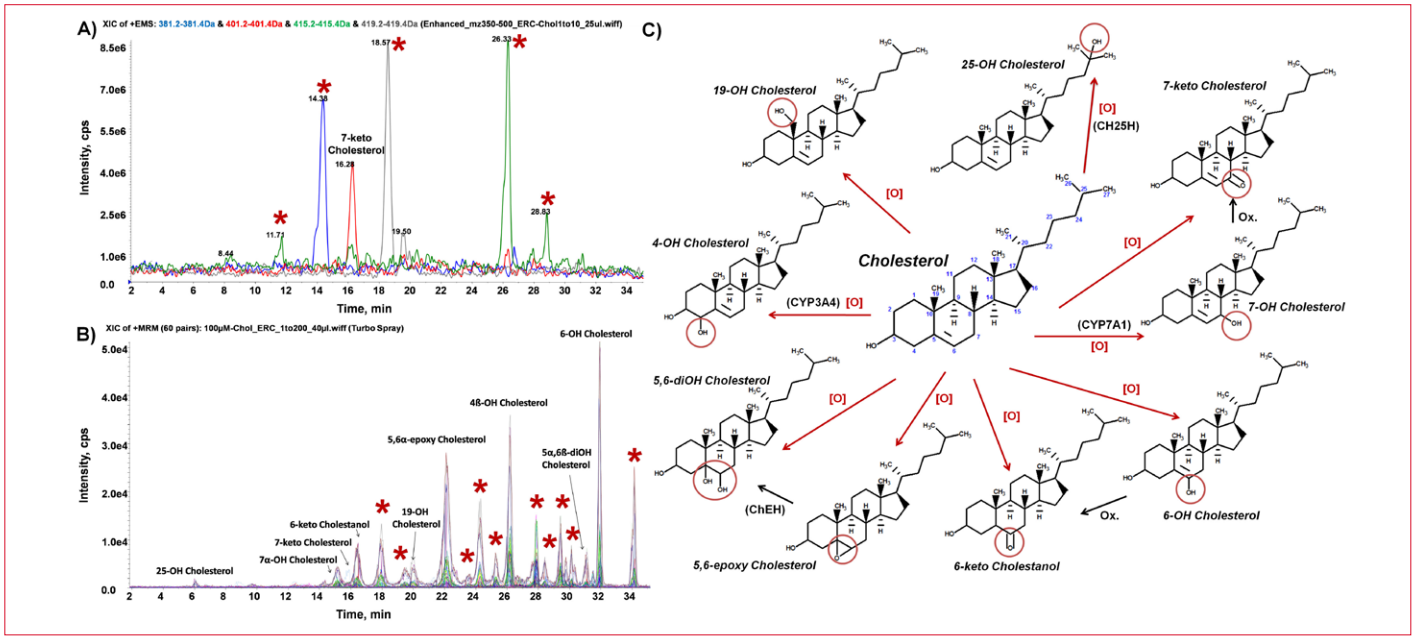


Figure 3: Analysis of the products generated by EC-oxidation of cholesterol by RP-HPLC-MS (extracted ion chromatograms from full scan MS (A) and MRM (B) on the QTRAP 4000). Unknown compounds are marked by asterisks. C: Cholesterol and the generated oxysterols identified by RP-HPLC-MRM. The carbon numbering at the cholesterol backbone is shown in blue. The sites of oxidation are highlighted by red circles. Indicated are the free radical driven (red arrows) and possible consecutive reactions (black arrows) leading to the formation of the identified compounds, as well as enzymes (in brackets) involved in the generation of oxysterols *in vivo*.

Results

The ROXY™ EC system (Antec, NL) equipped with the u-Prep-Cell™ allowed us to oxidize cholesterol yielding numerous oxidation products within short reaction times (Fig. 1C), which were analyzed by NP-TLC (Fig. 2) and RP-HPLC-MS (Fig. 3A, B) relative to 19 standard compounds. Besides the six oxysterols identified by both techniques, more than ten additional electrochemically generated compounds were detected. The identified products were mostly oxidized near the double-bound at the B-ring (and to a lower extent at the tertiary carbon in position 25), which is in agreement with susceptibility to free radical driven oxidation (Fig. 3C). Interestingly, some of the new electrochemically generated oxysterols were also present in lipid extracts obtained from cell culture models of nitrosative stress. Further investigation of electrochemically generated compounds (e.g. using SynthesisCell™ for higher production yields) will allow identification and characterization of new oxysterols in vivo.

Acknowledgment

All data of this application note were kindly provided by Dr. Maria Fedorova[1,2] et al., and will be presented at the 4th European Lipidomics Meeting, September 22-24, Graz, Austria and as poster at the SFRR 2014, September 5-7, 2014, Paris, France

1 Institute of Bioanalytical Chemistry, Faculty of Chemistry and Mineralogy,
2 Center for Biotechnology and Biomedicine (BBZ), University of Leipzig, Leipzig, Germany

Conclusion

By using an electrochemical flow-through cell, cholesterol can be easily oxidized to different oxysterols. The obtained oxysterols show excellent agreement with the known enzymatic biotransformation reactions and with some of the radical driven reactions.

Electrochemistry in combination with LC/MS and/or TLC/MS has great potential for the identification and discovery of oxysterols, thereby mimicking enzymatic and free radical reactions including nitrosative stress.

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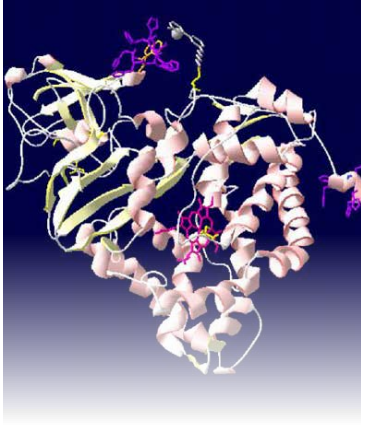
Figure 4: ROXY EC System consisting of ROXY Potentiostat, dual syringe pump and µ-PrepCell

Ordering information

210.0074A	ROXY EC system, incl. dual syringe pump, µ-PrepCell and electrodes. All parts included for described Electrochemical (EC) application.

Application Note
Proteomics & Protein Chemistry



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

Controlled Reduction of Disulfide Bonds in Proteins/Peptides using on-line EC/MS

- **Fast reduction of S-S bonds in an electrochemical flow-through cell**
- **Reagent free, no reducing chemicals, e.g., DTT, TCEP, etc.**
- **Ideal for TCEP resistant proteins**
- **Automated reduction by on-line EC/MS or LC/EC/MS**

Summary

The electrochemically-assisted reduction of disulfide bonds in peptides and proteins after HPLC and followed by on-line mass spectrometric detection is presented. The method is based on square-wave potential pulses applied on a new type of working electrode made from Titanium alloy. The method does not use any chemical agents and is purely instrumental resulting in a fully automated platform for fast assessment and characterization of S-S bonds in proteins/peptides [1-6].

ROXY Application Note # 210_006_03

Controlled Reduction of Disulfide Bonds in Proteins/Peptides using on-line EC/MS

Schematics Disulfide Bond Reduction

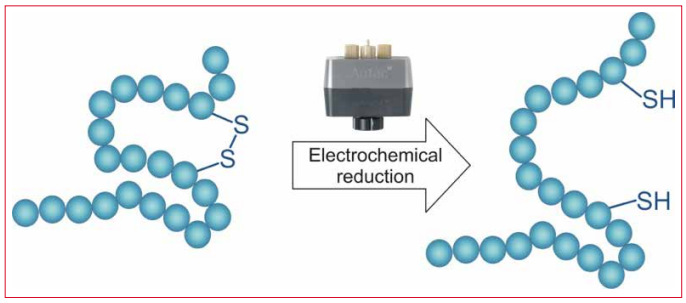


Figure 1: Schematics disulfide bond reduction: replacing interfering chemicals (e.g., DTT, TCEP) by an electrochemical reactor cell used on-line with LC/MS. Push-button reduction

Introduction

Reduction of disulfide bonds in peptides and proteins prior to MS analysis is done for several reasons. It is one of the steps in the determination of the disulfide bonds positions which is important for understanding the folding processes of a protein. Also disulfide bonds can hinder MS identification and their reduction is necessary for efficient sequence analysis in top-down proteomics, or prior to HDX exchange procedures. We developed an electrochemical (EC) method for the efficient and fast reduction of disulfide bonds in peptides and proteins. The method utilizes an electrochemical flow-through cell. The cell can be directly connected to the ESI-MS for flow injection analysis or after the HPLC column in case of complex sample analysis by LC/EC/MS. A complete or near to complete reduction of the disulfide bonds of the tested proteins and peptides has been demonstrated.

Method

All experiments were performed on a ROXY EC system (Antec, The Netherlands) consisting of a ROXY Potentiostat, equipped with a µ-PrepCell. The ROXY system was online hyphenated to a LTQ-FT mass spectrometer (Thermo Fisher Scientific, USA).

The thin-layer electrochemical reactor cell consisted of a titanium-based working electrode (WE) specifically optimized for efficient reduction [1-6], a titanium auxiliary (counter) electrode (AUX) and a Pd/H2 reference electrode (REF). A 100-µm spacer was used to separate the WE and the auxiliary electrode inlet block giving a cell volume of approximately 6 µL. The ROXY EC system was controlled by Dialogue software. An electrical grounding union was used to decouple the electrochemical cell from the ESI high voltage.

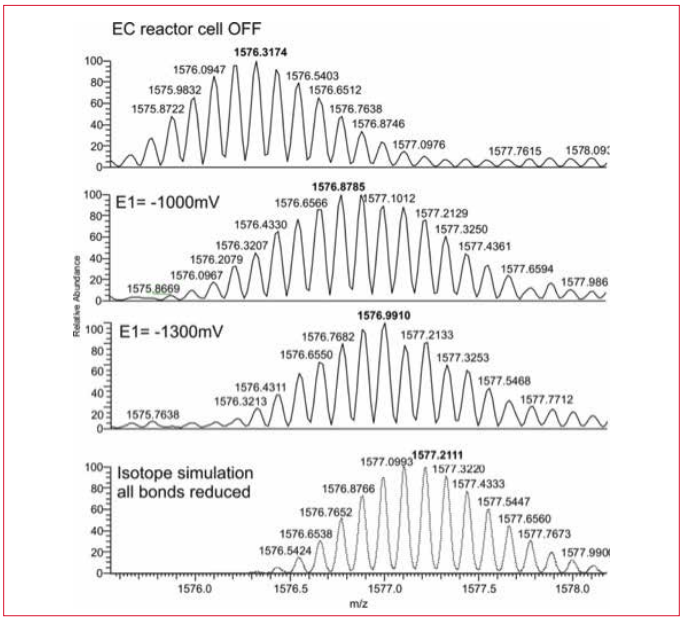


Figure 2: Partial and full reduction of disulfide bonds in α-lactalbumin. The overlapping isotopic pattern of the +9 ion measured with the EC reactor cell turned OFF (top), and ON at E1= -1000mV and E1= -1300mV.

Table 1

Conditions

HPLC	ROXY HPLC/EC system
Column	ALF C18, 150 x 1 mm, 3 um (Antec)
Mobile phase	1% Formic acid (250 mmol/L) in water with 5% acetonitrile. Gradient running from 10 – 50 % acetonitrile in 10 min.
Flow rate	50 µL/min
Vinjection	5 µL
Temperature	35 C
Flow cell	µ-PrepCell, Ti working electrode, HyREF electrode.
Potential waveform	E1, E2: -1.5, +1 V, t1, t2: 1990,1010 ms

In all experiments a square wave pulse was applied which has been described earlier [1].

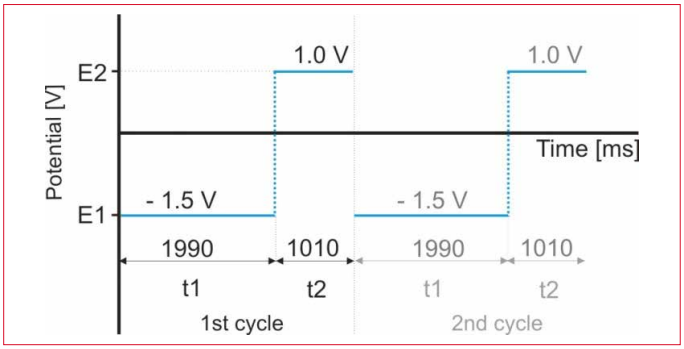


Figure 3: A schematic representation of the square-wave pulse. Under optimized conditions, the potentials were −1.5 V (E1) and +1.0 V (E2) and time intervals were 1,990 ms (t1) and 1,010 ms (t2), unless specified otherwise

Results

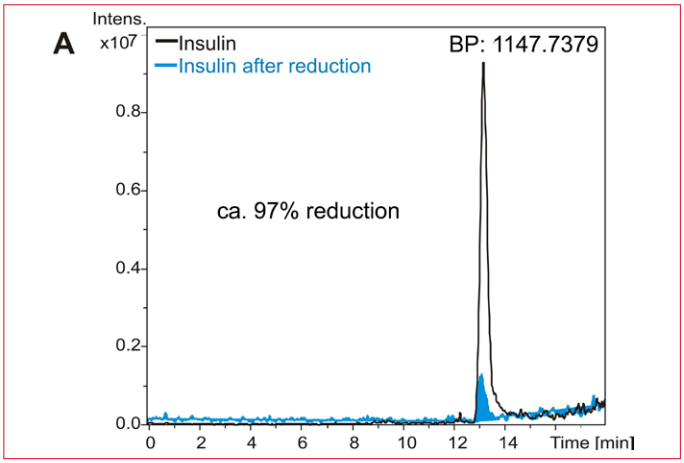


Figure 4: Reduction efficiency: Online HPLC/EC/MS analysis of a mixture of insulin (m/z 1147.7379) and somatostatin (m/z 819.3654). The overlays in A and B show the m/z traces of the non-reduced intact peptide with the µ-PrepCell "OFF" (main peak) and the almost fully reduced peptide with µ-PrepCell "ON" with reduction efficiencies of 95 and 97%.

Insulin, a small protein of 5733 Da containing 3 disulfide bridges, somatostatin with one disulfide bond (1638 Da), and α-lactalbumin with four bonds (14178 Da) were used as test substances to demonstrate the applicability of the method.

The reduction efficiency is affected by several parameters such as instrument set-up (i.e. infusion EC/MS vs. LC/EC/MS), flow rate, mobile phase composition (formic acid and acetonitrile content) peptide/protein concentration of the sample and the square-wave pulse settings (potential). By changing the applied potential the extent of disulfide bond cleavage is controlled. More negative potentials result in a shift of the charge state distribution indicating increased disulfide bond cleavage and unfolding of the protein (Fig. 2).

By increasing the flow rate and thereby shortening the residual time of the chromatographic peak in the electrochemical cell, a decrease of the reduction efficiency is inevitable.

Consequently, the flow rate can also be used to control the reduction efficiency beside the applied potential (square-wave pulse). Thus, by proper selection of the flow rate or potential it becomes possible to switch between complete and partial disulfide bond reduction.

Partial reduction is of particular importance to localize disulfide bonds and to study the impact of individual disulfide bonds on peptide and protein structures.

The effects of different experimental parameters are tested and the optimized protocol for the electrochemical reduction of disulfide bonds by online LC/EC/MS has been described in details elsewhere [1].

Conclusion

An electrochemical Flow-Through Cell for efficient reduction of disulfide bonds in proteins and peptides has been demonstrated. The new proprietary [6] titanium based working electrode provides high reduction efficiencies of 80 to almost 100% for the tested peptides. This electrochemical (EC) reduction can be done by direct infusion EC/MS or by LC/EC/MS. The applied potential can be used to control the degree of S-S bond reduction/cleavage and therefore offers new opportunities for faster and superior characterization of disulfide bonds in protein therapeutics. The chemical free EC reduction shows further great potential for the disulfide bond reduction of TCEP resistant proteins/peptides prior to HDX MS analysis.

Figure 5: ROXY EC system for S-S reduction.

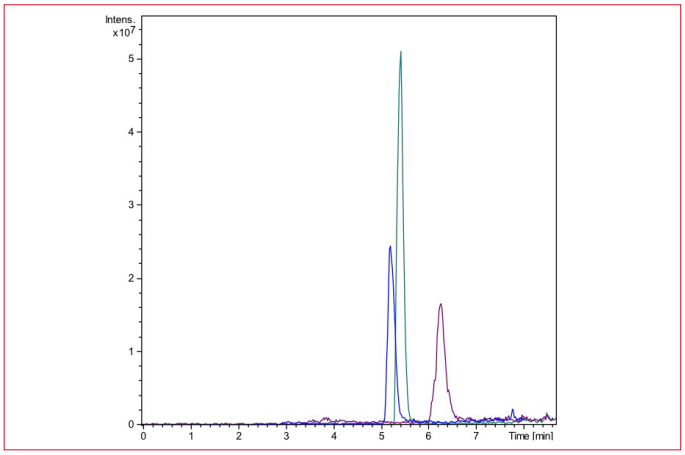


Figure 6: Peak broadening caused by the electrochemical cell. Separation of Insulin, without µ-PrepCell (green), and with µ-PrepCell: with a 100 µm spacer (blue) and with 150 µm spacer (purple). Flow rate was 50 (green) and 75 (blue, purple) µL/min.

The electrochemical cell was positioned post-column resulting in a fully automated platform for fast characterization of disulfide bonds in protein/peptide samples. HPLC mass spectra of two test substances are shown in Fig. 4. After separation the peptides are reduced in the µ-PrepCell. The peak broadening has been investigated by comparing analysis with and without the µ-Prepcell (Fig. 6). The plate numbers changed from 6000 to about 4000 when applying the µ-PrepCell. Changing the spacer from 150 to 100 µm improved the platenumber to 5000 and this configuration was used for further experiments. In Figure 6A the amino acid sequence of Insulin with its 3 disulfide bonds is shown. Figure 6B and C show the mass spectra of intact and reduced insulin and in Figure 6D the ions of the intact and reduced intrachain disulphide bond of chain A are shown.

References

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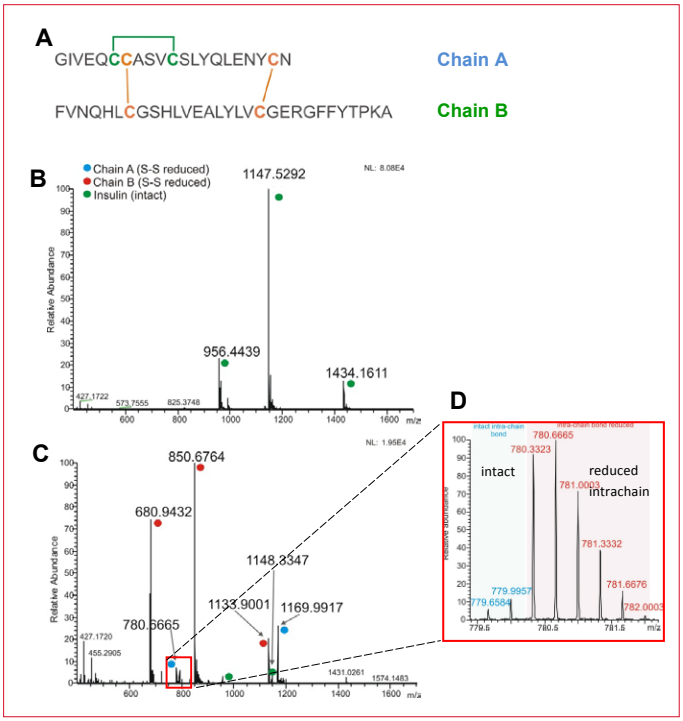

Figure 7:

A) Amino acid sequence of Insulin consisting of 51 amino acids with two interchain disulfide bonds between Chain A and B and one intrachain disulfide bond located in chain A.

B) Mass spectra of intact insulin with 3 major fragments (green dots) with m/z 1434.1611, m/z 1147.5292 and m/z 956.4439 for $[M+4H]^{4+}$, $[M+5H]^{5+}$, $[M+6H]^{6+}$ ions, respectively.

C) Mass spectra of reduced insulin with two low abundant fragments for chain A (blue dots): m/z 1169.9917 and m/z 780.6665 which are in agreement with the theoretical masses of reduced $[M+2H]^{2+}$ and $[M+3H]^{3+}$ ions of chain A, and three higher abundant fragments for chain B (red dot), m/z 1133.9001, m/z 850.6764 and m/z 680.9432, detected as $[M+3H]^{3+}$, $[M+4H]^{4+}$, $[M+5H]^{5+}$ ions of the reduced B chain.

D) Zoom of the $[M+3H]^{3+}$ ion with m/z 780.6667 of chain A of reduced insulin with its isotopic distribution showing ions from the intact and reduced intrachain disulphide bond.

Ordering information

210.0072A	ROXY EC system for S-S reduction
210.4300T	µ-PrepCell, Ti WE, HyREF

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].

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

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.

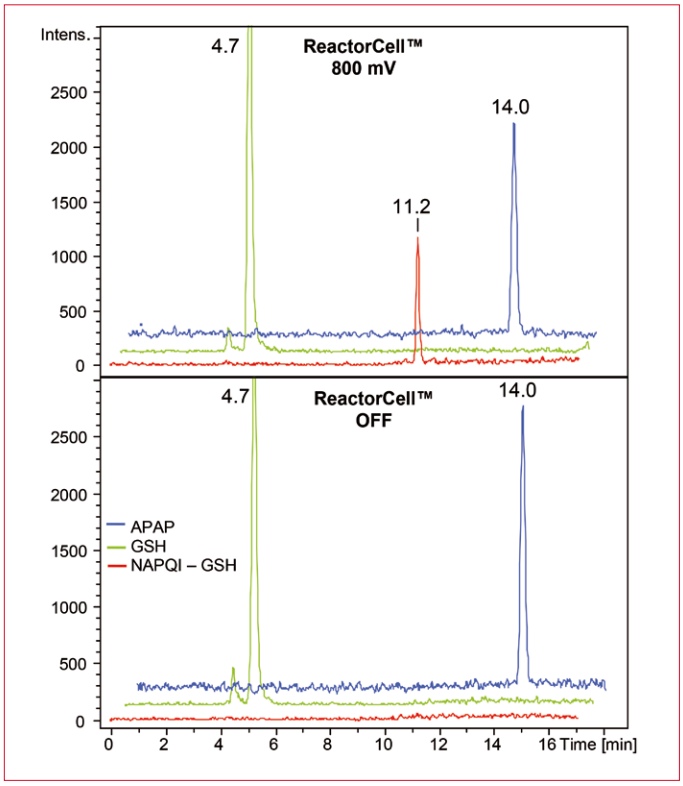

Figure 1: Automated generation of phase I and II metabolites of acetaminophen (APAP) using the ROXY™ EC/LC system: with Reactor Cell™ ON (top) immediate formation of the NAPQI—GSH conjugate (phase II reaction) is observed (red).

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.

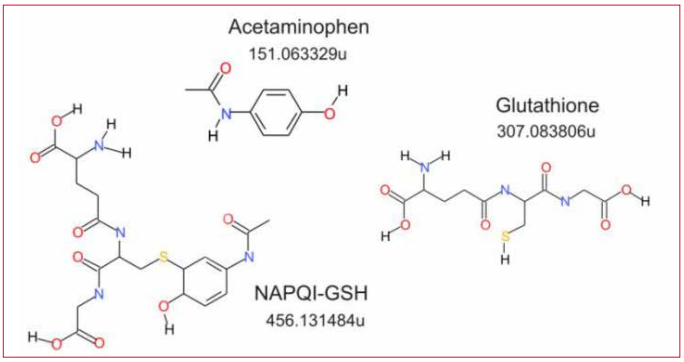

Figure 3: Structures and monoisotopic masses of acetaminophen, glutathione and conjugate of the reactive metabolite of acetaminophen (NAPQI-GSH).

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

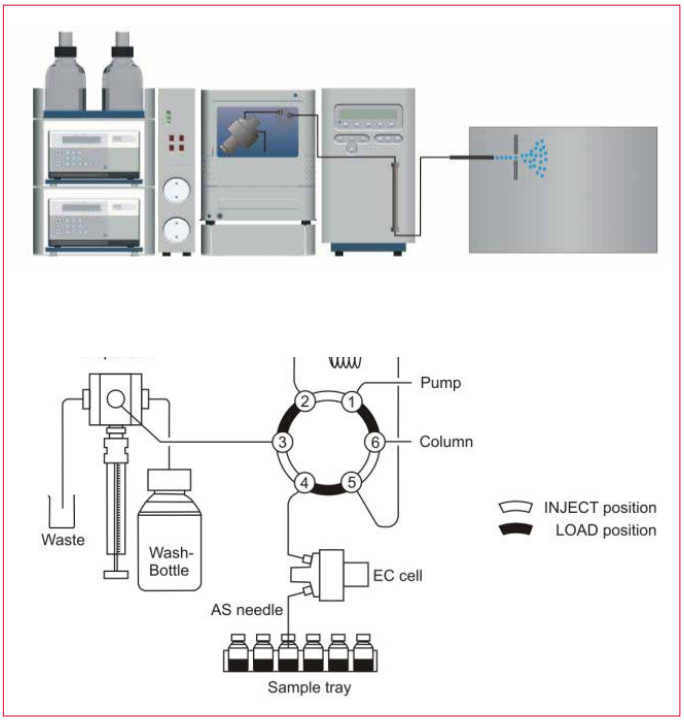

Figure 3: Top: ROXY™ EC/LC System including ReactorCell™ integrated in the AS110 autosampler flow path. Bottom: Detailed lay-out of autosampler flow path with 6-port injection valve and ReactorCell™

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).

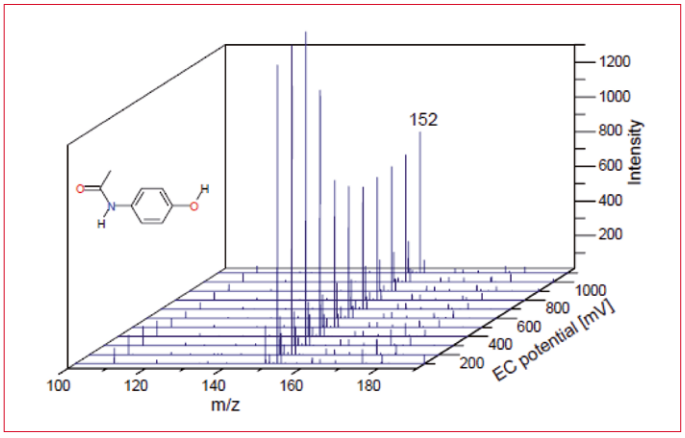

Figure 4: Mass voltammogram of acetaminophen.

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

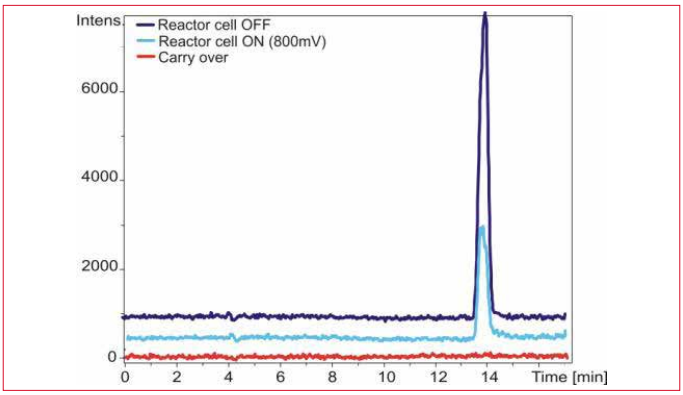

Figure 5: Extracted ion chromatograms (EIC) of APAP (m/z= 152 Th), eluted in 14 min. 65% conversion of the APAP is observed with ReactorCell™ ON (800mV; light blue). Dark blue trace corresponds to control measurements with ReactorCell™ OFF. Red trace is a mobile phase injection

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.

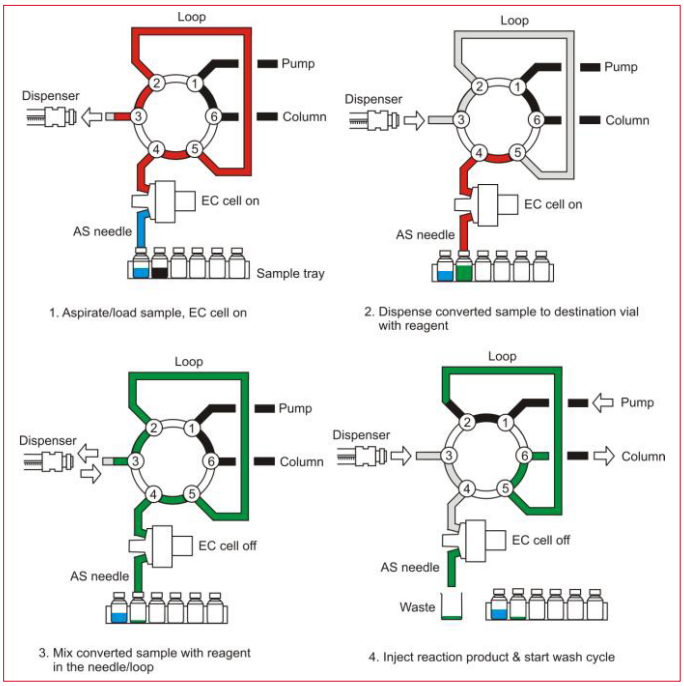

Figure 6: Simplified schematic representation of the automated Phase II injection routine. Blue:sample,red: oxidized sample, black: reagent and green: oxidized sample mixed with reagent.

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).

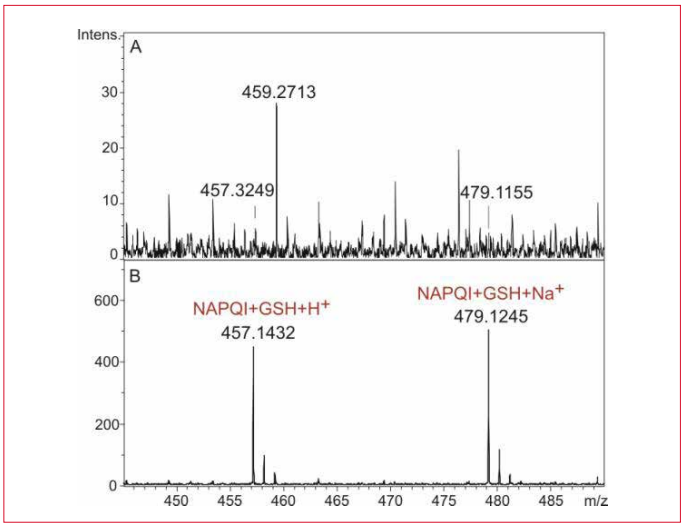

Figure 7: Result of conjugation of phase I metabolite of acetaminophen (NAPQI) and GSH. (A.) ReactorCell OFF, (B.) Reactor Cell EC = 800mV.

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).

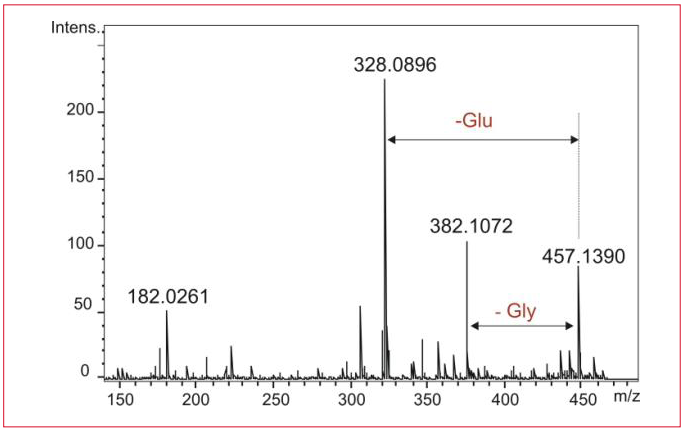

Figure 8: Fragmentation spectrum (MS/MS) of conjugation product.

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.

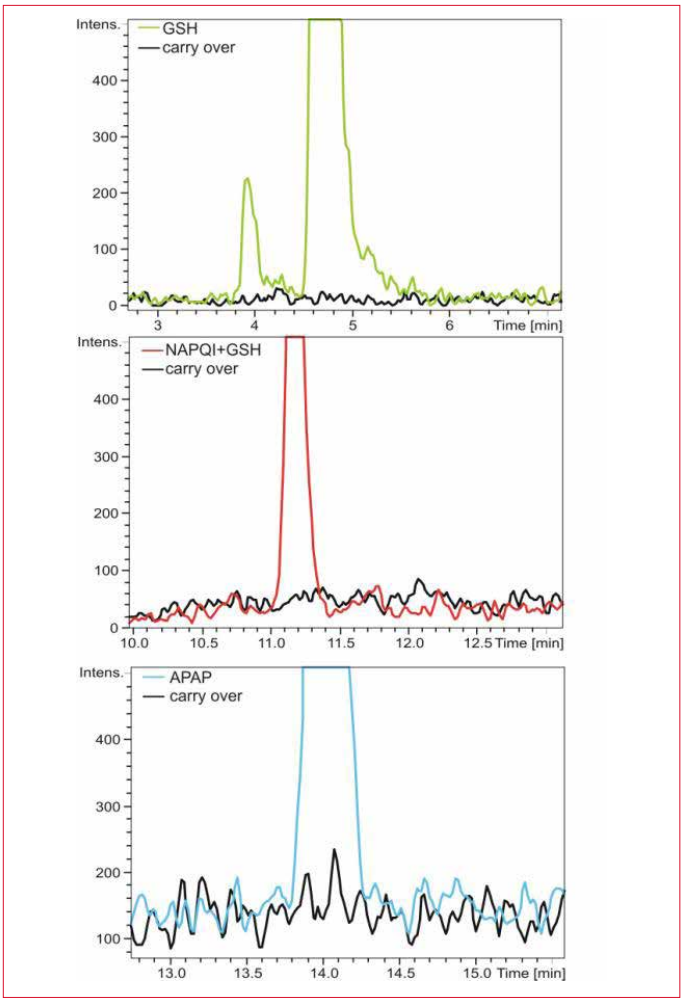

Figure 9: Carry-over experiment: Black traces in all panels correspond to EIC of 152 (acetaminophen); 457(conjugation product) and 308 (GSH), respectively, for injection of mobile phase A. No peaks were detected when mobile phase was injected.

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.

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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.

Application Note
Programming ROXY™
Autosampler

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

User-defined Programs for AS110 (ROXY Autosampler)

- **User-defined programs (UDPs) for optimal reaction control**
- **Pre-programmed, easy to use and modify**
- **EC reaction conditions independent from LC part**
- **Automated screeing of multiple samples**

Introduction

User-defined programs (UDPs) can be generated for an AS110 micro autosampler equipped with a 25µL syringe and 50µL buffer tubing to aspirate samples into an EC cell (Phase 1 programs) and then injected into the LC/MS system. In addition, programs can be written to aspirate both samples and reagents into the cell (Phase 2 programs) to generate products which are then injected into the LC/MS system.

In the phase I programs sample is aspirated via the EC cell directly into the sample loop (figure 2), and subsequently injected in the LC/MS system. In the method with Reactor-Cell™ ON, the sample is oxidized with an applied working potential and the ReactorCell™ is switched OFF direcly after the loop filling step. In the method with ReactorCell™ OFF sample is not oxidized and no working potential is applied. The ReatorCell is switched OFF in the first UDP step.

In phase II programs (Figure 3) the sample (e.g. drug) is transferred to a destination vial containing the reagent (e.g. protein) where the conjugation reaction occurs.

The conjugate together with excess reagent are aspirated into the loop and injected into the LC/MS system. In the method with ReactorCell™ ON, the sample is oxidized with optimal working electrode potential. The ReactorCell is switched OFF after dispensing oxidized sample in the destination vial and before mixing step to avoid substrate oxidation. In the method with Reactor-Cell™ OFF, sample is not oxidized and no working potential is applied. The ReactorCell is switched OFF in the first UDP step.

Figure 1: ROXY™ EC/LC System including ReactorCell™ and AS110 micro autosampler.

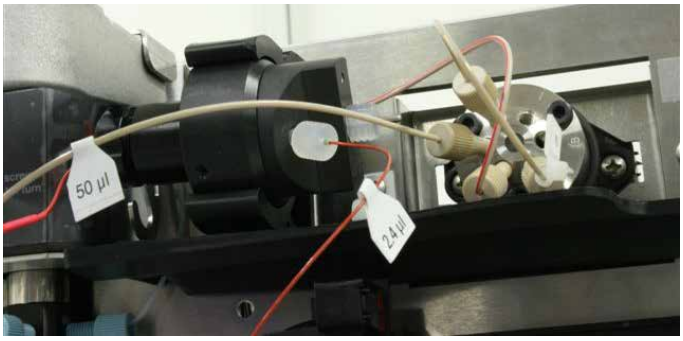


Figure 2: Picture of electrochemical ReactorCell (black) infront of the autosampler injection valve. All integarted in the AS110 austosampler.

The AS110 micro autosampler in the LC/EC system consists of:

- 2.4 µL injection needle
- 25 µL syringe
- 50 µL buffer tubing
- 10 µL sample loop
- 6-port micro bore valve

With this configuration it is possible to use aspiration flow rates as low as 3 µL/min for user-defined programs. It is important to use the lowest possible speed available in the UDPs to maximize the conversion. In the described UDPs the syringe speed is set to 2, which corresponds to 13µL/min, to balance between speed of analysis and conversion ratio. The UDPs can be easily adjusted to the customer needs.

The ROXY EC/LC system can be used for (1) automated formation of metabolites (phase I reaction; Fig. 3) or (2) automated metabolite formation and their conjugation with another compound of interest (phase II reaction; Fig. 4). The oxidation/conjugation products are injected to the LC system, detected and identified via MS equipped with ESI source. The ROXY EC/LC system is standard delivered with predefined Clarity configurations and methods containing the user-defined programs presented in this appendix.

(1) Phase I experiment

For phase I experiments (formation of metabolites) two user-defined programs have neen prepared and are presented, see Table 1 and 2.

The program with Reactor Cell OFF allows to perform control measurement in which the sample is not electrochemically oxidized. In the first step of this program ReactorCell is switched OFF. The program with ReactorCell ON will oxidize sample during the loop filling process. The Reactor Cell is automatically switched OFF after the loop has been filled with oxidation product. This step is important to avoid ReactorCell damage when no flow is passing through it or during a washing step where non-electrolyte solution is flushed through the cell. All metabolites that are created are directly injected onto the LC column and detected by means of mass spectrometry. UDPs can be easily adjusted by the user to change syringe speed, aspirated volume, needle height and wash volume.

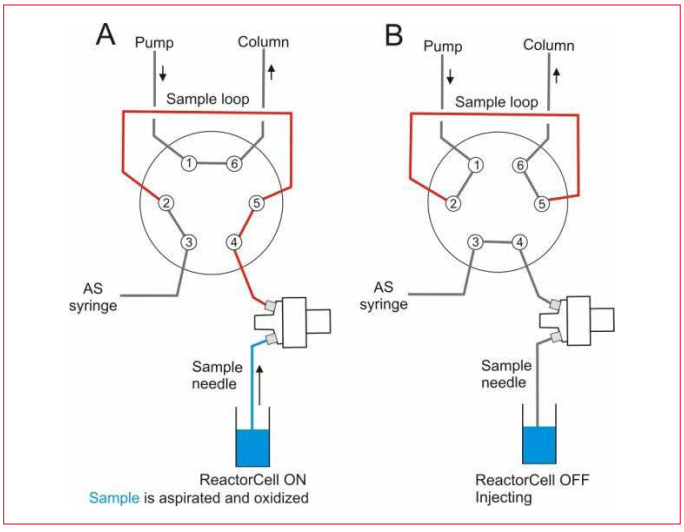


Figure 3: Principle of operation of the ROXY™ EC/LC System for phase I experiment. Blue – non oxidized sample; Red – oxidized sample. With ReactorCell ON, the sample is oxidized and transferred to the loop (A) and injected (B). The ReactorCell is washed after the injection of the sample.

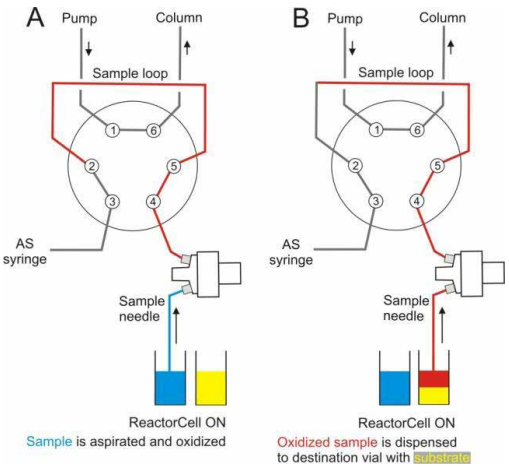


Figure 4: Principle of operation of the ROXY™ EC/LC System for phase II experiment. Blue – non oxidized sample; Red – oxidized sample; Yellow –substrate; Orange – conjugation reaction product.

When ReactorCell is ON sample is undergoing oxidation (A). Oxidized sample is transferred to the destination vial (B). The ReactorCell is OFF. Sample is mixed with the substrate and transferred to the loop. The last step is injecting (D). The ReactorCell is washed after the injection of the sample and stays OFF till next analysis.

(2) Phase II experiment

The equivalent programs (see Tables 3 and 4) were prepared for the phase II experiment. The program with ReactorCell OFF was written for system check (control experiment) and no oxidation can take place in this case, only substrates should be detected in MS and any conjugate formed.

For methods with Reactor Cell (RC) ON sample is aspirated and oxidized in the first step. Then with RC still ON sample is dispensed to the destination vial containing reaction substrate (e.g. peptide, protein etc.). The RC is switched OFF to prevent oxidation of reaction substrate in the next steps. The sample needle is washed and mixing performed by aspirating and dispensing of the oxidized sample and reaction substrate from destination vial. This step provides additional reaction time and the loop is then filled with conjugation reaction product, which is injected on the column. In the final step the washing of sample needle is programmed.

User-defined Programs (UDP)

The compressor (headspace pressure) step is used to assist transport of sample into the loop and a pressure of about 0.5 bar is applied on the head space of the sample vial via the pre-puncturing needle to 'push' the sample into the needle during the aspiration step.

The head space pressure should be OFF after aspiration, before the next step will be executed. The compress option should be used only with airtight vials.

Syr-valve → Waste: Syringe valve is switched to Waste position.
Syringe → Home: Syringe is placed in Home position, and buffer is dispensed to waste.
Syr-valve → Needle: Syringe valve is switched to Needle position.

In steps of all UDPs that include above commands (Syr-valve → Waste; Syringe → Home; Syr-valve → Needle) the syringe is prepared for repeated aspiration. The syringe valve should be switched to waste followed by placing the syringe in Home position. This will lead to dispense liquid from the syringe to waste. In step Syr-valve → Needle the syringe valve is switched to needle position and the syringe is ready to aspirate sample.

Without these steps, liquid from the syringe would be dispensed to sample/destination vial in case of repeated aspiration, and contaminate the vial content.

Table 1

AS110 UDP for phase I metabolism with ReactorCell OFF

Step	Action type	from / to position / speed	height (mm) amount(µL) / time (min)
Turning ReactorCell OFF			
001	Auxiliaries	Aux1	On
002	Wait	0.10	
003	Auxiliaries	Aux1	Off
Switching Injector valve to LOAD			
004	Valve	Injector	Load / 6-1
1st sample aspiration (no oxidation; ReactorCell OFF; loop filling)			
005	Compressor	On	
006	Syr.Speed/Height	2	4.0
007	Aspirate	Sample	0.00

User-defined Programs for AS110 (ROXY Autosampler)

Table 1 cont.

008	Wait	0.50	
009	Syr.Speed/Height	2	4.0
010	Aspirate	Sample	15.00
011	Compressor	Off	
012	Wait	0.50	
013	Syr.Speed/Height	2	4.0
014	Aspirate	Sample	0.00
015	Syringe valve	Waste	
016	Syringe	Home	
017	Syringe valve	Needle	
2nd sample aspiration (no oxidation; ReactorCell OFF; loop filling)			
018	Compressor	On	
019	Syr.Speed/Height	2	4.0
020	Aspirate	Sample	0.00
021	Wait	0.50	
022	Syr.Speed/Height	2	4.0
023	Aspirate	Sample	20.00
024	Compressor	Off	
025	Wait	0.50	
026	Syr.Speed/Height	2	4.0
027	Aspirate	Sample	0.00
Injection			
028	Valve	Injector	Inject / 1-2
Starting analysis (Clarity)			
029	Markers	Digital Inject	
Wash			
030	Wash		200
031	Wash		200

Table 2

AS110 UDP for phase I metabolism with ReactorCell ON

Step	Action type	from / to position / speed	height (mm) amount(µL) / time (min)
Switching Injector valve to LOAD			
001	Valve	Injector	Load / 6-1
1st sample aspiration (oxidation; ReactorCell ON; loop filling)			
002	Compressor	On	
003	Syr.Speed/Height	2	4.0
004	Aspirate	Sample	0.00
005	Wait	0.50	
006	Syr.Speed/Height	2	4.0
007	Aspirate	Sample	15.00
008	Compressor	Off	
009	Wait	0.50	
010	Syr.Speed/Height	2	4.0
011	Aspirate	Sample	0.00
012	Syringe valve	Waste	
013	Syringe	Home	
014	Syringe valve	Needle	
2nd sample aspiration (oxidation; ReactorCell OFF; loop filling)			
015	Compressor	On	
016	Syr.Speed/Height	2	4.0
017	Aspirate	Sample	0.00
018	Wait	0.50	
019	Syr.Speed/Height	2	4.0

Table 2 cont.

020	Aspirate	Sample	20.00
021	Compressor	Off	
022	Wait	0.50	
023	Syr.Speed/Height	2	4.0
024	Aspirate	Sample	0.00
Turning ReactorCell OFF			
025	Auxiliaries	Aux1	On
026	Wait	0.10	
027	Auxiliaries	Aux1	Off
Injection			
028	Valve	Injector	Inject / 1-2
Starting analysis (Clarity)			
029	Markers	Digital Inject	
Wash			
030	Wash		200
031	Wash		200

Table 3

AS110 UDP for phase II metabolism with Reactor Cell OFF

Step	Action type	from / to position / speed	height (mm) amount(µL) / time (min)
Turning ReactorCell OFF			
001	Auxiliaries	Aux1	On
002	Wait	0.1	
003	Auxiliaries	Aux1	Off
Switching Injector valve to LOAD			
004	Valve	Injector	Load / 6-1
1st Sample Aspiration (no oxidation; ReactorCell OFF)			
005	Compressor	On	
006	Syr.Speed/Height	2	2.0
007	Aspirate	Sample	0.00
008	Wait	0.30	
009	Syr.Speed/Height	2	2.0
010	Aspirate	Sample	10.00
011	Compressor	Off	
012	Wait	0.50	
013	Syr.Speed/Height	2	2.0
014	Aspirate	Sample	0.00
015	Syringe valve	Waste	
016	Syringe	Home	
017	Wait	0.30	
018	Syringe	valve Needle	Syringe
2nd Sample Aspiration (no oxidation; ReactorCell OFF)			
019	Compressor	On	
020	Syr.Speed/Height	2	2.0
021	Aspirate	Sample	0.00
022	Wait	0.30	
023	Syr.Speed/Height	2	2.0
024	Aspirate	Sample	25.00
025	Compressor	Off	
026	Wait	0.50	
027	Syr.Speed/Height	2	2.0
028	Aspirate	Sample	0.00

Table 3 cont.

Dispensing Not Oxidized Sample to Waste			
029	Syr.Speed/Height	1 (Slowest)	2.0
030	Dispense	Waste	3.00
031	Wait	0.30	
032	Syringe valve	Waste	
033	Syringe	Load	3.00
034	Wait	0.30	
035	Syringe valve	Needle	
Dispensing Not Oxidized Sample into Destination vial			
036	Syr.Speed/Height	1 (Slowest)	5.0
037	Dispense	Destination	25.00
038	Wait	2.00	
039	Syr.Speed/Height	1 (Slowest)	5.0
040	Dispense	Destination	0.00
Needle wash			
041	Needle wash		150.00
042	Wait	0.5	
Mixing			
043	Compressor	On	
044	Syr.Speed/Height	2	5.0
045	Aspirate	Destination	0.00
046	Wait	0.30	
047	Syr.Speed/Height	2	5.0
048	Aspirate	Destination	10.00
049	Compressor	Off	
050	Wait	0.50	
051	Syr.Speed/Height	2	5.0
052	Aspirate	Destination	0.00
053	Syr.Speed/Height	1 (Slowest)	5.0
054	Dispense	Destination	10.00
055	Wait	2.00	
1st Aspirating from Destination Vial (loop filling)			
056	Compressor	On	
057	Syr.Speed/Height	2	2.0
058	Aspirate	Destination	0.00
059	Wait	0.30	
060	Syr.Speed/Height	2	2.0
061	Aspirate	Destination	20.00
062	Compressor	Off	
063	Wait	0.50	
064	Syr.Speed/Height	2	2.0
065	Aspirate	Destination	0.00
066	Syringe valve	Waste	
067	Syringe	Home	
068	Wait	0.30	
069	Syringe valve	Needle	
2nd Aspirating from Destination Vial (loop filling)			
070	Compressor	On	
071	Syr.Speed/Height	2	2.0
072	Aspirate	Destination	0.00
073	Wait	0.30	
074	Syr.Speed/Height	2	2.0
075	Aspirate	Destination	20.00
076	Compressor	Off	
077	Wait	0.50	
078	Syr.Speed/Height	2	2.0
079	Aspirate	Destination	0.00
Injection			
080	Valve	Injector	Inject
Starting Analysis (Clarity)			
081	Markers	Digital Inject	
Wash			
082	Wash		200.00
083	Wash		200.00

Table 4

AS110 UDP for phase II metabolism with Reactor Cell ON

Step	Action type	from / to position / speed	height (mm) amount(µL) / time (min)
Switching Injector valve to LOAD			
001	Valve	Injector	Load / 6-1
1st sample aspiration (oxidation; ReactorCell ON)			
002	Compressor	On	
003	Syr.Speed/Height	2	2.0
004	Aspirate	Sample	0.00
005	Wait	0.30	
006	Syr.Speed/Height	2	2.0
007	Aspirate	Sample	10.00
008	Compressor	Off	
009	Wait	0.50	
010	Syr.Speed/Height	2	2.0
011	Aspirate	Sample	0.00
012	Syringe valve	Waste	
013	Syringe	Home	
014	Wait	0.30	
015	Syringe valve	Needle	
2nd sample aspiration (oxidation; ReactorCell ON)			
016	Compressor	On	
017	Syr.Speed/Height	2	2.0
018	Aspirate	Sample	0.00
019	Wait	0.30	
020	Syr.Speed/Height	2	2.0
021	Aspirate	Sample	25.00
022	Compressor	Off	
023	Wait	0.50	
024	Syr.Speed/Height	2	2.0
025	Aspirate	Sample	0.00
Dispensing not oxidized Sample to Waste			
026	Syr.Speed/Height	1 (Slowest)	2.0
027	Dispense	Waste	3.00

Table 4 cont.

028	Wait	0.30	
029	Syringe valve	Waste	
030	Syringe	Load	3.00
031	Wait	0.30	
032	Syringe valve	Needle	
Dispensing Oxidized Sample into Destination Vial			
033	Syr.Speed/Height	1 (Slowest)	5.0
034	Dispense	Destination	25.00
035	Wait	2.00	
036	Syr.Speed/Height	1 (Slowest)	5.0
037	Dispense	Destination	0.00
Turning ReactorCell OFF			
038	Auxiliaries	Aux1	On
039	Needle wash	150.00	
040	Auxiliaries	Aux1	Off
041	Compressor	On	
042	Syr.Speed/Height	2	5.0
043	Aspirate	Destination	0.00
044	Wait	0.30	
045	Syr.Speed/Height	2	5.0
046	Aspirate	Destination	10.00
047	Compressor	Off	
048	Wait	0.50	
049	Syr.Speed/Height	2	5.0
050	Aspirate	Destination	0.00
051	Syr.Speed/Height	1 (Slowest)	5.0
052	Dispense	Destination	10.00
053	Wait	2.00	
1st Aspirating from Destination Vial (loop filling)			
054	Compressor	On	
055	Syr.Speed/Height	2	2.0
056	Aspirate	Destination	0.00
057	Wait	0.30	
058	Syr.Speed/Height	2	2.0
059	Aspirate	Destination	20.00
060	Compressor	Off	
061	Wait	0.50	
062	Syr.Speed/Height	2	2.0
063	Aspirate	Destination	0.00
064	Syringe valve	Waste	
065	Syringe	Home	
066	Wait	0.30	
067	Syringe valve	Needle	
2nd Aspirating from Destination Vial (loop filling)			
068	Compressor	On	
069	Syr.Speed/Height	2	2.0
070	Aspirate	Destination	0.00
071	Wait	0.30	
072	Syr.Speed/Height	2	2.0
073	Aspirate	Destination	20.00
074	Compressor	Off	
075	Wait	0.50	
076	Syr.Speed/Height	2	2.0
077	Aspirate	Destination	0.00
Injection			
078	Valve	Injector	Inject
Starting Analysis (Clarity)			
079	Markers	Digital Inject	
Wash			
080	Wash		200.00
081	Wash		200.00

Clarity configuration

The ROXY EC/LC system includes an Antec Clarity installer, which contains specific predefined configuration and method files for easy installation and system start-up.

The installer contains the hardware configuration (Fig. 5), user-defined programs for phase I and II metabolism studies and examples of sample queue for phase I, phase II and optimization for the experiment (mass voltammogram acquisition). The user-defined programs can be easy modified by the end user to meet the precise needs for the analysis.

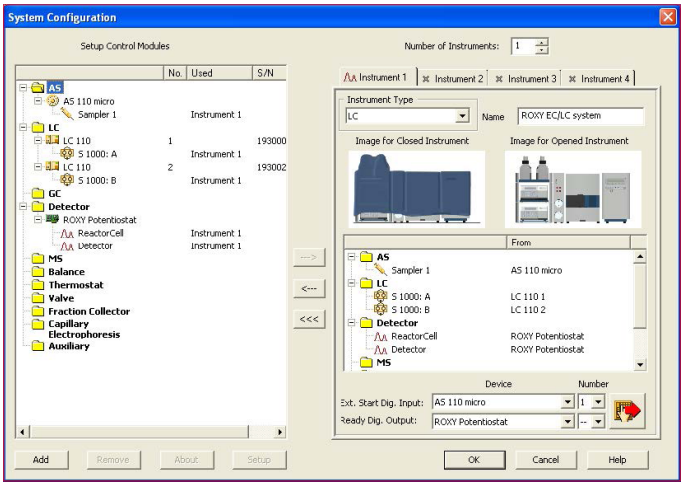


Figure 5: Configuration of the ROXY EC/LC in the Clarity System Configuration. AS110 micro is chosen as external Start digital input device.

Methods prepared in the installer allow automatic triggering of the mass spectrometer via contact closure (Fig. 7). The signal is provided by the ReactorCell. The MS acquisition time is determined by mass spectrometer software and it is important to set correct analysis time for MS measurement (should be set in MS controlling software) and for Clarity controlled analysis (time of LC run is set in Method Measurement window).

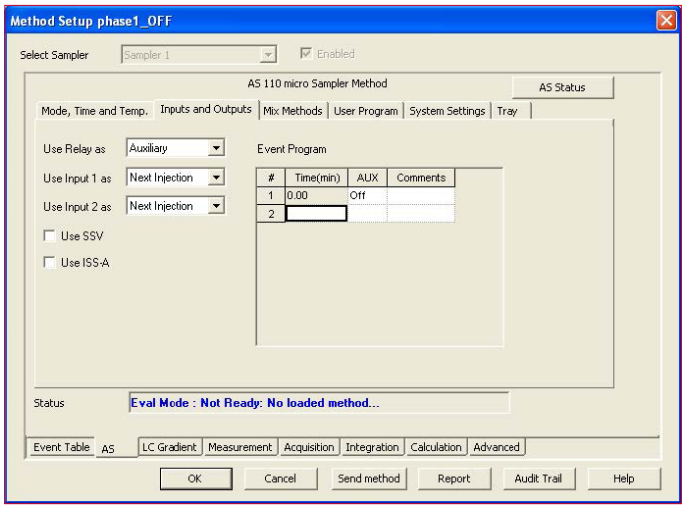


Figure 6: Configuration of the Inputs in the ROXY EC/LC system with AS110 micro autosampler. Use Relay as Auxiliary is set for this configuration.

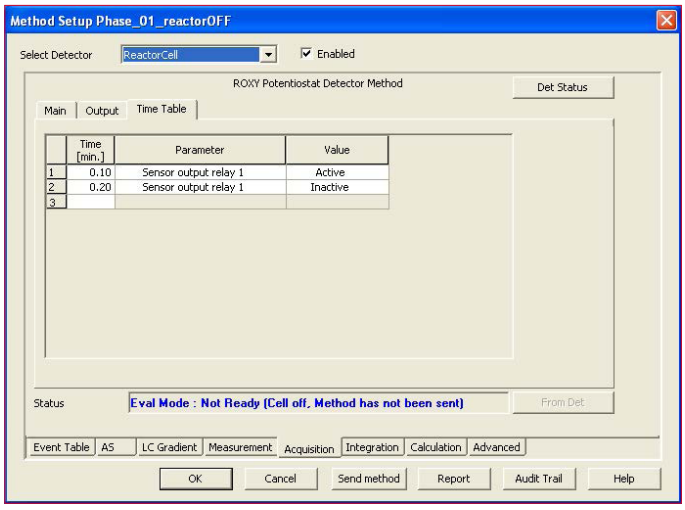


Figure 7: MS trigger programming.

The installer contains examples of the sequences:

(1) Phase I.seq

This sequence contains an example of sample queue with gradient and different working potential applied to the electrode. The potential is in range of 200-1200mV and is suitable for Glassy Carbon electrode. If a Magic Diamond electrode is used, it is recommended that the voltages is set to higher value. The example contains the method when ReactorCell is OFF for control measurement. The total run time for LC is 17 min.

(2) Phase II.seq

In this sample queue the methods for automated conjugation reaction are applied. The AS100 UDP method with ReactorCell ON includes compound oxidation, mixing with substrate (e.g. GSH) in the destination vial, loop filling with conjugate and injection. The voltage applied to ReactorCell is 800mV as Glassy Carbon electrode is used and can be adjusted by end user (e.g., with use of the MD electrode the voltage value should be higher). The run time for LC is 17 min.

The gradient composition and run time depend on type of column, mobile phase composition and type of the analyzed compound and should be adjusted by end user.

(3) Voltammogram.seq

The sequence allows to execute the set of quick measurements with ramped working electrode potential from 200 – 2000 mV with incremental steps of 200 mV. Additionally, the control measurement with ReactorCell switched off is included. Based on direct infusion measurement (no LC separation)

Conclusion

The ROXY EC/LC System with the AS110 autosampler allows for fully automated screening of numerous samples on REDOX reactions. Various user-defined programs (UDP) are available to perform phase I reactions, such as oxidation or reduction, followed by phase II reactions such as adduct formation, conjugation, detoxification reactions, etc.

The UDP are pre-configured, easy in use and can be easily modified to perform more demanding reactions unattended.

the optimal potential to convert drug or any compound of interest can be estimated. To perform this experiment LC column should be bypassed, e.g. with union. In presented methods the isocratic flow of 50% mobile phase A and B is applied and can be adjusted by the user. The flow rate is 100µL/min. The sample is transported by AS110 micro to the loop passing the ReactorCell and the hole procedure is executed automatically. After each injection the flow path is washed.

Run time is set to 3 minutes and data will be collected in separate files, to make easier to create 3-D mass voltammogram (Fig. 8) and keep track of the voltage changes.

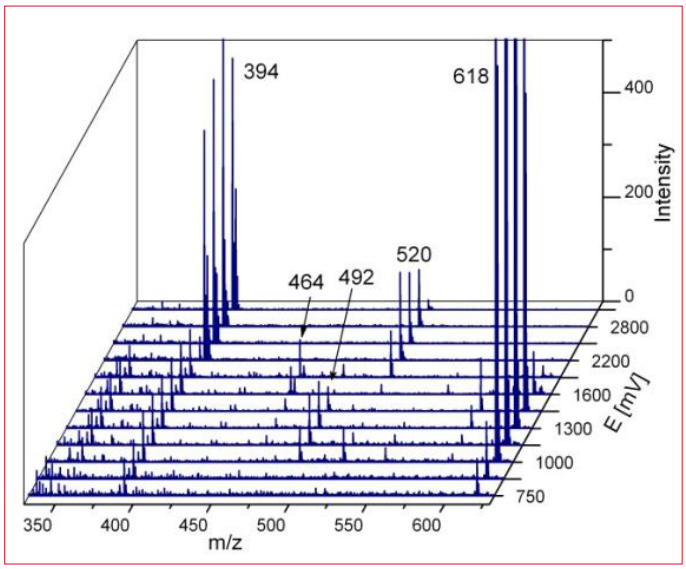


Figure 8: Example of 3-D mass voltammogram. Amiodarone.

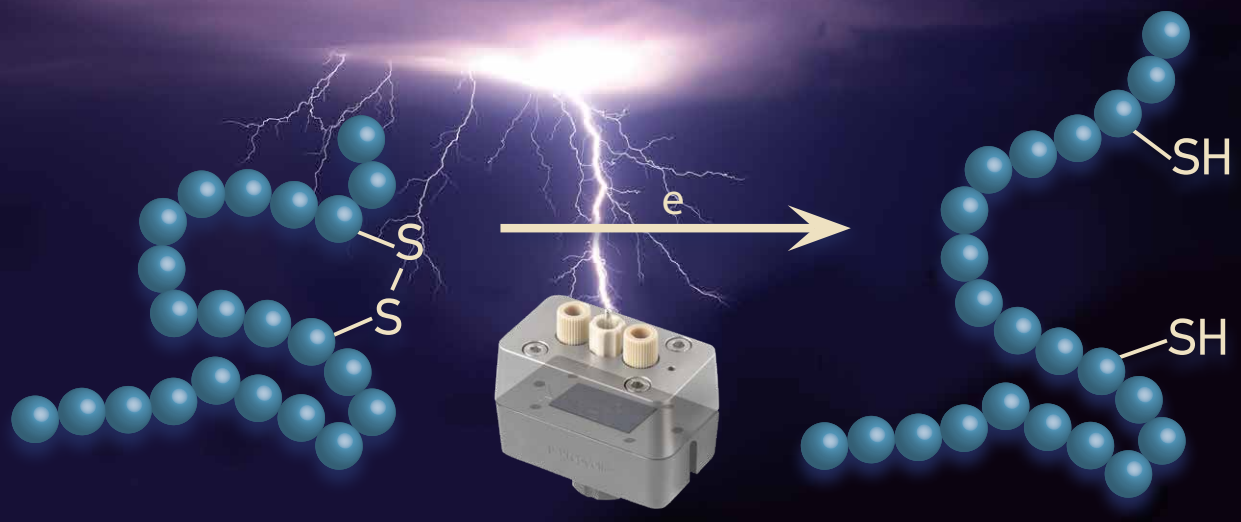
(4) Voltammogram_syringe_pump.seq

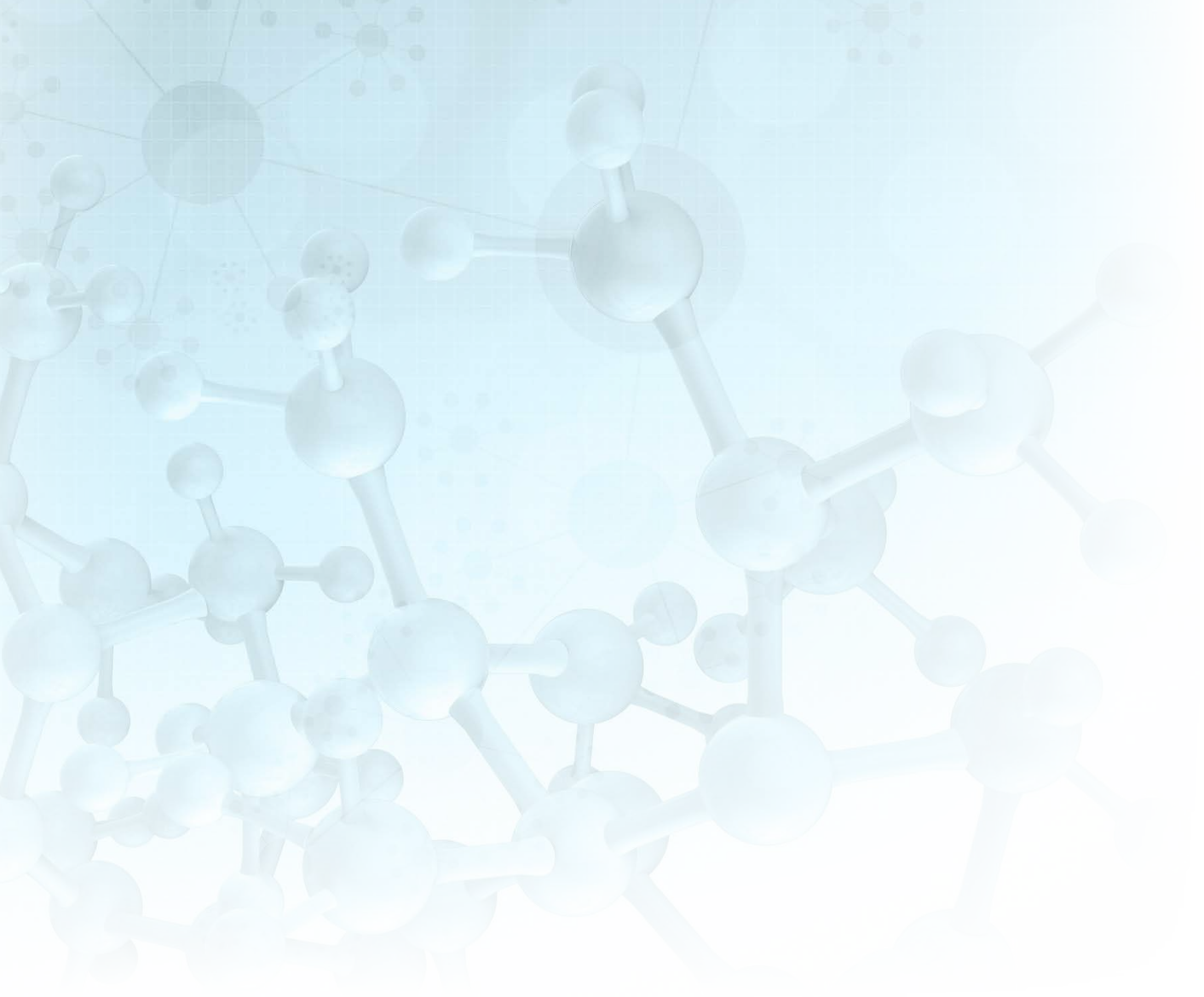
In this file method, off-line mass voltammogram data acquisition is prepared. The measurement starts with ReactorCell OFF followed by the change in the working electrode potential from 200 – 2000 mV via with incremental steps of 200 mV (the potentiostat is controlled by Clarity software). The sample is delivered with the syringe pump at flow rate of 10µL/min. The ReactorCell is connected with MS source with 1m red striped PEEK tubing. The analysis is started by the AS110 autosampler, and while an injection is done, the flow rate from the LC pumps is 0 µL/min. The total run time is 7 min, including delay related to dead volume (PEEK tubing, RC itself). For each voltage 30 s measurements is conducted and only one MS file will be collected.

It is important to set the MS in remote mode (to allow for an external trigger) and to program the appropriate time for data acquisition e.g. MS measurement time should not be longer then run time of the gradient or method in the ROXY EC/LC system.

Electrochemical reduction of disulfide bonds in proteins a truly complementary approach to chemical or enzymatic reduction.

Jean-Pierre Chervet





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