

Fast and Easy HPLC Method Development: Application of an Automated Method Scouting System

Susanne Fabel and Michael Heidorn
Thermo Fisher Scientific, Germering, Germany

Key Words

Column Switching, Custom Injection Variables, Intelligent Run Control (IRC), Queries, Spectral Library, Solvent Selection Valve

Introduction

Whenever a method is developed, the analyst should know certain physicochemical properties of the sample compounds and various stationary phases in order to pre-select columns which promise the best selectivity for effective analysis. When appropriate columns are selected, a number of variable method parameters have to be studied to optimize the selectivity of the chosen stationary phases. For this purpose, basic information about physicochemical properties of the sample compounds is now required to pre-select mobile phase conditions and, hence, limit the amount of scouting tests that needs to be performed. Variable method parameters with reversed-phase columns include the separation temperature, a selection of the organic modifier, the use of buffer to obtain a certain pH range or ionic strength, and the need and nature of a mobile phase gradient. For ionizable compounds, for example, the pH range and ionic strength of the mobile phase strongly influences the selectivity. For very polar compounds, a significant selectivity variation may be observed with C18 columns from different column manufacturers. Thus, a selection of different C18 columns can help to determine the best separation of very polar compounds.

If no information is available about the sample compounds, a broad range of different stationary phases as well as basic and acidic conditions with different organic modifiers can help to determine preliminary conditions. Then, a very large number of tests are required until a desired method is obtained. The *Automated Method*



*Scouting Solution*¹ combines Thermo Scientific™ Dionex™ UltiMate™ 3000 HPLC hardware with the power of Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) software, version 7.2,² making the task of method scouting fast and easy. A quaternary gradient pump provides four different mobile phases which can be mixed with each other at the same time. With the *Solvent Selection Valve (SSV)* of the *Extension Kit for Automated Method Scouting*, up to thirteen different mobile phases can be mixed. The use of two 6-position 7-port valves allows to select between up to six columns with one LC system setup. Each column can be tested with particularly chosen mobile phases and flushed with a designated washing-liquid after the method scouting procedure to lengthen its lifetime. The Chromeleon CDS software automatically presents those methods that provide the best resolution between a chosen critical peak pair or the best average resolution.

This Technical Note demonstrates automated method scouting for a simultaneous separation of amlodipine besylate (a dihydropyridine calcium-channel blocker), hydrochlorothiazide (a diuretic) and valsartan (an angiotensin II receptor antagonist). A combination of these substances is given to treat essential hypertension, e.g. the therapeutics Copalia HCT or Dafiro HCT.^{3,4} For finding the best method, combinations of two different column types, two different organic solvents, and four different aqueous eluents were applied following a method scouting workflow described within this note.

Experimental

System Equipment

UltiMate 3000 RS System, consisting of:

- Solvent Rack with Degasser (SRD-3400)
- Quaternary Low-Pressure Gradient Pump (LPG-3400RS)
- Thermostatted Autosampler (WPS-3000TRS)
- Thermostatted Column Compartment (TCC-3000RS) with two 6-position 7-port valves
- Diode Array Detector (DAD-3400RS) with semi-micro flow cell (SST, 2 μ L)
- Viper Automated Method Scouting Kit, RS system (P/N 6040.2807)
- Extension Kit for Automated Method Scouting (P/N 6040.0100)
- Chromeleon CDS software, version 7.2

Consumables

Reagents and Chemicals

- Valsartan (SIGMA-ALDRICH™ P/N SML0142)
- Amlodipine besylate (SIGMA-ALDRICH P/N A5605)
- Hydrochlorothiazide (SIGMA-ALDRICH P/N H4759)
- Ammonium acetate (Fisher Scientific™ P/N A114-50)
- Formic acid (Fisher Scientific P/N F/1900/PB15)
- Ortho-Phosphoric acid (85%) (Fisher Scientific P/N O/0515/PB08)
- Methanol OPTIMA™ LC/MS (Fisher Scientific P/N A456-212)
- Acetonitrile OPTIMA LC/MS (Fisher Scientific P/N A955-212)
- Ultra-pure lab water, 18.2 M Ω -cm at 25 °C

Conditions

Columns:	1. Thermo Scientific™ Accucore™ aQ, 2.6 μ m, 2.1 \times 100 mm (P/N 17326-102130) 2. Thermo Scientific™ Synchronis™ C18, 1.7 μ m, 2.1 \times 100 mm (P/N 97102-102130)
Eluents:	A – defined by solvent selection valve: 1. Water 2. 0.1% phosphoric acid in water 3. 0.1% formic acid in water 4. 20 mM ammonium acetate in water, pH 4 with acetic acid B – Acetonitrile C – Methanol D – Water/Acetonitrile (50/50% v/v) for column cleaning
Gradient:	0–6.5 min 20–80% B, 6.5–7.5 min 80% B, 7.5–7.6 min 80–20% B, 7.6–10.5 min 20% B
Flow Rate:	0.8 mL/min (for column 1), 0.4 mL/min (for column 2)
Pressure:	From 450 to 800 bar depending on flow rate and column
Temperature:	40 °C
Injection:	1.0 μ L
Detection:	214 nm, 240 nm, 260 nm, 280 nm, 50 Hz, 0.1 s response time, slit width wide, 4 nm bandwidth, 3D-field 190–400 nm

Standard Preparation

For amlodipine besylate, valsartan and hydrochlorothiazide, single stock solutions of 2 mg/mL were prepared by dissolving the appropriate amount in methanol. The single standard solutions were mixed and diluted with water to yield a standard mixture containing 40 μ g/mL amlodipine besylate, 320 μ g/mL valsartan and 50 μ g/mL hydrochlorothiazide. The concentrations of these three components refer to their typical ratio in hypertension drug formulations.

Table 1: Custom Injection Variables used for method scouting.

Name (Type)	Description	Instrument Method Entry	Ref. Figure 1
ColumnSelector (Numeric)	Right and left TCC valve position	Command: ColumnOven.ValveRight	A
		Value: System.Injection.CustomVariables.ColumnSelector	
		Command: ColumnOven.ValveLeft	
		Value: System.Injection.CustomVariables.ColumnSelector	
ColumnName (Text)	Describes active column material	Command: Log	B
		Value: System.Injection.CustomVariables.ColumnName	
ColumnOvenTemp (Numeric)	Sets nominal column thermostat temperature	Command: ColumnOven.Temperature.Nominal	C
		Value: System.Injection.CustomVariables.ColumnOvenTemp	
FlowRate [mL/min] (Numeric)	Sets nominal flow rate	Command: PumpModule.Pump.Flow.Nominal	D
		Value: System.Injection.CustomVariables.FlowRate	
SSV_Position (Numeric)	Sets position of 10-port solvent selection valve	Command: Valve.Position	E
		Value: System.Injection.CustomVariables.SSV_Position	
BufferName (Text)	Describes selected buffer	Command: Log	F
		Value: System.Injection.CustomVariables.BufferName	

Method Scouting Workflow

Less Instrument Methods with Custom Variables

To reduce workload and prevent the operator from programming a multitude of different instrument methods, Chromeleon CDS provides Custom Variables. A Custom Variable is associated with an injection list. In a variable manner, a specific value can be set to such a Custom Variable in the injection list. One or more Custom Variables can be applied to set instrument method parameter values for a sample run. These specific values are then used within the corresponding commands in the instrument method as soon as it is executed and their values are logged in the Chromeleon CDS audit trail. With one single instrument method only, it is possible to screen multiple combinations of different buffers or organic modifiers, flow rates, columns, and column compartment temperatures when Custom Variables are applied. Only when different mobile phase gradients need to be screened, additional instrument methods are required as gradients cannot be altered using Custom Variables.

In this example, the injection list includes six different Custom Variables (A-F, see Table 1 and Figure 1). The Custom Variable *ColumnSelector* (A) defines the thermostating column compartment (TCC) valve position and therefore the active column. The second Custom Variable, *ColumnName* (B), allows logging of the active column material (depending on the TCC valve position). This

Custom Variable is not used as a method variable, but stores column information in the audit trail to relate the TCC valve position to the column which is connected to this position. The third Custom Variable, *ColumnOvenTemp* (C), defines the temperature setting of the column compartment. The Custom Variable D, *FlowRate*, defines the flow rate, whereas the following Custom Variables refer to the position of the solvent selection valve (E), or logs some descriptive information about the selected buffer (F) to the Chromeleon CDS audit trail.

#	UV_VIS_1	*ColumnS	*ColumnName	*ColumnOve	Name	*FlowRate (m	*SSV_Pos	BufferName
28		4	Accucore aQ	40	Standard	0.800	2	0.1% H3PO4
29		4	Accucore aQ	40	Blank, 0.1% formic acid	0.800	3	0.1% formic acid
30		4	Accucore aQ	40	Blank Injection	0.800	3	0.1% formic acid
31		4	Accucore aQ	40	Standard	0.800	3	0.1% formic acid
32		4	Accucore aQ	40	Standard	0.800	3	0.1% formic acid
33		4	Accucore aQ	40	Blank, 20 mM NH4Ac...	0.800	4	20 mM NH4Ac, pH4
34		4	Accucore aQ	40	Blank Injection	0.800	4	20 mM NH4Ac, pH4
35		4	Accucore aQ	40	Standard	0.800	4	20 mM NH4Ac, pH4
36		4	Accucore aQ	40	Standard	0.800	4	20 mM NH4Ac, pH4
37		4	Accucore aQ	40	Blank	0.800	1	H2O
38		4	Accucore aQ	40	Blank Injection	0.800	1	H2O
39		2	Synchronis, C18	40	Column Switching	0.400	1	H2O
40		2	Synchronis, C18	40	Blank	0.400	1	H2O
41		2	Synchronis, C18	40	Blank Injection	0.400	1	H2O
42		2	Synchronis, C18	40	Standard	0.400	1	H2O
43		2	Synchronis, C18	40	Standard	0.400	1	H2O
44		2	Synchronis, C18	40	Blank, 0.1% H3PO4	0.400	2	0.1% H3PO4
45		2	Synchronis, C18	40	Blank Injection	0.400	2	0.1% H3PO4
46		2	Synchronis, C18	40	Standard	0.400	2	0.1% H3PO4

Figure 1: Injection list with six custom injection variables for easy method scouting. For a more detailed description please refer to the text.

Method Scouting eWorkflow from AppsLab

For a smooth method scouting approach, the injections should be ordered specifically (see Figure 1). For example, during a column switching, the flow rate can be reduced in a dedicated instrument method to avoid a system pressure increase when the valve switches. After switching the column and/or changing the eluent composition, an equilibration run and blank injection needs to be made for reliable data generation. The sample should be injected at least twice for each combination of changing parameters. Here, the sequence was generated by an eWorkflow™ downloaded from Thermo Scientific AppsLab Library of Analytical Applications.⁵ AppsLab Library is a web-based analytical method repository which is fully accessible online at www.thermoscientific.com/appslab.

Identification of the Best Method

The *Intelligent Run Control* (IRC) feature of Chromeleon CDS is used to check if a sample run passes certain criteria. Concerning these criteria, IRC applies a global result (passed or failed) to each finished sample run. The number of tests which can be performed on each sample run and, therefore, the number of criteria for finding the best method is unlimited. Modifications of IRC are easily made by applying the *New Test Case* wizard in Chromeleon 7.2 CDS.⁶ The processing method template *SCOUTING* of the *Automated Method Scouting Solution CD* or the *Automated Method Scouting eWorkflow* on AppsLab Library already include several common test cases for IRC such as the total number of detected peaks in the chromatogram or the maximum peak asymmetry. In Figure 2, IRC is passed for separations resulting in a chromatogram with at least three peaks, resolutions higher than two for each peak pair, and peak asymmetries lower than two.

#	Name	Statistics	Operator	Ref.Value 1	Ref.Value 2	Channel	Peak	N.A.	Inj. Condition	Result
1	Number of Peaks	None	>=	3		UV_VIS_1		Failed	Injection Type: Calibration Standard	Passed
2	Resolution next 1	None	>=	2		UV_VIS_1	Number: 1	Passed	Injection Type: Calibration Standard	Passed
3	Resolution previous 2	None	>=	2		UV_VIS_1	Number: 2	Passed	Injection Type: Calibration Standard	Passed
4	Resolution next 2	None	>=	2		UV_VIS_1	Number: 2	Passed	Injection Type: Calibration Standard	Passed
5	Resolution previous 3	None	>=	2		UV_VIS_1	Number: 3	Passed	Injection Type: Calibration Standard	Passed
6	Resolution next 3	None	>=	2		UV_VIS_1	Number: 3	Passed	Injection Type: Calibration Standard	NA -> Passed
7	Resolution previous 4	None	>=	2		UV_VIS_1	Number: 4	Passed	Injection Type: Calibration Standard	NA -> Passed
8	Peak Asymmetry	None	<=	2		UV_VIS_1	All Identified Peaks	Passed	Injection Type: Calibration Standard	NA -> Passed

Figure 2: IRC with common test cases (number of peaks, resolution, and peak asymmetry).

To easily find sample runs which pass all IRC criteria, the powerful Injection Query tool of Chromeleon CDS pulls the most promising candidates according to the search criteria together in a virtual sequence. Here, the sequence *Valsartan_Scouting* is chosen in the Chromeleon CDS local Data Vault by a quick search criterion of the Injection Query (Figure 3). As computed criteria, only sample runs of the injection type calibration standard and which do not fail any IRC are listed in the virtual sequence.

Figure 3: Query that extracts most promising method candidates to a virtual sequence.

Sample runs of the virtual sequence are accessed via the Chromeleon CDS Studio. Subsequently, data is visualized by opening the Report Designer. Figure 4 shows a run time and resolution map for sample runs that met the IRC criteria. The shorter the elution time of the last peak, the lower is the bubble on the y-axis. The diameter of the bubble corresponds to the peak resolution of the critical peak pair. The bigger the bubble, the higher is the resolution. The results are also listed in the table below the graph, including injection number, sample name, elution time of last compound, and the resolution of the critical peak pair. Depending on the preference for a fast method or a method that provides a better resolution, the best method(s) can be selected from this report. In this example, injections 8 and 9 are judged to result from the best method. Unsurprisingly, both results were obtained by two consecutive sample runs with same instrument method settings. Providing a resolution of 18 for the critical peak pair and an elution time below 4 minutes, this method is best for a UHPLC separation.

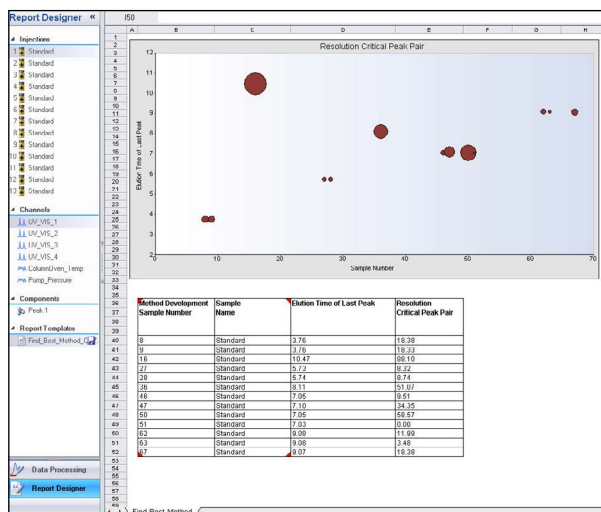


Figure 4: The query results are visualized with the "Find best method" report.

Spectral Library for Compound Identification

When the best method was found, individual peaks have to be assigned to their corresponding compounds. UV spectra of single standard sample runs have been acquired and stored in a spectra library. In the component table section of the processing method, the *Chromeleon Component Table Wizard* is started. Peaks are assigned to sample components, according to the information in the *Spectral Library Screening*.

Method Scouting Result

In this example, the automated method scouting workflow results in the best separation of hydrochlorothiazide (1), amlodipine (2), and valsartan (3) on an Accucore aQ column, 2.1 × 100 mm, 2.6 μm with 0.1% phosphoric acid in water and acetonitrile as eluents in terms of resolution and elution time (Figure 5). This method could be further optimized regarding the mobile phase gradient and, thereby, elution time which requests knowledge from the chromatographer. On the other hand, an analysis time of five minutes is already fast and the scouted method provides robust and reproducible results. Hence, in this case, the method development can also be seen as finished at this stage after the method scouting step without any improvements or adjustments.

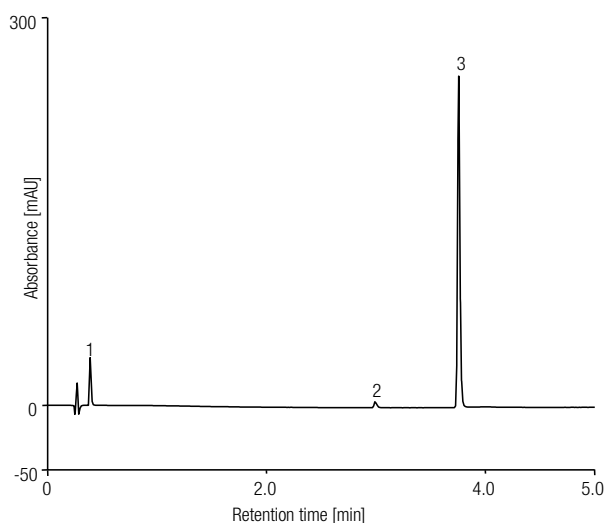


Figure 5: Best separation of hydrochlorothiazide (1), amlodipine (2) and valsartan (3) in terms of resolution and elution time.

Summary

The *Automated Method Scouting Solution* with Chromeleon CDS 7.2 allows an efficient and easy screening of methods, which was demonstrated with the separation of a drug mixture. The user-friendly solution requires no manual interaction and runs unattended. By applying the Custom Injection Variables the needed instrument methods for altering chromatographic conditions is decreased to a minimum. In addition, it can be easily overseen from the sequence, if all conditions to test are met. Otherwise very descriptive instrument names have to be used to manage multiple test conditions. Easy generation of a method scouting sequence is provided by a dedicated eWorkflow which already contains Custom Injection Variables and related instrument methods. Predefined test case criteria and queries help to extract the most promising methods within seconds.

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