Automated In-Needle Derivatization Applying a User-Defined Program for the Thermo Scientific Dionex WPS-3000 Split-Loop Autosampler

INTRODUCTION

The Thermo Scientific Dionex UltiMate® 3000 autosampler series provides sample preparation commands that help define individual sample preparation steps. User-defined programs (UDPs) allow the use of several micro-robotic features of the autosampler, such as diluting or mixing of the sample with reagents. The Program Wizard in the Thermo Scientific Dionex Chromeleon® Chromatography Data System (CDS) software assists in creating a UDP, thus making it easy to specify the single steps for sample preparation.

One interesting field of application for automated in-sampler preparation steps is the determination of amino acids. Most amino acids lack a good chromophore. Amino acids are amperometrically detected using either Thermo Scientific Dionex *AAA-direct*™ Amino Acid Analysis System,[1,2] detected by charged aerosol detection,[3] or they must be derivatized before spectroscopic detection. The derivatization can be performed either precolumn or postcolumn.[4] A fast and sensitive precolumn derivatization reaction is achieved using *o*-phthaldialdehyde (OPA) as the derivatization reagent[5,6] followed by separation on a C18 reversed-phase column. The complete derivatization procedure is rapid and can easily be automated using the micro-robotic features of the Thermo Scientific Dionex WPS-3000RS autosampler.

The autosampler allows the customized determination of five different positions for reagent vials (Reagent Vial A to D and an additional PrepVial position). The PrepVial may also be used as a target vial for mixing sample and reagent. This procedure is called in-vial mixing. The fastest and most sample-saving method of reagent mixing is the in-needle mixing procedure. It is performed directly in the autosampler needle.

Here, step-by-step description explains how to create a UDP for an automated precolumn derivatization. The capability of the autosampler programming is demonstrated by peak area evaluation of an amino acid analysis performed on an UltiMate 3000 Rapid Separation LC (RSLC) system.

EQUIPMENT

UltiMate 3000 RSLC System including:

- Solvent Rack with Degasser SRD-3000
- Binary Rapid Separation Pump HPG-3200RS (1034 bar) with 200 µL mixing volume
- Rapid Separation Well Plate Sampler with thermostatting WPS-3000TRS
- Rapid Separation Thermostatted Column Compartment TCC-3000RS
- Rapid Separation Fluorescence Detector FLD-3400RS with micro flow cell (2 µL, SST)

Chromeleon CDS software, 6.80, SR10

CHROMATOGRAPHIC CONDITIONS

Column:	Macherey-Nagel, Nucleodur® C18 Gravity, 2.0 × 100 mm, 1.8 µm with OptiSolve Inline Filter with 0.2 µm frit
Mobile Phase A:	10 mM Na_2HPO_4, 10 mM $Na_2B_4O_7 \cdot 10\ H_2O$, adjusted to pH 7.8 with hydrochloric acid (fuming), filtered through a 0.2 µm membrane
Mobile Phase B:	Acetonitrile/methanol/water (45/45/10 v/v/v)
Wash Solvent:	Methanol in water (10%)
Flow Rate:	0.7 mL/min

Gradient:

Time (min)	*Eluent B (%)*	*Curve*
0	2	5
0.1	10	5
1.5	20	6
6.5	38	6
8.2	57	5
8.3	100	5
10.0	100	5
10.5	2	5
16.0	2	5

Temperature.:	40 °C
Max. Backpressure:	810 bar (12,150 psi)

Detector Settings

Data Collection Rate:	25 Hz
Response Time:	0.8 s
Excitation Wavelength:	337 nm
Emission Wavelength:	442 nm
Filter Wheel Position:	280 nm
Sensitivity:	6
Lamp Mode:	High Power

Reagents and Standards

Water, 18 MΩ.cm deionized

Acetonitrile, HPLC grade (JT Baker P/N 9017)

Methanol, HPLC grade (JT Baker P/N 8402)

Kit of 21 L-Amino Acids (Sigma Aldrich P/N LAA21)

o-Phthaldialdehyde (OPA) (Fluka P/N 79760)

3-Mercapto-propionic acid (MPA) (Fluka P/N 63768)

Sodium phosphate dibasic anhydrous, ≥99.5% (Fluka P/N 71639)

Sodium tetraborate decahydrate, 99.5-105.0% (Sigma Aldrich P/N S9640)

Hydrochloric acid, fuming (Merck P/N 1.00317)

Sample

Amino acid standard including asparagine (Asp), glutamic acid (Glu), serine (Ser), histidine (His), glycine (Gly), threonine (Thr), arginine (Arg), alanine (Ala), cystine (Cys-Cys, 1.25 nmol/µL), tyrosine (Tyr), valine (Val), methionine (Met), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), lysine (Lys), tryptophan (Trp), asparagine (Asn), and glutamine (Gln) with a concentration of 2.5 nmol/µL. This stock solution was diluted with purified lab water to prepare the respective concentrations.

Injection Volume

The volume is defined by UDP settings. With the described UDP, a volume of 10 µL is injected onto the column, originating from a drawn sample volume of 1 µL.

DERIVATIZATION REAGENT

Borate Buffer (Reagent A):*	0.1 M $Na_2B_4O_7 \cdot 10\ H_2O$ pH 10.0 adjusted with 5 M NaOH (7.6 g $Na_2B_4O_7 \cdot 10\ H_2O$ in 200 mL H_2O)
OPA Solution:	50 mg/mL *o*-phthaldialdehyde in methanol
OPA/MPA Reagent (Reagent B):**	7.5 mM *o*-phthaldialdehyde, 225 mM 3-mercapto-propionic acid in 0.1 M borate buffer, pH 10.0 (980 µL borate buffer, 20 µL) OPA solution, 20 µL MPA, freshly mixed before measurements
Inj. Diluent (Reagent C):*	1 M acetic acid

**Filled in 1.8 mL vial (P/N 6000.0072) with crimp cap and slotted septum (P/N 6000.0061)*

***Filled in 1.8 mL vial (P/N 6000.0072) with crimp cap and septum (P/N 6000.0071)*

EXPERIMENTAL

Automatically perform the precolumn derivatization in the WPS-3000RS with a UDP. Mix small volumes of OPA/MPA reagent, borate buffer, and sample. All mixing steps take place in the needle without transfer to an additional preparation vial. After 60 s, stop the derivatization reaction by mixing with injection diluent. Add this injection diluent to decrease the pH of the derivatization mixture prior to injection for enhanced column lifetime. The successful neutralization of the basic derivatization mixture by adding the injection diluent can be checked in preliminary experiments using standards. Prior to starting an experiment in which samples are analyzed, check the pH values of a completed derivatization of one of the samples, and the same solution treatment with the injection diluent. The pH after mixing sample and derivatization buffer should be basic at pH 10.0. The pH of the derivatization solution after adding the injection diluent should be in the neutral range. An adjustment of buffer concentration and injection diluent strength might be necessary to ensure the needed pH values depending on sample properties. Nevertheless, the column lifetime depends on the composition of the sample: for example, salt concentration and impurities.

In addition, longevity may be reduced with injection of in-needle derivatization mixtures compared to the usual longevity that can be expected by applying the normal injection mode of the autosampler.

Set the global settings of the autosampler at 2 µL/s for the draw speed and 10 µL/s for the dispense speed. A proper flushing of the autosampler fluidics prior to the experiments is mandatory for reproducible results. Set the sample height to 0.00 mm.

USER-DEFINED PROGRAM

The commands of the UDP may be either inserted via the device view of the PGM Editor (or module view of Instrument Method in Chromeleon 7.1 software), or directly by entering the commands into the PGM Editor (or Script Editor in Chromeleon 7.1 software). With Chromeleon software, the commands can be filtered to show only entries of interest for a certain user level. The UDP commands are available for Advanced level and higher in the PGM Editor (Chromeleon 6.8 software) or Script Editor (Chromeleon 7.1 software). Table 1 provides a step-by-step explanation of the UDP of the WPS-3000 autosampler for in-needle derivatization.

Table 1. Step-by-Step Description of Commands Applied in the UDP for Automated In-Needle Derivatization

Action	UDP Command	UDP Parameter/comment
Activate the UDP mode of the autosampler. *This command activates the UDP mode of the autosampler. In this mode, every single movement of the autosampler has to be programmed. Chromeleon software ignores the injection volume of the sequence table and uses the value provided in the UDP.*	InjectMode=UserProg	
Define positions for derivatization reagents. *The WPS-3000 autosampler allows definition of up to four reagent vial positions and an additional PrepVial position. The experiment described here uses three reagent vials.*	ReagentAVial=RA1 ReagentBVial=RA2 ReagentCVial=RA3	Position of borate buffer Position of OPA/MPA-reagent Position of acetic acid
Draw air for separation of mobile phase and derivatization mixture. *To separate the derivatization mixture from the mobile phase in the sample loop, an air bubble is drawn after the inject valve switches into loading position. This step is not mandatory and depends on the application. The needle moves over the needle seat and the defined volume is drawn into the sample loop.*	UdpDraw	From=Air, Volume=1.000, SyringeSpeed=1.000
Draw borate buffer.	UdpDraw	From=ReagentAVial, Volume=5.000, SyringeSpeed=1.000
Draw sample.	UdpDraw	From=SampleVial, Volume=1.000, SyringeSpeed=1.000
Draw air bubble for mixing spacing and mix three times.	UdpDraw UdpMoveSyringe UdpMoveSyringe UdpMoveSyringe UdpMoveSyringe UdpMoveSyringe	From=Air, Volume=6.000, SyringeSpeed=1.000 Unload=6.000, SyringeSpeed=33.000 Load=6.000, SyringeSpeed=33.000 Unload=6.000, SyringeSpeed=33.000 Load=6.000, SyringeSpeed=33.000 Unload=6.000, SyringeSpeed=33.000

Action	UDP Command	UDP Parameter/comment
Wait 15 seconds to allow equilibration of the liquids in the needle.	UdpMixWait	Duration=15
The outer surface of the needle is washed with 100 µL needle wash solution.	UdpMixNeedleWash	Volume=100.000
Draw derivatization reagent.	UdpDraw	From=ReagentBVial, Volume=1.000, SyringeSpeed=1.000
Draw air bubble for mixing spacing and mix six times.	UdpDraw UdpMoveSyringe UdpMoveSyringe UdpMoveSyringe UdpMoveSyringe UdpMoveSyringe UdpMoveSyringe UdpMoveSyringe UdpMoveSyringe UdpMoveSyringe UdpMoveSyringe UdpMoveSyringe	From=Air, Volume=7.000, SyringeSpeed=1.000 Unload=7.000, SyringeSpeed=33.000 Load=7.000, SyringeSpeed=33.000 Unload=7.000, SyringeSpeed=33.000 Load=7.000, SyringeSpeed=33.000 Unload=7.000, SyringeSpeed=33.000 Load=7.000, SyringeSpeed=33.000 Unload=7.000, SyringeSpeed=33.000 Load=7.000, SyringeSpeed=33.000 Unload=7.000, SyringeSpeed=33.000 Load=7.000, SyringeSpeed=33.000 Unload=7.000, SyringeSpeed=33.000
Allow reaction of reagent mixture for 60 s.	UdpMixWait	Duration=60
The outer surface of the needle is washed with 100 µL needle wash solution.	UdpMixNeedleWash	Volume=100.000
Draw injection diluent for pH decrease.	UdpDraw	From=ReagentCVial, Volume=3.000, SyringeSpeed=5.000
Draw air bubble for mixing spacing and mix four times.	UdpDraw UdpMoveSyringe UdpMoveSyringe UdpMoveSyringe UdpMoveSyringe UdpMoveSyringe UdpMoveSyringe UdpMoveSyringe	From=Air, Volume=10.000, SyringeSpeed=5.000 Unload=10.000, SyringeSpeed=33.000 Load=10.000, SyringeSpeed=33.000 Unload=10.000, SyringeSpeed=33.000 Load=10.000, SyringeSpeed=33.000 Unload=10.000, SyringeSpeed=33.000 Load=10.000, SyringeSpeed=33.000 Unload=10.000, SyringeSpeed=33.000
Generate an inject marker pulse. *This command is required in UDPs. The injection can be performed only after this pulse.*	UdpInjectMarker UdpInjectValve	 Position=Inject
Reset the syringe after injection. *After the injection valve switches to inject, the syringe plunger has to be moved into the home position.*	UdpSyringeValve UdpMoveSyringeHome	Position=Waste SyringeSpeed=GlobalSpeed
Wash buffer loop with 100 µL to allow the next injection of sample.	UdpDraw UdpDispense	From=Wash, Volume=100.000, SyringeSpeed=10.000 To=Drain, Volume=100.000, SyringeSpeed=10.000,

RESULTS

The WPS-3000TRS autosampler performed an automated in-needle derivatization of amino acid-containing samples with OPA/MPA reagent. The duration of the automated derivatization program described in Table 1 is approximately 4.5 min. Derivatized samples were separated on a sub-2 µm column with the UltiMate 3000 RSLC system. Figure 1 shows the overlay of five consecutive analyses with automated precolumn derivatization of the same sample.

The comparison of the detected peak areas for 19 amino acids of six consecutively injected samples of the same concentration results in an average RSD for the area of 1.06% (see Table 2 for details). The peak area of every single amino acid derivative directly depends on the reaction yield of the sample preparation step. The good result of the average RSD for peak area demonstrates the excellent precision of the automated in-needle derivatization procedure. The linearity test was performed by injection of mixed amino acid standards of 0.1, 0.25, 0.67, 1, and 2 pmol/µL concentrations with three replicates for each concentration. The coefficients of determination are listed in Table 2. The linearity of the derivatized amino acids is excellent, showing an average coefficient of determination of r^2=0.99915.

Table 2. Retention Time, Area Precision, and Coefficient of Determination of the Calibration Curve

Amino Acid	Retention Time Average (min)	Area Average (counts)	Area RSD (%) n=6	r^2
Asp	0.58	41693	1.27%	0.99930
Glu	1.01	52250	0.44%	0.99978
Asn	1.60	48655	0.41%	0.99949
Ser	1.71	82193	0.49%	0.99935
Gln	1.89	56809	0.43%	0.99971
His	1.99	44476	0.62%	0.99916
Gly	2.09	84604	0.61%	0.99981
Thr	2.16	43852	0.77%	0.99936
Arg	2.51	63186	0.55%	0.99981
Ala	2.61	63187	0.49%	0.99978
Tyr	3.13	58381	0.48%	0.99961
Cys-Cys	3.78	11377	3.67%	0.99593
Val	4.22	62721	0.98%	0.99924
Met	4.40	60925	1.01%	0.99783
Trp	5.18	57043	0.66%	0.99966
Phe	5.47	59493	0.76%	0.99958
Ile	5.62	62375	4.76%	0.99711
Leu	6.17	75007	0.23%	0.99991

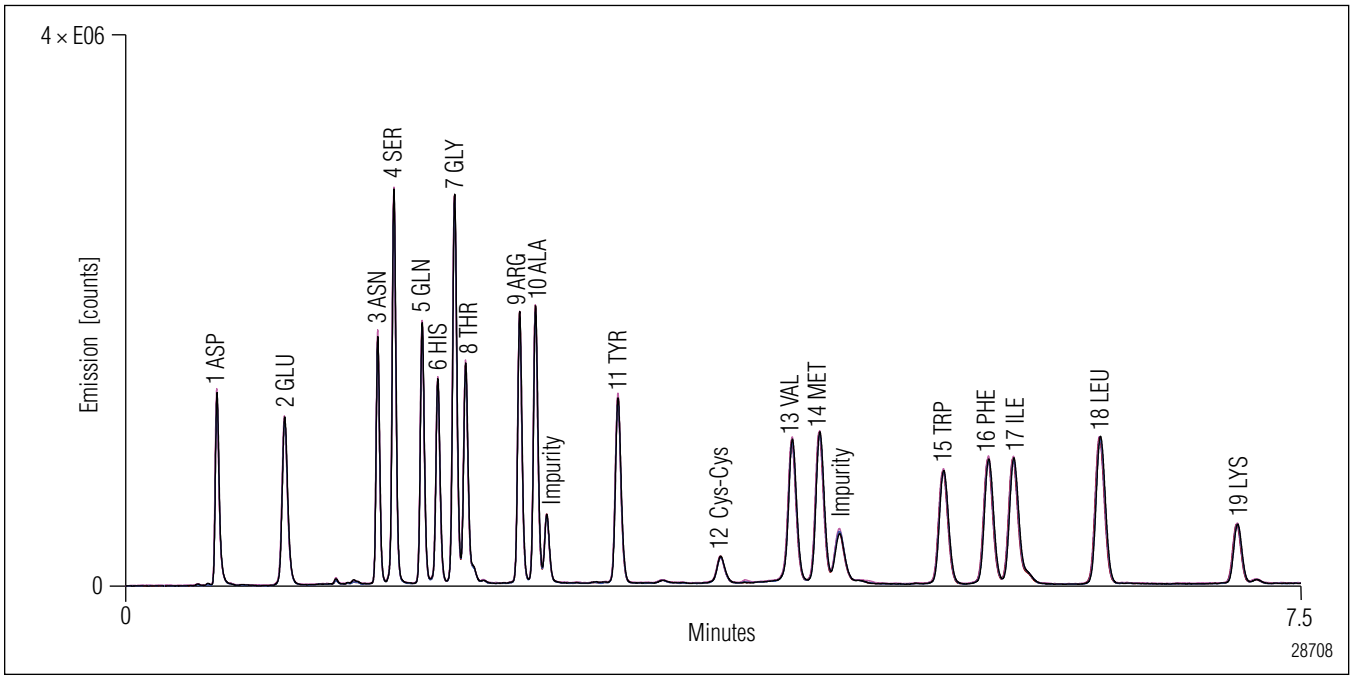

Figure 1. Overlay of five injections of an amino acid standard (0.67 pmol/µL) after in-needle derivatization with o*-phthaldialdehyde.*

ADVANCED FEATURES IN USER-DEFINED PROGRAMS

Variable Location of PrepVial

The WPS-3000 autosampler allows the definition of up to four reagent vial positions and one additional PrepVial position. In UDP mode, the location of the PrepVial can be set fixed or relative to the position of the SampleVial. The relative distance of the PrepVial to the SampleVial is defined by the property PositionCalculator. The size of the installed solvent racks is then considered in the calculation. The following example sets the PrepVial to a position of five positions after the sample vial.

```
PositionCalculator = Sampler.Position
IncrementPositionCalculator By = 5
PrepVial = PositionCalculator
```

Applying User-Defined Columns in UDPs with Chromeleon 6.8 Software

In the Chromeleon 6.8 software, the experienced analyst can draw parameters from the sequence table for module control. Almost any parameter can be entered in a so-called user-defined column (UDC). For example, with the creation of a UDC diluent_volume, the volume of injection drawn by the UDP may be changed from injection to injection. To achieve this, the name of the UDC replaces the fixed volume of the UDP command as demonstrated in Table 3. Please note that a change in the drawn volumes may cause changes in the derivatization yield, resulting in the need for further optimization of the reagent concentrations and volumes.

Table 3. UDC Included in UDP with Chromeleon 6.8 Software

UDP Command	UDP Parameter (Incl. UDC Value)
UdpDraw	From=ReagentCVial, Volume=Sample.diluent_volume, SyringeSpeed=5.000

CONCLUSION

The WPS-3000 split-loop autosampler series offers versatile possibilities in sample preparation by applying UDPs. A practical example for automated sample preparation illustrates the capability of autosampler programming:

- Various sample preparation steps handled through UDPs.
- Precise and effective sample preparation, as demonstrated with amino acid analysis.
- Advanced programming steps expand the options for sample preparation.

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LPN 2849 PDF 08/16