



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 screening 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 directly after the loop filling step. In the method with ReactorCell™ OFF sample is not oxidized and no working potential is applied. The ReactorCell 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.

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)

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.

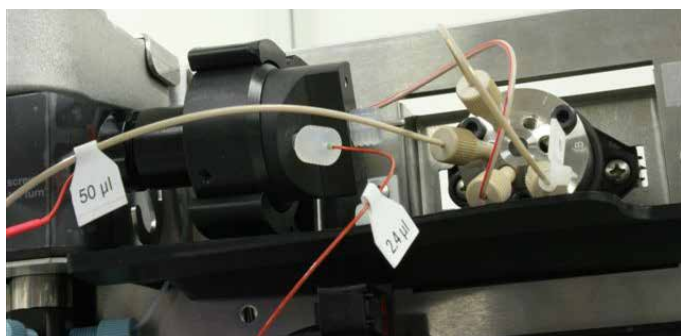


Figure 2: Picture of electrochemical ReactorCell (black) in front of the auto-sampler injection valve. All integrated in the AS110 auto-sampler.

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 13 µL/min, to balance between speed of analysis and conversion ratio. The UDPs can be easily adjusted to the customer needs.

Principle of operation

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 been 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.

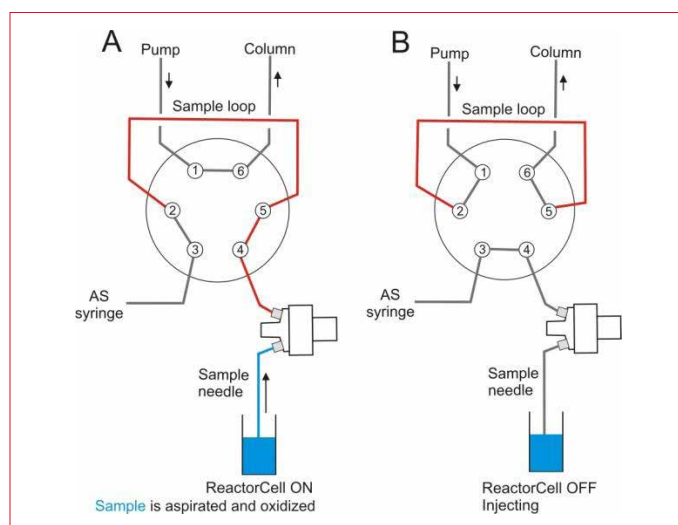


Figure 3: Principle of operation of the ROXY™ EC/LC System for phase I experiment. Blue – non oxidized sample; Red – oxidized sample. With Reactor-Cell ON, the sample is oxidized and transferred to the loop (A) and injected (B). The ReactorCell is washed after the injection of the sample.

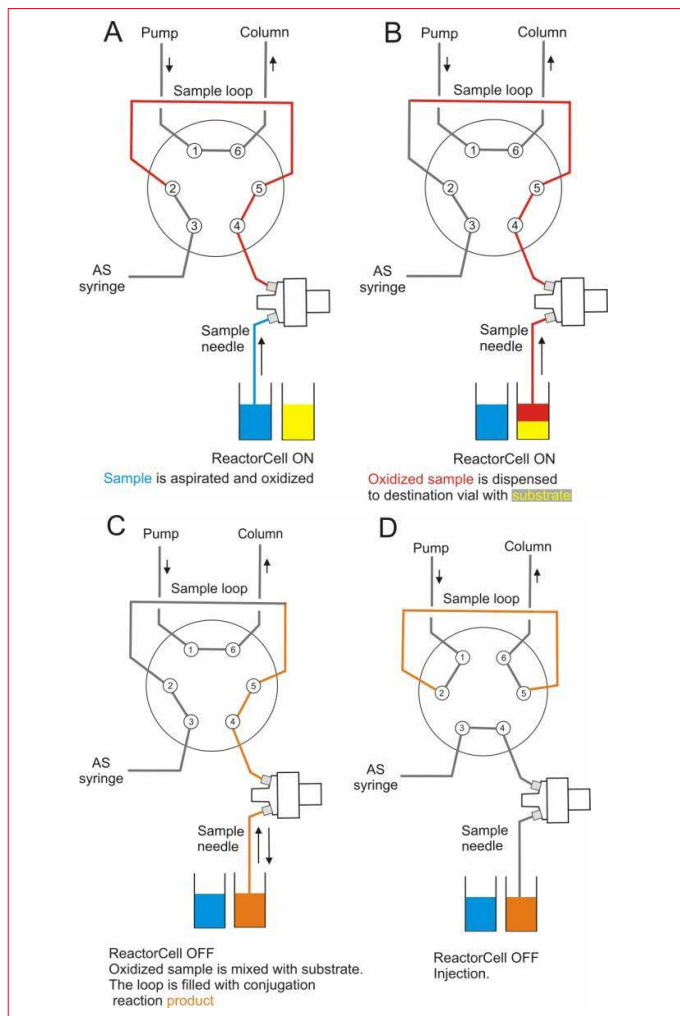


Figure 4: Principle of operation of the ROXY™ EC/LC System for phase II experiment. Blue – non oxidized sample; Red – oxidized sample; Yellow – substrate; Orange – conjugation reaction product.

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
1 st sample aspiration (no oxidation; ReactorCell OFF; loop filling)			
005	Compressor	On	
006	Syr.Speed/Height	2	4.0
007	Aspirate	Sample	0.00
008	Wait	0.50	

User-defined Programs for AS110 (ROXY Autosampler)

Table 1 cont.

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
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Starting analysis (Clarity)

029	Markers	Digital Inject	
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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)
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Switching Injector valve to LOAD

001	Valve	Injector	Load / 6-1
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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
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Starting analysis (Clarity)

029	Markers	Digital Inject	
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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)
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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
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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	



Table 3 cont.

026	Wait	0.50	
027	Syr.Speed/Height	2	2.0
028	Aspirate	Sample	0.00

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
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Starting Analysis (Clarity)

081	Markers	Digital Inject	
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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)
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Switching Injector valve to LOAD

001	Valve	Injector	Load / 6-1
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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

Table 4 cont.

025	Aspirate	Sample	0.00
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Dispensing not oxidized Sample to Waste

026	Syr.Speed/Height	1 (Slowest)	2.0
027	Dispense	Waste	3.00
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
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Starting Analysis (Clarity)

079	Markers	Digital Inject	
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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 easily modified by the end user to meet the precise needs for the analysis.

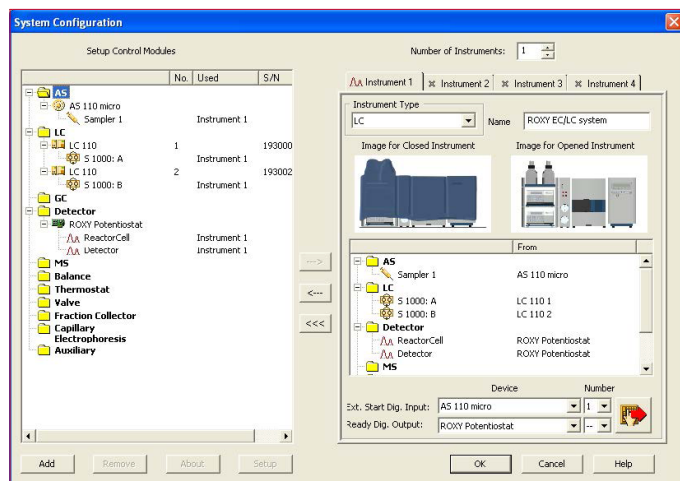


Figure 5: Configuration of the ROXY EC/LC in the Clarity System Configuration. AS110 micro is chosen as external Start digital input device.

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 impor-

tant 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).

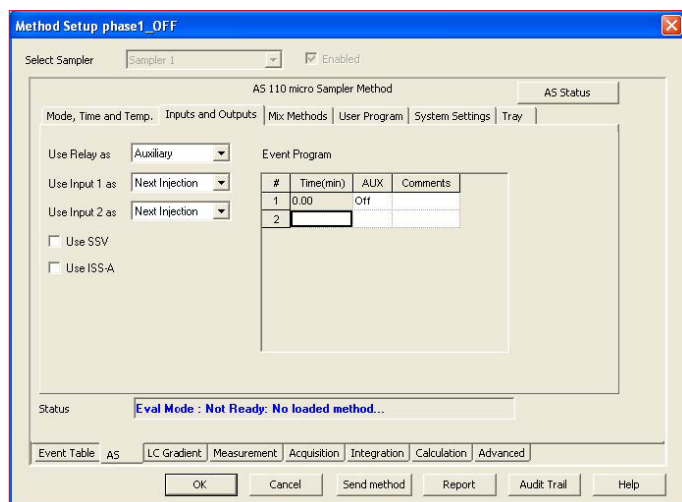


Figure 6: Configuration of the Inputs in the ROXY EC/LC system with AS110 micro autosampler. Use Relay as Auxiliary is set for this configuration.



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 100 μ L/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.

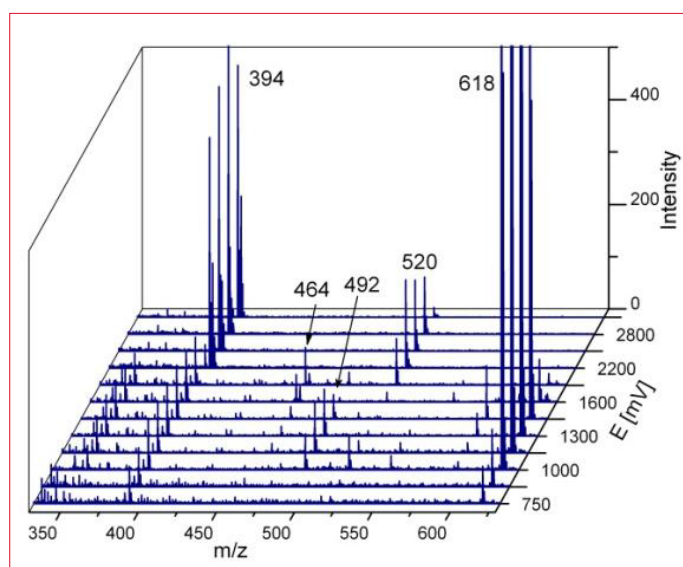


Figure 8: Example of 3-D mass voltammogram. Amiodarone.

(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 10 μ L/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 μ L/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 than run time of the gradient or method in the ROXY EC/LC system.

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

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