Automated Two-Dimensional Separation of Peptides by Ion-Pair Reversed-Phase High-Performance Liquid Chromatography-Electrospray Ionization-Mass Spectrometry at High and Low pH

**INTRODUCTION**

Protein samples of biological origin can be very complex, and require powerful separation tools like multi-dimensional liquid chromatography (MDLC) to resolve components of interest. MDLC can be performed using on-line and off-line approaches. In the off-line MDLC approach, due to a fractionation step, a high flexibility in terms of column dimensions, optimization of flow rate, buffer composition, and pH can be realized for each separation dimension independently. The optimization of both separation dimensions can increase peak capacity, although to fully exploit the peak capacity, the separation mechanism used in the first-dimension should be orthogonal to that of the second-dimension separation. The combination of strong-cation-exchange and reversed-phase chromatography (SCX/$\times$RP) is known for its high degree of orthogonality, but can have difficulties resolving higher valence tryptic peptides. However, reversed-phase HPLC using low and high pH (RP/$\times$/RP) offers an alternative approach.

In this technical note, the off-line RP/$\times$/RP 2-D LC approach is examined in terms of selectivity, separation efficiency, orthogonality, repeatability, and assessment as to how complementary it is to SCX/$\times$/RP.

**EQUIPMENT**

Automated off-line two-dimensional chromatographic experiments were performed using an UltiMate® 3000 Proteomics MDLC system (Dionex Corporation) consisting of a dual ternary gradient pump (DGP-3600) with membrane degasser (SRD-3600), flow manager module (FLM-3100) equipped with a 1:100 flow splitter, autosampler with integrated micro-fraction collector (WPS-3000), and two UV detectors (VWD-3400). The second-dimension separation was performed using the reduced-solvent-consumption option. With this option, solvent consumption is reduced by a factor of 10, allowing continuous operation for up to one month.

**Fluidic Connections**

The first-dimension SCX and RP columns, the Acclaim® PA2 trap column, the second-dimension Acclaim PA2 nano column, and the connection tubing to the FLM switching valves are connected as described in Table 1.
First-Dimension Separation: SCX Performed at pH = 3

Column: PolySULFOETHYL Aspartamide, 300 µm i.d. × 15 cm, 5 µm, 300 Å (P/N 164263)

Mobile Phase A: 95/5 water/CH₃CN, 5 mM NaH₂PO₄ buffer pH = 3

Mobile Phase B: Mobile Phase A + 1 M NaCl

Gradient: 0–60% B in 15 min, 100% B for 5 min, 25 min equilibration

Flow Rate: 6 µL/min

UV Detection: 214 nm, 45 nL flow cell

Samples: Six-protein digest mixture (PMD, P/N 161088) containing the following digested proteins:

- Cytochrome-C
- Lysozyme
- Alcohol dehydrogenase
- Bovine serum albumin
- Apo-transferrin
- Beta-galactosidase

Tryptic digest of bovine serum albumin 1 pmol/µL

Tryptic digest of an *Escherichia coli* (*E. coli*) protein extract (BioRad) concentration = 1 µg/µL

Loop: 20 µL

Inj. Volume: 10 µL PMD solution

2 µL *E. coli* digest solution

Fractionation: 10 fractions of 1 min in a 384-well micro plate, V-Bottom (P/N 6820.4114)

Oven Temp.: 30 ºC

Sampler Temp.: 5 ºC
First-Dimension Separation: RP at pH = 9.6

Column: Acclaim PA2, 300 µm i.d. × 15 cm (P/N 164149)

Mobile Phase A: 100% water,
1% (72 mM) triethylamine (TEA) titrated to pH = 9.6 with acetic acid.

Mobile Phase B: 20/80 water/CH₃CN,
1% (72 mM) triethylamine (TEA), titrated to pH = 9.6 with acetic acid.

Gradient: 4–60% B in 15 min, 90% B for 5 min, 25 min equilibration

Flow Rate: 6 µL/min

UV Detection: 214 nm, 45 nL flow cell

Sample: Six-protein digest mixture (PMD) containing the following digested proteins:
- Cytochrome-C
- Lysozyme
- Alcohol dehydrogenase
- Bovine serum albumin
- Apo-transferrin
- Beta-galactosidase

Tryptic digest of an Escherichia coli (E. coli) protein extract (BioRad) concentration = 1 µg/µL.

Inj. Volume: 10 µL PMD solution

Fractionation: 10 fractions of 1 min in a 384-well micro plate, V-Bottom (P/N 6820.4114)

Oven Temp.: 30 ºC

Sample Treatment after Fractionation, prior to Injection onto the Second-Dimension

Fractions from the first-dimension separation were collected in a 384-well micro plate, V-Bottom (P/N 6820.4114). Fractions were diluted with 20 µL of 0.1% TFA in water by the autosampler prior to injection onto the second-dimension to increase sample volume and to lower the pH from pH = 9.6 to pH = 2. The 20 µL sample was then injected onto the second-dimension column. Drying the sample completely between the two dimensions increases hydrophobic absorption to the well plate and is therefore not advisable.

Second-Dimension Separation: RP at pH = 2

Column: Acclaim PA2, 75 µm i.d. × 15 cm (P/N 164504)
Acclaim PA2, 300 µm i.d. × 5 mm, 5 µm (P/N 164503)

Mobile Phase A: 100% water, 0.05% TFA

Mobile Phase B: 20/80 water/CH₃CN, 0.04% TFA

Loading Solvent: 99/1 water/CH₃CN, 0.1% TFA

Flow Rate: 300 nL/min (Micro Pump), CAP splitter
20 µL/min (Loading Pump)

Loading Time: 5 min

Gradient: 4–60% B in 30 min, 9% B for 5 min, 25 min equilibration

UV Detection: 214 nm, 3 nL flow cell

Sample: First-dimension fractions

Inj. Loop: 20 µL

Inj. Volume: Whole first-dimension fraction

Oven Temp.: 30 ºC

MS: HCTultra™ ion-trap mass spectrometer (Bruker Daltonics). ESI-MS/MS, positive ion mode, mass range 300–1600 m/z, dry-gas stream 4 L/min, nebulizer gas 138 kPa, drying temperature of 300 ºC, and a target mass set to m/z = 800. Peptides were considered positively identified when the correlation between measured and simulated MS/MS spectrum yielded Mowse scores of ≥15

Valve Switching

All experiments were performed with the set-up shown in Figure 1. The loading pump and the micro pump are both connected to the right valve and use the same splitter. The right valve controls which dimension the sample is injected into for separation. The valve positions for various stages in the experiment are described below and listed in Table 2. As an example, the first-dimension and second-dimension program files, including a User Defined Program (UDP), are presented in Appendix I.
• Valve Left position 1_2, Valve Right position 1_2; the loading pump is in-line with the splitter and the peptides are separated on the first-dimension separation column. Injection of sample is performed by a UDP and transported onto the column by the loading pump which also delivers the gradient for the first-dimension separation through channels A and B. The effluent from the column is directed to the injection needle for fractionation.

• Valve Left position 10_1, Valve Right position 10_1; The first-dimension fractions are injected by a UDP injection program and transported onto the RP trap column using loading solvent, which consists of water + 0.1% TFA, delivered by channel C of the loading pump. The fractions are desalted and/or pre-concentrated for 5 min.

• Valve Left position 1_2, Valve Right position 10_1; The peptides are eluted onto the second-dimension separation column by the micro pump, which also delivers the gradient for elution of the peptides through channels A and B.

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**Workflow for Automated Off-Line 2-D LC**

The workflow and LC conditions for automated off-line 2-D LC included the following:

- 2–10 µL injection of a six-protein digest (PMD) or *E. coli* digest (1 µg/µL)
- A first-dimension separation with automated fraction collection, either a:
  - Strong-cation-exchange separation at pH = 3, applying a linear NaCl salt gradient, or
  - Reversed-phase separation at pH = 9.6, applying a linear water/CH₃CN gradient.
- Repeated cycles of injections, desalting/pre-concentration, and second-dimension reversed-phase separations (at pH 2) in the reduced-solvent-consumption mode, with UV detection at 214 nm and MS/MS detection. Proteins were considered positively identified when the correlation between measured and simulated MS/MS spectrum yielded Mowse scores of ≥15 for at least two peptides.

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**RESULTS**

In order to achieve the maximum peak capacity in 2-D LC, a high degree of orthogonality must be achieved. In a truly orthogonal 2-D LC system, separation is based on two different retention mechanisms. In the off-line 2-D LC approach, where the first-dimension separation is fractionated, both the separation efficiency and the distribution of the peptides over the collected fractions plays an important role. SCX/×/RP chromatography is known for its high degree of orthogonality. However, one of the known drawbacks is an uneven distribution of peptides over the gradient. Tryptic peptides are mostly 2+ and 3+ charged, and since retention is driven by charge, these peptides will elute in a small retention window, whereas the other peptides will elute in the remaining parts of the chromatogram. To evenly distribute the peptides over the fractions, either the gradient or the fraction-collection time can be optimized.

In ion-pair chromatography (RP/×/RP), applying a high pH in the first-dimension separation, the retention mechanism is mostly driven by hydrophobic interactions, which results in a more even distribution of peptides over the gradient.

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**Table 2. Valve Positions During Automated Off-Line 2-D LC**

<table>
<thead>
<tr>
<th></th>
<th>Injector Valve</th>
<th>Left Valve</th>
<th>Right Valve</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First-Dimension Separation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Loop fill and effluent to waste</td>
<td>1-2</td>
<td>1-2</td>
<td>1-2</td>
</tr>
<tr>
<td>First-dimension separation and fractionation</td>
<td>8-1</td>
<td>1-2</td>
<td>1-2</td>
</tr>
<tr>
<td>Wash first dimension</td>
<td>8-1</td>
<td>1-2</td>
<td>1-2</td>
</tr>
<tr>
<td><strong>Second-Dimension Separation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Loop fill</td>
<td>1-2</td>
<td>10-1</td>
<td>10-1</td>
</tr>
<tr>
<td>Inject fractions and wash free of salt</td>
<td>8-1</td>
<td>10-1</td>
<td>10-1</td>
</tr>
<tr>
<td>Elute and separate fractions</td>
<td>8-1</td>
<td>1-2</td>
<td>10-1</td>
</tr>
<tr>
<td>Equilibrate RP trap</td>
<td>8-1</td>
<td>10-1</td>
<td>10-1</td>
</tr>
</tbody>
</table>

The SCX/×/RP and the RP/×/RP experiments were performed according the schematic shown in Figure 1. For switching from the SCX/×/RP configuration to the RP/×/RP configuration, the first-dimension separation column was exchanged and the ion-exchange buffers pH = 3 (loading pump A and B) were replaced with reversed-phase buffers pH = 9.6. The flow manager was equipped with a 1:100 CAP flow splitter resulting in a pump flow of 30 µL/min for the second-dimension separation.
Peptide Selectivity in RP-/×RP 2-D LC

The retention mechanism of the RP column at low pH is mainly driven by hydrophobic interactions and secondarily by electrostatic interactions resulting from the addition of amphiphilic ions to the mobile phase.

To show the difference in peptide selectivity in ion-pair chromatography, a tryptic digest from BSA was separated on a reversed-phase column applying a pH of 9.6 and a pH of 2. The peptides were detected by MS in the positive ionization mode. Figure 2 shows the separation of a BSA tryptic digest at pH = 2 and pH = 9.6 (A and B). Extracted-ion chromatograms were used to determine the elution order and retention time of four individual peptides (C–F).

As shown in Figure 2, the peptides were separated under basic as well as under acidic conditions. The difference in selectivity was evaluated by the elution order of four individual peptides. The two peptides LVTDLTK and AEFVEVTK showed a reversed elution order under basic conditions compared with acidic conditions. The two peptides RHPEYAVSVLLR and TVMENFVAFVDK were separated under acidic conditions and co-eluted under basic conditions.

Figure 2. Tryptic digest of BSA separated at A) pH = 2, and B) pH = 9.6; C–F) extracted-ion chromatograms of four selected peptides. The observed decrease in detectability of peptides at high pH is mainly due to the higher ionic strength of the alkaline eluent.
These results indicate electrostatic interactions play a minor role in high-pH separations. The higher charged, more hydrophilic peptides like AEFVEVTK eluted earlier under basic conditions compared to acidic conditions. Based on these observations, the application of buffers with different pH-values provides enough selectivity changes in the elution of tryptic peptides to serve as a basis for an orthogonal 2-D LC method.

To investigate the applicability of the RP/×/RP approach in an automated off-line 2-D set-up, a six-protein digest mixture (PMD) was analysed and compared with the SCX/×/RP approach. For both techniques a fraction collection time of 1 min was applied.

The six-protein digest mixture (PMD) was separated successfully using RP/×/RP as well as the SCX/×/RP approach. As shown in the 2-D-retention map, a higher degree of orthogonality was observed with the SCX/×/RP approach, although the distribution of peptides over the fractions was not optimal. The RP/×/RP approach showed a lower degree of orthogonality compared to the SCX/×/RP approach, but peptides were distributed more evenly over the fractions.

**RP/×/RP 2-D LC Analysis of Tryptic Peptides from *E. coli***

To demonstrate the applicability of the RP/×/RP approach for the analysis of complex proteomic samples, *E. coli* tryptic peptides were separated. Since an even distribution of peptides over the gradient was observed in Figure 3, the first-dimension in the RP/×/RP approach was fractionated with a 1 minute time interval. Figure 4 shows the obtained 2-D retention map with the corresponding second-dimension separations of the first-dimension fractions.

The automated off-line 2-D LC separation of *E. coli* peptides in the RP/×/RP approach was successful. As previously observed in Figure 3, the RP/×/RP approach showed an even distribution of peptides over the fractions.

**Repeatability of Automated Off-Line RP/×/RP 2-D LC**

To allow the comparison of two samples analyzed by an entire 2-D LC analysis, retention-time fluctuations must be very small. Three second-dimension fractions obtained from three subsequent 2-D LC experiments were compared for retention time repeatability. The chromatograms shown in Figure 5 were obtained with a 12 hours time difference that was needed for a complete 2-D LC analysis experiment.

![Figure 3. 2-D retention map for the separation of a six-protein digest mixture (PMD) peptides using the SCX/×/RP approach (left) and the RP/×/RP approach (right).]
Excellent 2-D LC retention time repeatability was observed for the RP/$\times$/RP approach, characterized by an RSD value below 0.3%. In addition, the highly similar peak profiles of the RP chromatograms indicate that the first-dimension separation and fractionation were highly reproducible.

**Complementary SCX/$\times$/RP and RP/$\times$/RP 2D-LC**

To demonstrate the complementary nature of the SCX/$\times$/RP and RP/$\times$/RP approaches, the *E. coli* peptide mixture was separated successfully using the SCX/$\times$/RP as well as the RP/$\times$/RP 2-D LC methods.

For the SCX/$\times$/RP method, the fraction-collection time was adjusted from 2 min at the beginning and the end of the first-dimension separation to 1 min during the elution of the 2+ and 3+ charged peptides. However, it must be noted that even with adjusting the fraction-collection time during the first-dimension SCX separation, the peptide distribution over the fractions was less compared to the first-dimension high-pH RP separation. The SCX separation or fraction-collection time was not further optimized to get a better distribution of peptides in the SCX/$\times$/RP approach.
The peptides separated using the SCX/×/RP and RP/×/RP approaches were identified by MS/MS and were searched against the Mascot® database. The complementary nature of both setups is presented in the Venn diagram in Figure 6.

Due to the higher degree of orthogonality previously observed in the SCX/×/RP approach where the peptides elute over the entire second-dimension gradient, an increase in peptide identification could be expected. However, as shown in Figure 6, more peptides were identified using the RP/×/RP approach. This can be explained by the better distribution of peptides over the fractions in the first-dimension separation that results in a decreasing number of co-elution of peptides per fraction in the second-dimension, and consequently lower ion-suppression in the mass-spectrometer.

46% of the peptides were only identified in the RP/×/RP approach, 32% were only identified in the SCX/×/RP approach and 22% of the peptides were identified in both 2D-LC methods. The corresponding number of identified proteins were 171 (RR×RP), 82 (SCX×RP) and 270 for both techniques giving a total of 623 proteins. From these results, it can be concluded that the 2D-LC techniques are complementary to a significant extent.

CONCLUSIONS
For the separation of highly complex peptide samples, the RP/×/RP approach was applied as an alternative for the frequently applied SCX/×/RP approach. The UltiMate 3000 Proteomics MDLC system equipped with the automated off-line 2-D-LC solution provided a versatile and easy-to-use solution. The RP/×/RP approach showed high chromatographic resolution separations and flexibility towards sample handling. Excellent retention time repeatability was obtained, characterized by an RSD value below 0.3% for three consecutive 2-D-LC experiments. The SCX/×/RP approach showed a higher degree of orthogonality compared to the RP/×/RP method, however, the RP/×/RP approach was found to be complementary to the SCX/×/RP approach in terms of peptide identification.

MATERIALS
Standard System
Proteomics MDLC (Contact local Dionex representative for P/N)
Micro Fraction Collection Option, WPS-3000PL including hardware, software (Chromelone Extended Fraction Suite), documentation, for use with Chromelone 6.8 or higher (P/N 6820.0050)
Micro Fraction Collection Option, WPS-3000PL for use with DCMSlink™ V2.5 (P/N 6820.0051)
Off-line 2DLC kit for Peptides, CAP RP/NAN RP Acclaim PA2 (P/N6720.0115)

Biocompatible System
Proteomics MDLC, biocompatible (Contact local Dionex representative for P/N)
Micro Fraction Collection Option, WPS-3000PL, biocompatible, Including hardware, software (Chromelone Extended Fraction Suite), documentation, for use with Chromelone 6.8 or higher (P/N 6821.0050)
Micro Fraction Collection Option, WPS-3000PL biocompatible, for use with DCMSlink™ V2.5 (P/N6821.0051)
Off-line 2-D LC kit for Peptides, biocompatible, CAP RP/NAN RP Acclaim PA2 (P/N 6721.0115)
### Table 2. Required Materials Which are Not Provided in the Automated Off-Line Application Kit

<table>
<thead>
<tr>
<th>Off-Line 2-D LC Kit for Peptides, CAP RP/NANO RP Acclaim PA2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Part Number</strong></td>
</tr>
<tr>
<td>6720.3150A</td>
</tr>
<tr>
<td>6721.3150A</td>
</tr>
<tr>
<td><strong>VWD-3x00 - Flow Cells</strong></td>
</tr>
<tr>
<td>6074.0280</td>
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<tr>
<td>6074.0270</td>
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<tr>
<td><strong>UVD-3000 - Flow Cells</strong></td>
</tr>
<tr>
<td>6073.0003</td>
</tr>
<tr>
<td>6073.0002</td>
</tr>
</tbody>
</table>

*For the generation of 2-D retention plots, two UV detectors are required.

### REFERENCES


### APPENDIX I

First-dimension program file including a User Defined Program (UDP) for RP/×/RP automated off-line 2-D LC experiments.

```plaintext
;============================================================
; First Dimension RP Program
; ------------------------------------------------------------
; PGM-Version January 2009
; Sample: Protein Mix Digest (PMD)
;=============================================================

;Settings for Pump
LoadingPump.Pressure.LowerLimit =  0 [bar]
LoadingPump.Pressure.UpperLimit =  400 [bar]
LoadingPump.MaximumFlowRampDown =  600 [µl/min]
LoadingPump.MaximumFlowRampUp =  600 [µl/min]
LoadingPump.%A.Equate =  "%A"
LoadingPump.%B.Equate =  "%B"
LoadingPump.%C.Equate =  "%C"

MicroPump.Pressure.LowerLimit =  0 [bar]
MicroPump.Pressure.UpperLimit =  350 [bar]
MasterPressure.LowerLimit =  0 [bar]
MasterPressure.UpperLimit =  500 [bar]
MicroPump.MaximumFlowRampDown =  0.300 [µl/min]
MicroPump.MaximumFlowRampUp =  0.300 [µl/min]
MicroPump.%A.Equate =  "%A"
MicroPump.%B.Equate =  "%B"
MicroPump.%C.Equate =  "%C"
MicroPump.Flow =  0.000 [µl/min]
MicroPump.%B =  0.0 [%]
MicroPump.%C =  0.0 [%]
MicroPump.Curve =  5

LoadingPump_Pressure.Step =  Auto
LoadingPump_Pressure.Average =  On
MicroPump_MasterPressure.Step =  Auto
MicroPump_MasterPressure.Average =  On

;Settings for UV Detectors
;
In the Server Configuration the first UV Detector
was named UV with Signal name UV_VIS_1
;
In the Server Configuration the second UV Detector
was named UV_2 with Signal name UV_VIS_1_2

UV_2.Data_Collection_Rate =  2 [Hz]
UV_2.TimeConstant =  1 [s]
UV.TimeConstant =  1 [s]
UV_VIS_1.Wavelength =  214 [nm]
UV_VIS_1.Step =  Auto
UV_VIS_1.Average =  On
UV_VIS_1_2.Wavelength =  214 [nm]
UV_VIS_1_2.Step =  Auto
UV_VIS_1_2.Average =  On

;Settings for Columnoven

ColumnOven.TempCtrl =  On
ColumnOven.Temperature.Nominal =  30.0 [°C]
ColumnOven.Temperature.UpperLimit =  45.0 [°C]
ColumnOven.Temperature.LowerLimit =  5.0 [°C]
ColumnOven.Temperature.UpperLimit =  85.0 [°C]
ColumnOven.ReadyTempDelta =  1.0 [°C]
ColumnOvenTemp.Step =  Auto
ColumnOvenTemp.Average =  On
ColumnPressure.Step =  0.20 [s]
ColumnPressure.Average =  Off
ColumnOven_FC_BridgeFlow.Step =  Auto
ColumnOven_FC_BridgeFlow.Average =  On
ColumnOven_FC_Stepper.Step =  Auto
ColumnOven_FC_Stepper.Average =  On
ColumnOven_FC_Filter.Step =  Auto
ColumnOven_FC_Filter.Average =  On
ColumnOven_FC_Deviation.Step =  Auto
ColumnOven_FC_Deviation.Average =  On
ValveLeft =  1_2
ValveRight =  1_2
FlowSplitter_1.FSControlMode =  Auto

;Settings for Autosampler

Sampler.TempCtrl =  On
Sampler.Temperature.Nominal =  30.0 [°C]
Sampler.Temperature.UpperLimit =  45.0 [°C]
Sampler.Temperature.LowerLimit =  4.0 [°C]

DrawSpeed =  200 [nl/s]
DrawDelay =  5000 [ms]
DispSpeed =  2000 [nl/s]
DispenseDelay =  2000 [ms]
WasteSpeed =  4000 [nl/s]
WashSpeed =  6000 [nl/s]
SampleHeight =  3.000 [mm]
PunctureDepth =  9.000 [mm]
10 Automated Two-Dimensional Separation of Peptides by Ion-Pair Reversed-Phase High-Performance Liquid Chromatography-Electrospray Ionization Mass Spectrometry at High and Low pH

WashVolume = 25.000 [µl]  
SyncWithPump = On  
Sampler.TubePosition = Ral  
FractionCollection.TubePosition = Sampler.TubePosition  
Sampler.TubePosition = Ral  
SyncWithPump = On  
SyncWithPump = On  
Sampler.TubePosition = Ral  
FractionCollection.TubePosition = Sampler.TubePosition  
Sampler.TubePosition = Ral  
SyncWithPump = On  
Sampler.TubePosition = Ral  
FractionCollection.TubePosition = Sampler.TubePosition  
ReagentAVial= R1  
UdpDispense To=Drain, Volume=0.000, SyringeSpeed=GlobalSpeed, SampleHeight=GlobalHeight  
UdpDispense To=Drain, Volume=0.000, SyringeSpeed=GlobalSpeed, SampleHeight=GlobalHeight  
UdpDispense To=Drain, Volume=0.000, SyringeSpeed=GlobalSpeed, SampleHeight=GlobalHeight  
UdpDispense To=Drain, Volume=0.000, SyringeSpeed=GlobalSpeed, SampleHeight=GlobalHeight  

dDelayTime = 30.0 [s]  
); Settings for Fraction Collection  
);=============================================================  
); Definition of triggers for fraction collection starts here.  
); Vials have to be uncapped  
(Command: StartFraction/ChangeTube: VialType=Uncapped)  
); Needle remains outside the puncture  
(e.g. 10 mm -> NeedleExtension)  
); during fractionation / vial change  
);=============================================================  

; Definition of triggers for fraction collection starts here.  
); Vials have to be uncapped (Commands: StartFraction/ChangeTube: VialType=Uncapped)  
); Needle remains outside the puncture (e.g. 10 mm -> NeedleExtension)  
); during fractionation / vial change  

Trigger FracStart FracStartDetected  
Sampler.TubePosition = FractionCollection.TubePosition  
StartFractionUncapped NeedleExtension=10000, fractionheight=6000  
EndTrigger  

Trigger FracChange FracChangeDetected  
Sampler.TubePosition = FractionCollection.TubePosition  
ChangeTubeUncapped NeedleExtension=10000, fractionheight=6000  
EndTrigger  

Trigger FracEnd FracEndDetected  
EndFraction  

); Definition of triggers for fraction collection ends here.  
)}>=
Second dimension program file including a User Defined Program (UDP) for RP/x/RP automated off-line 2D experiments

; Second Dimension RP Program
; PGM-Version January 2009
; Sample: First Dimension Fractions

;=============================================================
; Second Dimension RP Program
;=============================================================

AcquireExclusiveAccess

;Settings for Pump
LoadingPump.Pressure.LowerLimit = 0 [bar]
LoadingPump.Pressure.UpperLimit = 400 [bar]
LoadingPump.Pressure.LowerLimit = 0 [µl/min]
LoadingPump.MaximumFlowRampDown = 50 [µl/min]
LoadingPump.MaximumFlowRampUp = 1000 [µl/min]
LoadingPump.%A.Equate = "%A"
LoadingPump.%B.Equate = "%B"
LoadingPump.%C.Equate = "100% water + 0.05% HFBA"
MicroPump.Pressure.UpperLimit = 350 [bar]
MicroPump.Pressure.LowerLimit = 0 [bar]
MasterPressure.LowerLimit = 0 [bar]
MasterPressure.UpperLimit = 400 [bar]
MicroPump.MaximumFlowRampDown = 0.300 [µl/min]
MicroPump.MaximumFlowRampUp = 0.300 [µl/min]
MicroPump.%A.Equate = "100% water + 0.05% TFA"
MicroPump.%B.Equate = "20/80 water/ACN + 0.04% TFA"
MicroPump.%C.Equate = "%C"

LoadingPump_Pressure.Step = Auto
LoadingPump_Pressure.Average = On
MicroPump_MasterPressure.Step = Auto
MicroPump_MasterPressure.Average = On

LoadingPump.Flow = 20 [µl/min]
LoadingPump.%B = 0.0 [%]
LoadingPump.%C = 100.0 [%]

;Settings for UV Detector
UV.Data_Collection_Rate = 2 [Hz]
UV.TimeConstant = 1 [s]
UV_2.TimeConstant = 1 [s]
UV_VIS_1.Wavelength = 214 [nm]
UV_VIS_1.Step = Auto
UV_VIS_1.Average = On
UV_VIS_1_2.Wavelength = 214 [nm]
UV_VIS_1_2.Step = Auto
UV_VIS_1_2.Average = On

;Settings for ColumnOven
ColumnOven.TempCtrl = On
ColumnOven.Temperature.Nominal = 30.0 [°C]
ColumnOven.Temperature.LowerLimit = 5.0 [°C]
ColumnOven.Temperature.UpperLimit = 85.0 [°C]
ColumnOven.ReadyTempDelta = 1.0 [°C]

ValveLeft = 10_1
ValveRight = 10_1
ColumnOven.Temp.Step = Auto
ColumnOven.Temp.Average = On
ColumnPressure.Step = Auto
ColumnPressure.Average = On
ColumnOven.FC_BridgeFlow.Step = Auto
ColumnOven_FC_BridgeFlow.Average = On
ColumnOven.FC_Stepper.Step = Auto
ColumnOven.FC_Stepper.Average = On
FlowSplitter_1.FSCtrlMode = On
FlowSplitter_1.FSControlMode = Auto
FlowSplitter_1.FSControlMode = Auto
FlowSplitter_1.FSControlMode = Auto

;Settings for Autosampler
DrawSpeed = 300 [nl/s]
Delay = 5000 [ms]
DispSpeed = 2000 [nl/s]
DispenseDelay = 2000 [ms]
WasteSpeed = 4000 [nl/s]
SampleHeight = 1.000 [mm]
PunctureDepth = 1.000 [mm]
WashVolume = 100.000 [µl]
SyncWithPump = On
Sampler.PumpDevice = "MicroPump"
LowDispersionMode = Off
InjetcMode = UserProg
ReagentBVial = R2
UdpDispense To=Drain, Volume=0.000,
UdpDispense To=SampleVial,
UdpDispense To=SampleVial,
UdpDispense To=SampleVial,
UdpDispense To=SampleVial,
UdpDispense To=SampleVial,
UdpDispense To=SampleVial,
UdpDispense To=SampleVial,
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UdpDispense To=SampleVial,
UdpDispense To=SampleVial,
UdpDispense To=SampleVial,
UdpDispense To=SampleVial,
UdpDispense To=SampleVial,
Sampler.TempCtrl = On
Sampler.Temperature.Nominal = 10.0 [°C]
Sampler.Temperature.UpperLimit = 45.0 [°C]
Sampler.ReadyTempDelta = 5.0 [°C]

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MicroPump.%B = 4.0 [%]  
MicroPump.%C = 0.0 [%]  
Wait  
UV.Ready and  
ColumnOven.Ready and  
Sampler.Ready  
Inject  
LoadingPump_Pressure.AcqOn  
MicroPump_MasterPressure.AcqOn  
ColumnOven_Temp.AcqOn  
ColumnPressure.AcqOn  
ColumnOven_FC_BridgeFlow.AcqOn  
ColumnOven_FC_Stepper.AcqOn  
CollectFractions = No  
UV_VIS_1.AcqOn ; UV_VIS_1_2.AcqOn ; Select the correct UV detector  
MicroPump.Flow = 0.300 [µl/min]  
MicroPump.%B = 4.0 [%]  
MicroPump.%C = 0.0 [%]  
1.000 Sampler.Wash  
5.000 ValveLeft = 1_2  
6.000 MS_Start.State = On  
30.000 MicroPump.Flow = 0.300 [µl/min]  
MicroPump.%B = 60.0 [%]  
MicroPump.%C = 0.0 [%]  
30.100 MicroPump.Flow = 0.300 [µl/min]  
MicroPump.%B = 90.0 [%]  
MicroPump.%C = 0.0 [%]  
35.000 MicroPump.Flow = 0.300 [µl/min]  
MicroPump.%B = 60.0 [%]  
MicroPump.%C = 0.0 [%]  
35.100 MicroPump.Flow = 0.300 [µl/min]  
MicroPump.%B = 4.0 [%]  
MicroPump.%C = 0.0 [%]  
55.000 ValveLeft = 10_1  
57.000 MS_Start.State = Off  
60.000 LoadingPump_Pressure.AcqOff  
MicroPump_MasterPressure.AcqOff  
ColumnOven_Temp.AcqOff  
ColumnPressure.AcqOff  
ColumnOven_FC_BridgeFlow.AcqOff  
ColumnOven_FC_Stepper.AcqOff  
UV_VIS_1.AcqOff ; UV_VIS_1_2.AcqOff ; Select the correct UV detector  
MicroPump.Flow = 0.300 [µl/min]  
MicroPump.%B = 4.0 [%]  
MicroPump.%C = 0.0 [%]  
ReleaseExclusiveAccess  
End

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