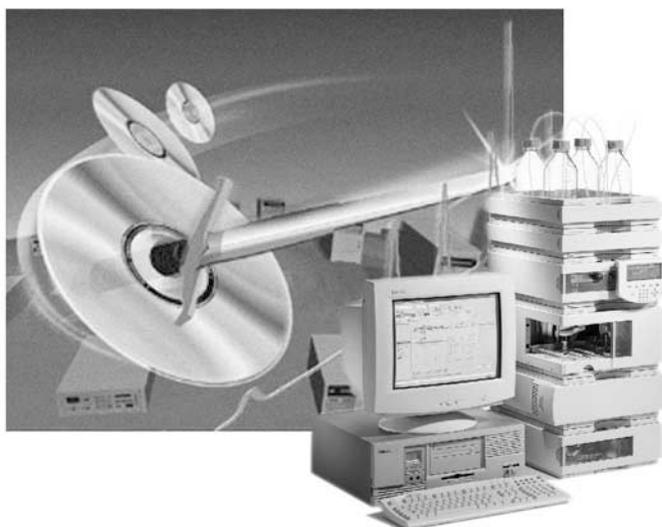


Automated method development using Agilent 1100 Series HPLC systems, Agilent ChemStation and ChromSword® software

Application

Sergey Galushko
Ralf Honsberg



Abstract

The development of an HPLC method is invariably a repetitive and time-consuming process of method planning, development, execution and interpretation. Analysts are often forced to spend a good portion of their effort on these routine tasks rather than concentrating on more valuable work in the laboratory. Today, many tools are available to help automate the method development process. This Application Note demonstrates the use of ChromSword Auto software with Agilent 1100 Series HPLC systems and Agilent ChemStation software.

Introduction

ChromSword is a software pack that supports rapid development of separation methods for liquid chromatography, whereby a minimum number of experiments are required. ChromSword can be used for optimizing separations in reversed-phase, normal-phase and ion-exchange liquid chromatography.

ChromSword can be used offline as standalone software for computer-assisted HPLC method development or, in combination with Agilent 1100 Series HPLC systems and Agilent ChemStation software, it creates a powerful, specialized method development system capable of developing new methods or improving existing methods fully automatically. The following types of optimization and computer simulation can be performed using the software in offline mode.

For reversed-phase chromatography:

- composition of the mobile phase (isocratic, linear and multi-step gradients)
- pH value, pK determination
- temperature
- 2-dimensional optimization: concentration isocratic/temperature, gradient profile/temperature, concentration/pH value, temperature/pH value, ternary solvent mixtures using one column or column combinations, (column combinations/concentration, gradient profile, temperature, pH value)

For normal phase chromatography:

- composition of the mobile phase (isocratic, linear and multi-step gradients)
- temperature

For ion-exchange chromatography:

- buffer concentration
- temperature

One approach to develop or optimize reversed-phase methods is to use virtual chromatography based on structural formula and column/solvent type. The ChromSword software contains a database of properties with more than 60 different commercial reversed-phase columns that allows to simulate retention behavior of compounds in isocratic and gradient mode. When ChromSword and ChemStation software work together online HPLC method development for reversed-phase chromatography can be performed in unattended mode and can optimize:

- composition of the mobile phase (isocratic, linear and multi-step gradients)
- column temperature

If the Agilent 1100 Series system is equipped with a quaternary pump and a column thermostat with a column selection valve, ChromSword and ChemStation can perform fully automatically HPLC optimization with different columns and up to three different organic modifiers.

ChromSword has different functions in a HPLC laboratory:

- to develop completely new methods

- to improve existing methods
- to optimize separation only target compounds
- to find conditions for separation of main components, excipients and impurities

Analysts need only to specify their objectives, for example, find isocratic or gradient method condition, separate only target compounds or unknown compounds such as impurities, and so on, and then press the GO button.

Configuring ChromSword for automated method development

Before ChromSword can be used for automated method development with an Agilent 1100 Series HPLC system and the Agilent ChemStation software (revision A.09.01 or higher), it's necessary to configure ChromSword correctly using the HPLCAdmin program, see figure 1

There are several parameters, which need to be configured:

- Current Instrument — specifies the number of instruments connected to ChemStation. A new instrument can be added by pressing the + (plus) button. The – (minus) button deletes the last instrument from the list. The Current Instrument dialog box allows the analyst to choose the current instrument, for which the other parameters can then be specified.
- Basic Directory — selects a directory where ChemStation will store the data (for example, C:\cstest2\) during method development. All data and method files will be stored in this directory.

The subdirectories corresponding to each instrument will be created in this directory. For example, if the basic directory is C:\Test\ and 2 instruments were specified, the directories C:\cstest2\Instr1\ and C:\cstest2\Instr2\ will be created by the administration program, see figure 2.

- **Default Project** — specifies projects and choose the default project. Projects can be added by the + (plus) and deleted by the - (minus) buttons. Each project is the name of the subdirectory where the results and methods will be stored. This subdirectory may appear in any instrument directory. For example, if the project ADrugs is selected for instrument 1, the data and methods will be stored in the C:\cstest2\Instr1\ADrugs directory. Removing a project with the Delete button will only make the project unavailable for further usage, however all data and methods created remain on disk.
- **Template Method** — ChromSword uses a ChemStation method as a template for generating new methods during the method development process. Any valid existing ChemStation method can be used as a template. The administration program will automatically copy the template method to the destination directory (into the TPL directory with the name Template.m. Multiple instruments require separate templates, because their configurations may be different. The Save&Exit function in the File menu is used to save the

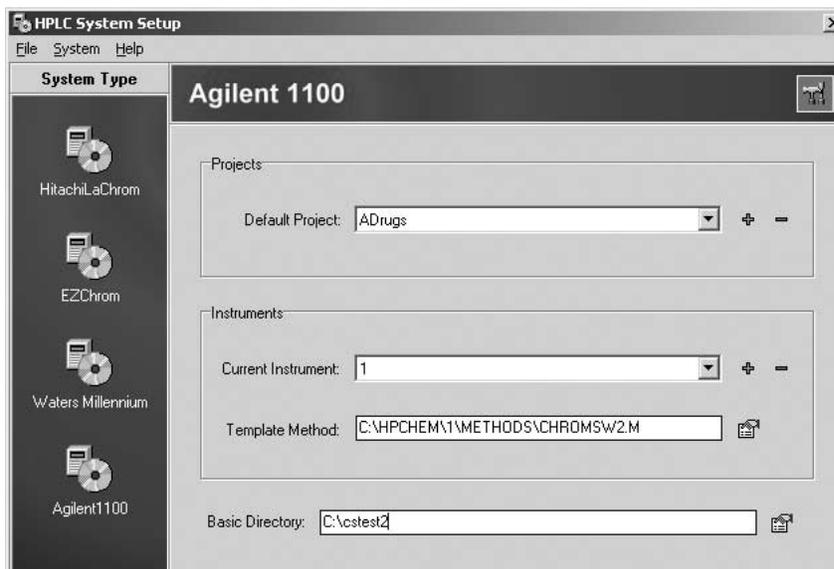


Figure 1
HPLCAdmin program used to configure the data-system connection for ChromSword

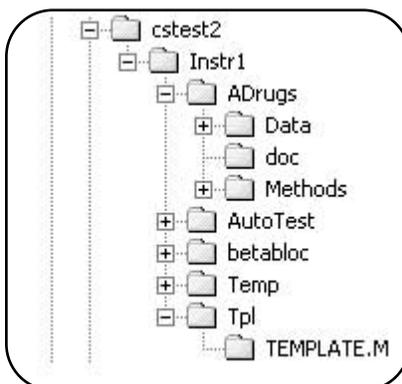


Figure 2
ChromSword data path

changes and close the HPLC Administration program.

It is also possible to select a template method. For this a method has to be saved with the name Template.m in the TPL subdirectory of the ChromSword instrument directory. To prepare a template method the following steps are needed.

- 1 Start the ChemStation in offline or online mode.
- 2 Load any valid method for the Agilent 1100 Series HPLC system that was used before with the instrument. Choose the Save Method as function from the Method menu and save the method it as Template.m in the corresponding directory <BasicDirectory>\Instr<n>\tpl\.

Tips and tricks for operation of ChromSword with ChemStation

- The Sequence subdirectory field in the Sequence parameters window of the ChemStation has to be empty (loading the sequence DEF_LC.S sets the correct default parameters).
- ChromSword resets the data paths of the ChemStation to the project directory. If samples are

run without ChromSword, the data might be saved in the wrong directory. Loading a method, sequence and data file from the default path (for example, \HPCHEM\1\DATA) resets the path to the correct directory.

- During the automated development process ChromSword creates new sequence files. These should not be used for creating a normal sequence in the ChemStation. As a solution the DEF_LC.S or any other known sequence should be loaded before doing analysis with the ChemStation only.
- ChromSword revision 2.1 currently supports the following Agilent 1100 Series HPLC modules: binary pump, quaternary pump, standard autosampler, column compartment including the built-in column selection valve, variable wavelength detector, multi-wavelength detector and diode array detector.

Automatic method optimization

This section gives a short description of how to set up a simple method development experiment. The following Agilent 1100 Series HPLC system was used for all experiments: quaternary pump, degasser, standard autosampler, column compartment with built-in column selection valve and diode array detector.

Preparing a method development experiment

Configure the ChromSword software as described in the previous section. The following procedure describes how to start the automatic experiment.

1. Start ChromSword.
2. Select Compounds tab (normally default).
3. Fill in compound names (vial number in the autosampler and compound number are the same).
4. Double-click on the compound name to activate the chemical formula program and draw the molecular structure for each compound, if it is available (alternatively formulas can be loaded from .sdf or .mol files).
5. Select the First Guess and find a column and an organic solvent according to the available columns in your laboratory.
 1. Prepare all standards (one compound per vial) plus one vial with a mixture of all standards. The concentration used is typically 1 mg/ml.
 2. Prepare the Agilent 1100 Series HPLC system according to the proposed parameters such as mobile phase and column.
 3. Select the Auto-Wizard function and follow dialogs.
 4. Make sure the Agilent 1100 Series HPLC system is set up properly, for example, no air in the system, lamp(s) on for 0.5 h, oven temperature ready, all standard vials in the predefined position, solvent bottles all filled to maximum (you can also use 2-L bottles, if necessary).
 5. Select Go from the Auto-wizard.If no structural formulas are available, fill in the list of compound names (step 3), skip steps 4 through 6, and then prepare the Agilent 1100 Series HPLC system. You can use phosphate buffers as aqueous component (solvent A) but use low concentrations (<20 mM) to avoid plugging a pump through precipitation.

Reviewing results and generating a report

After the experiment is finished successfully the following steps are needed to get a report print-out, with results tables and chromatograms.

6. Start the Rptview program.
7. Select the appropriate .txt file under for example C:\Program files\Merck\ChromSword 2.1\- 8. Now the results can be reviewed, a report can be printed, copied and saved in Microsoft® Word format.

Using created data sets for further optimization

The data (sets) created by the automatic experiment can be used for further optimization using the calculation procedures available in ChromSword. To take advantage of this feature the following steps should be followed (Goal: Further optimization of resolution based on the already available automatically processed data by changing % B):

9. Open appropriate .smp file in the ChromSword main screen, for example C:\Program files\Merck\ChromSword 2.1\- 10. Double-click on Concentration under Available Data.
- 11. Select, for example, Gradient and move line to see separation at different % B concentrations.

How to run a method development experiment

Figure 3 shows the screen that appears after starting ChromSword. Here the names and the sequence of compounds are entered first followed by the molecular structure of the molecules (if available), see figure 4. A chemical editor program ChromDraw is included in the ChromSword package and also offers information about sum formula and mass under the CHEM menu. The drawn molecular structure can also be saved so that it can be used in other applications.

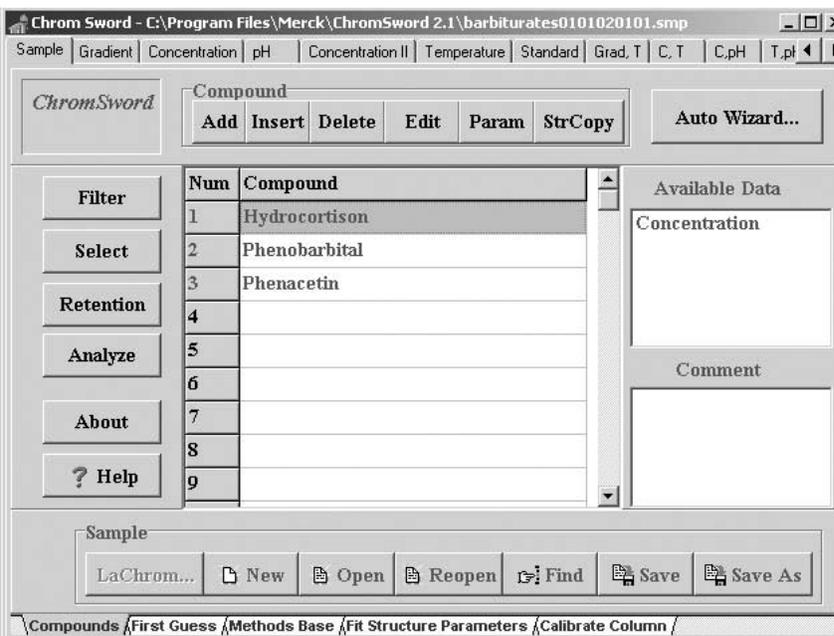


Figure 3
Start screen for setting up an experiment

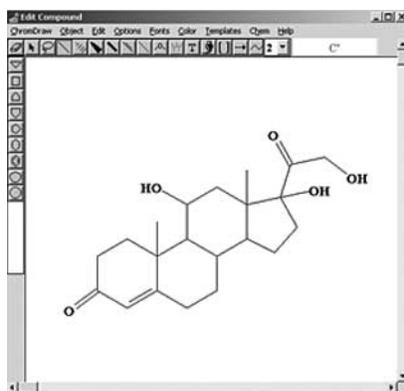


Figure 4
Drawing a molecular structure using ChromDraw

If names and structures are entered, the First Guess function of the main screen can be selected to get a proposal for column, mobile phase, type and concentration of an organic solvent, see figure 5.

Having selected the column and the mobile phase, the Agilent 1100 Series HPLC system should be set up and equilibrated as described above. During equilibration of the system the Auto Wizard dialog from the main screen can be followed and conditions for the experiments can be selected and saved, see figure 6.

In the first dialog, it is determined whether one or several columns should be tested, and whether one mobile phase set or another mobile phase set should be used. In the third dialog, it is determined whether standards are available. In the last screen, flow, temperature, injection volume, column parameters, elution test parameters, and so on are selected. For the elution test a high concentration of an organic modifier should be used that provides low or no retention of all the components of the mixture to be separated (the washing concentration). After all parameters have been set and if the Agilent 1100 Series HPLC system is equilibrated the GO button can be selected and the automated experiment starts.

Reporting

The history of a method development project that ChromSword has performed is saved automatically in a history file. After the optimization

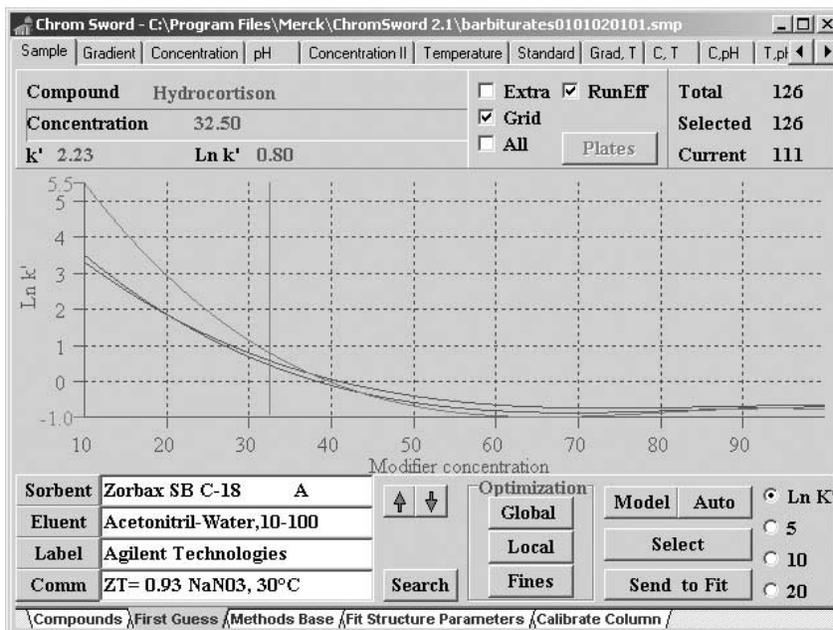


Figure 5
First proposal from the software based on the molecules and the molecular structure

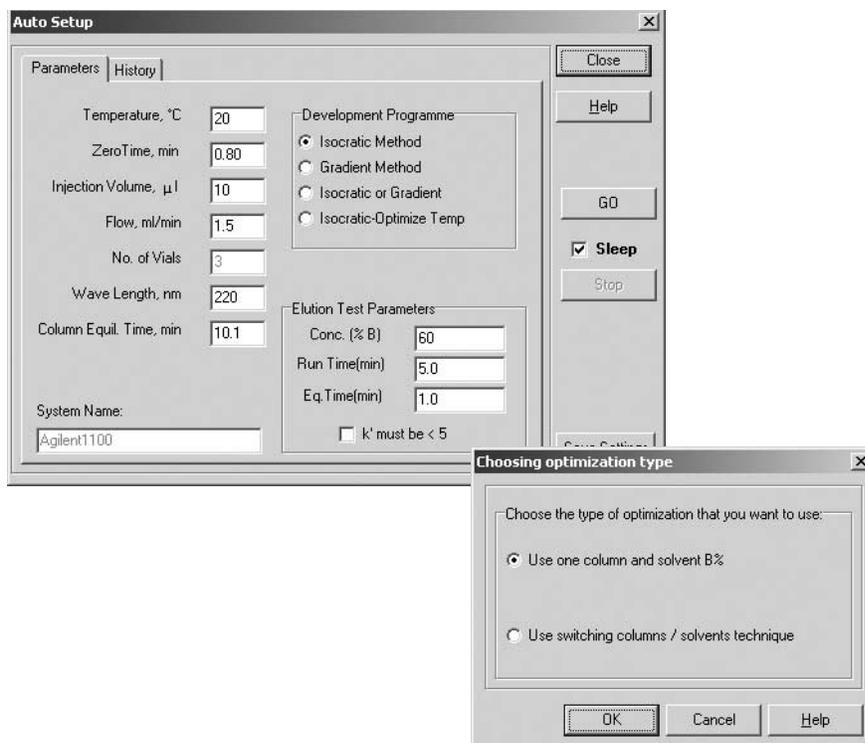


Figure 6
Auto Wizard screens

experiment is finished the report viewer (RptView) can be activated and the appropriate .txt file can be selected, see figure 7.

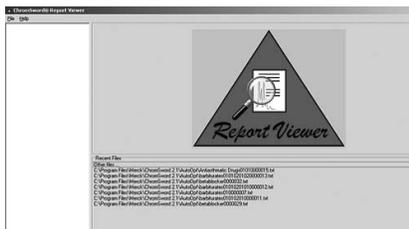


Figure 7
Report viewer start screen

Report Viewer is a ChromSword tool to navigate quickly within the results of a method development project.

After the appropriate .txt file has been loaded the results and report appears, see figure 8. This report can now be printed, copied and saved using the icons at the bottom of the screen.

Further optimization using a existing data set

The automatically created data set can be used for further optimization, for example, for increasing the resolution because more peaks are expected in the real life sample. In this case the .smp file is reopened and data set listed in Available Data listed can be selected by a double-click, see figure 1.

Here is an example where Concentration can be used for further optimizing the gradient and for improving the resolution between three peaks. After the data set has been selected, the screen shown in figure 9 appears.

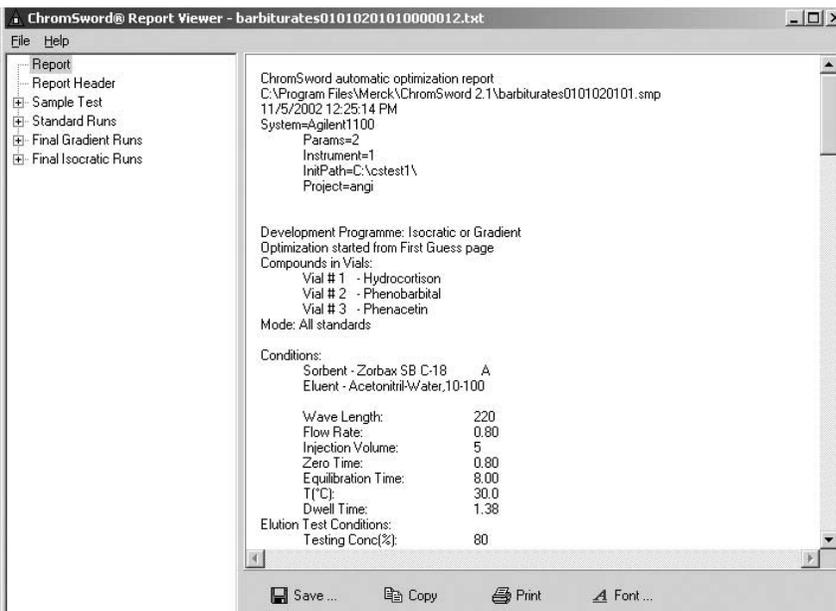


Figure 8
Example of a ChromSword report

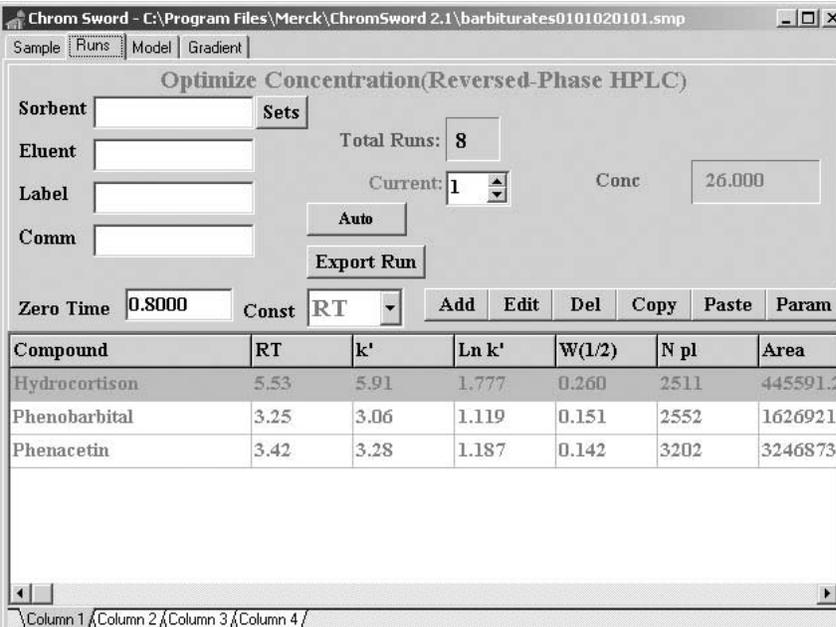


Figure 9
Screen for using available data set for further optimization

Selecting the Model as next screen, see figure 10, the influence of a different organic phase concentration on the separation of the three peaks can be simulated using the vertical cursor in the left diagram. To simulate the influence of different gradients on the separation the Gradient page can be opened. In this screen complete chromatograms can be displayed and the peaks are shown as black bars. By adding more Nodes even very complicated gradients can be simulated, predicting the influence on the resolution, see figure 11. In the diagram the Nodes appear as crosses. By clicking on a cross and moving it, the gradient profile can be altered. The Peaks move accordingly.

An alternative and a rapid option to optimize linear and multi-segment gradients is to choose the Optimize page tab. In this case the program optimizes a gradient profile automatically and displays parameters of the optimizing process.

Example experiments

Several experiments were done to test the performance and usability of the ChromSword software revision 2.1.

- Analysis of a mixture of hydrocortisone, phenobarbital and phenacetin
- Analysis of a mixture of beta blocking drugs: Pindolol, Timolol, Metoprolol and Propranolol
- Analysis of antiasthmatic drugs

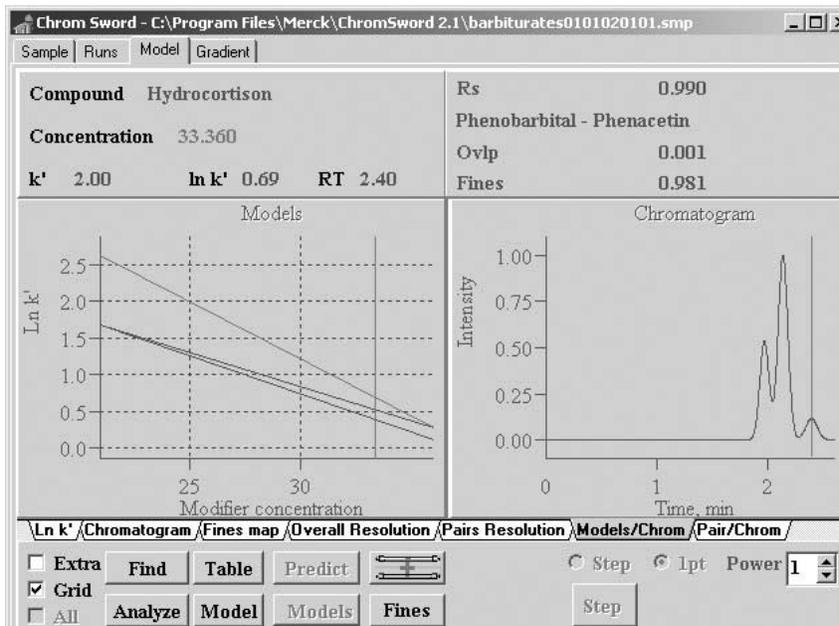


Figure 10
Influence of a different organic phase concentration on the separation

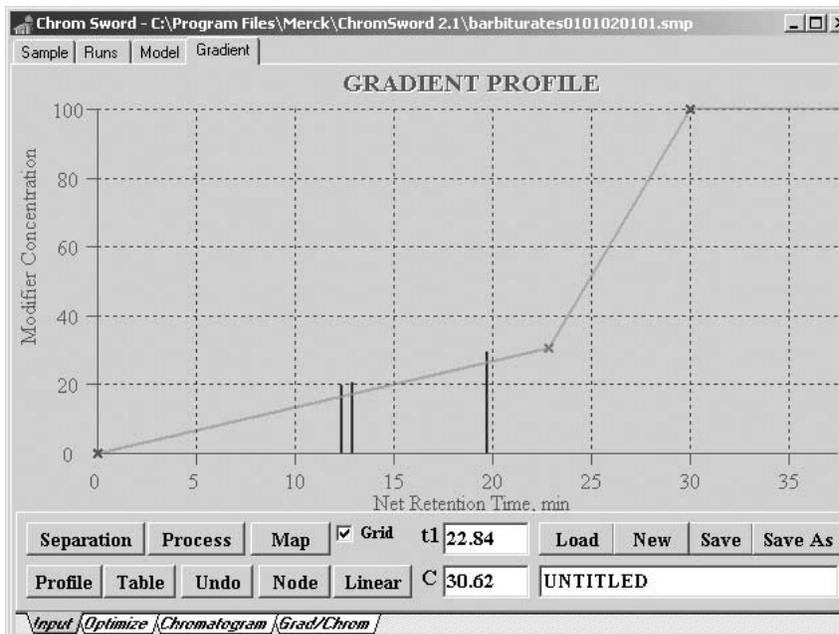


Figure 11
Optimizing the gradient profile for better resolution using the available data set

Experiment 1: Analysis of a mixture of hydrocortisone, phenobarbital and phenacetin

The chemical structures of all compounds were entered and the ChromSword software (First Guess function) proposed to use a C-18 stationary phase and methanol or acetonitrile as mobile phase. Based on this the following setup was selected using the method development wizard.

- Column: 3 x 150 mm ZORBAX SB C-18 phase
- Mobile phases: water-methanol for the first experiment and water-acetonitrile for the second experiment
- Flow rate: 0.8 mL/min
- Detection wavelength: 220 nm
- Column temperature: 30°C
- Injection volume: 5 µL
- Zero time: 0.80 min
- Equilibration time: 8.00 min
- Dwell time: 1.38 min

The Elution test conditions were set to:

- Concentration of % B: 80%
- Run time: 3 min
- Equilibration time: 1 min

When all conditions were set up the experiments were started and after approximately 9 h all experiments were finished, giving the following results.

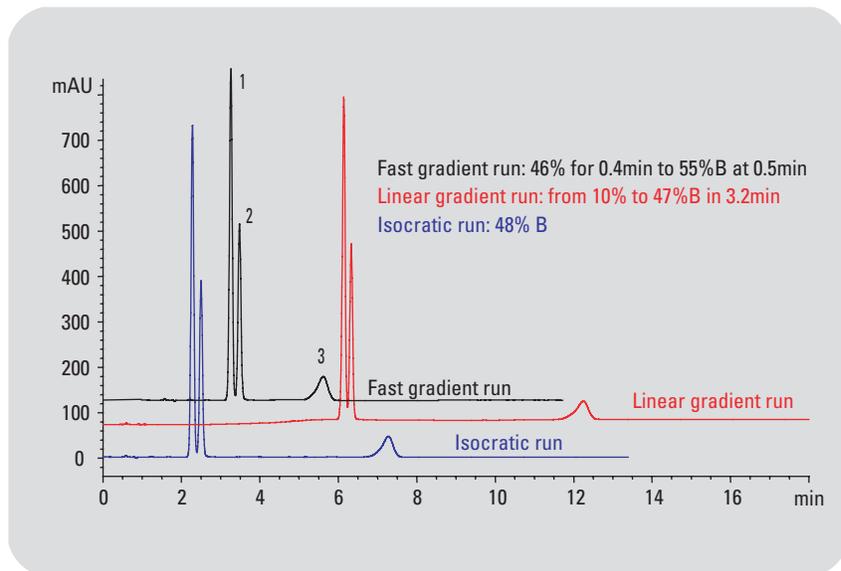


Figure 12
Analysis of phenobarbital (1), phenacetin (2) and hydrocortisone (3) using water-methanol as mobile phase

Results with water-methanol as mobile phase

The best conditions for an isocratic and a gradient runs were evaluated, see figure 12.

The best isocratic conditions with near-baseline separation of all peaks were at 48% methanol. The best linear gradient conditions involved a gradient from 10 %B to 47 %B in 3.2 min. Shortest run times were obtained starting with 46% Acetonitrile, holding it until 0.4 min and increasing the percentage of Acetonitrile then to 55% at 0.5 min.

The isocratic method elutes all peaks earlier than the linear gradient method. For real-life samples it might be of advantage to rinse the column after peak elution with a higher percentage of organic phase. This can help to avoid problems with matrix peaks, which might co-elute with peaks of interest after several isocratic runs.

Results with water-acetonitrile as mobile phase

After completion of the runs using water and methanol as mobile phase, the organic phase was switched to

acetonitrile for the following runs. The best conditions for an isocratic and gradient analysis of the mixture were also evaluated. Figure 13 shows both results, in which the isocratic run also provides near-baseline separation for all peaks and shorter retention times.

For high throughput analysis the isocratic method is best suited, whereas for real-life sample the gradient run with water and methanol as mobile phase might be the better choice. Finally a comparison was made between the method developed by an experienced user and the methods proposed by ChromSword, see figure 14. In this case the user did not need much time to develop the method. This was mainly based on the fact that column and mobile phase were given as prerequisites.

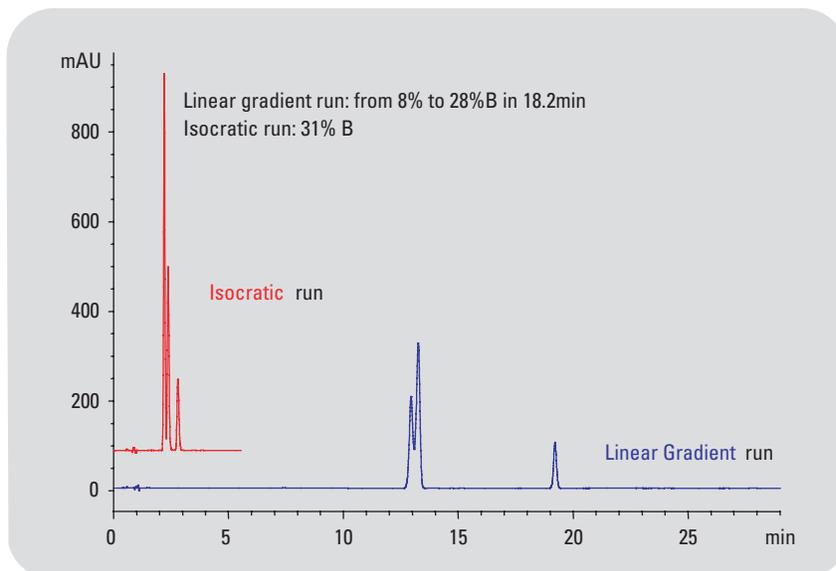


Figure 13
Analysis of phenobarbital, phenacetin and hydrocortisone using water-acetonitrile as mobile phase

Gradient method developed by user in 0.5 h:
 at 0 min 20% acetonitrile
 at 1 min 20% acetonitrile
 at 5 min 80% acetonitrile
 Isocratic method developed by ChromSword in 4.5 h:
 31% acetonitrile
 Linear gradient method developed by ChromSword in 4.5h:
 10 to 47% methanol in 3.2min

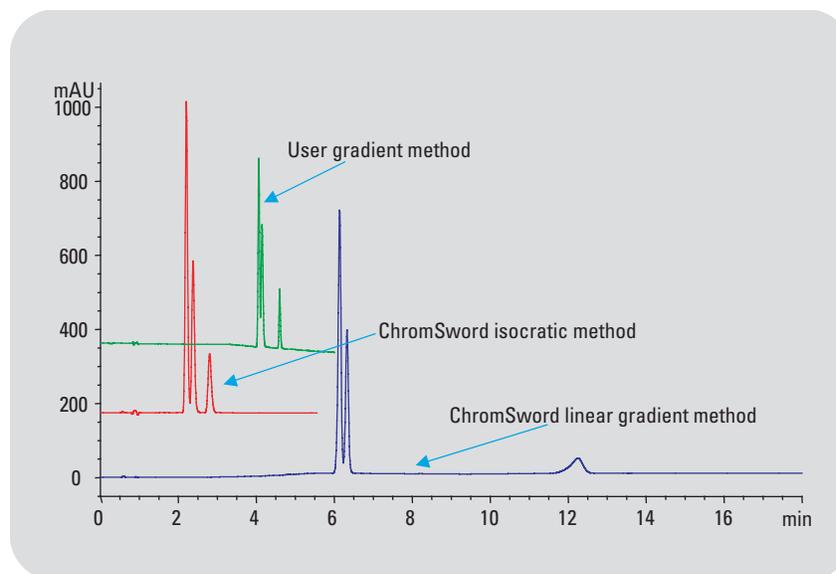


Figure 14
Comparison between ChromSword and method developed by an experienced user

All evaluated methods are applicable depending on the needs of the real-life samples to be analyzed. The benefit of the ChromSword method development software is that reasonable results were developed overnight, without any further user interaction. The evaluated methods can be used immediately or they can be optimized further with real-life samples

Experiment 2: Analysis of beta-blocking drugs Pindolol, Timolol, Metoprolol and Propranolol

The chemical structures of the beta-blocking drugs were entered and ChromSword (First Guess function) made a proposal for column and mobile phases. In this case no reasonable results were obtained. This was mainly due to the fact that software is not able to recommend a modifier, if this is needed.

From former experiments it was known that the analysis of beta-blocking drugs can be done on a C-8 stationary phase using water-acetonitrile as mobile phase. Both solvents must contain trifluoroacetic acid (TFA) as modifier for good separation, see figure 15.

These experiments were done using 0.3 x 150 mm capillary columns. However, transfer to a column with an internal diameter of 4.6 mm was required. It has to be considered that the limit of detection is much lower on a capillary column and that the injected concentration should be adjusted for the 4.6-mm id column. This ensures reasonable peak heights for method development as shown in figure 16.

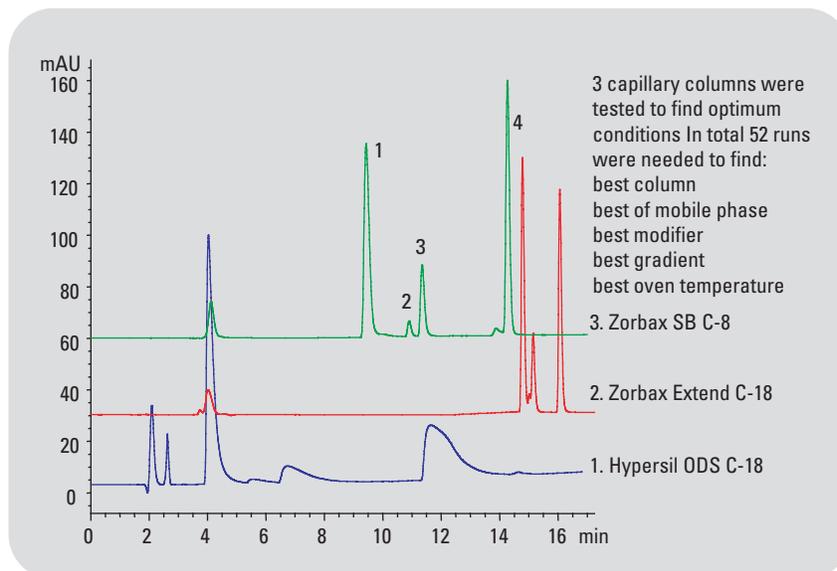


Figure 15
 Method development for beta-blocking drugs Pindolol (1), Timolol (2), Metoprolol (3), Propranolol (4) on three different capillary columns

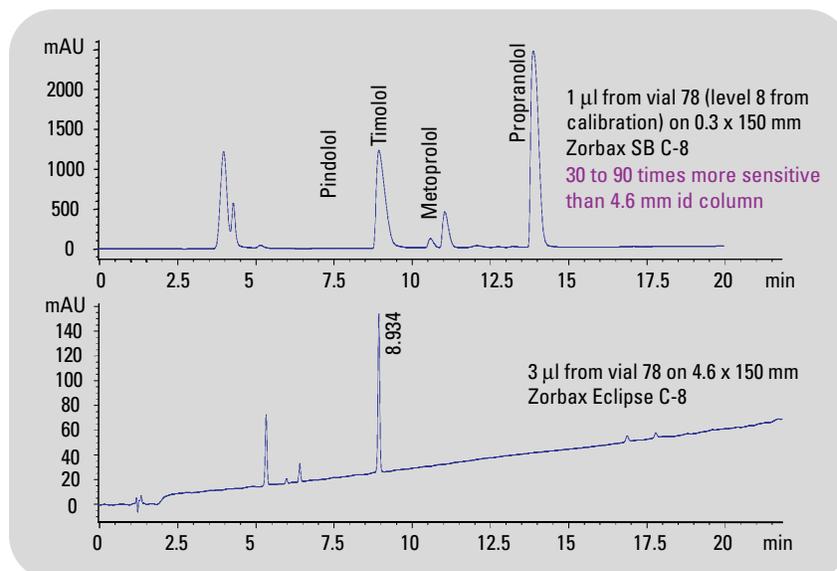


Figure 16
 Method transfer from a 0.3-mm id column to a 4.6-mm id column

The method transfer required about six runs and the following conditions were evaluated:

- Column: 4.6 x 150 mm ZORBAX Eclipse C-8 phase
 - Mobile phase: water-acetonitrile
 - Flow rate 1.5 mL/min
 - Detection wavelength: 220 nm
 - Column temperature: 20 °C
 - Injection volume: 10 µL
 - Zero time: 0.87 min
 - Equilibration time: 10.10 min
 - Dwell time: 0.80 min
- The elution test conditions were set to:
- Concentration of %B: 60 %
 - Run time: 5 min
 - Equilibration time: 1 min

These evaluated conditions were used to start the method development of the ChromSword software for further optimization of chromatographic parameters. Figure 17 shows the results that were obtained within about 9.3 h.

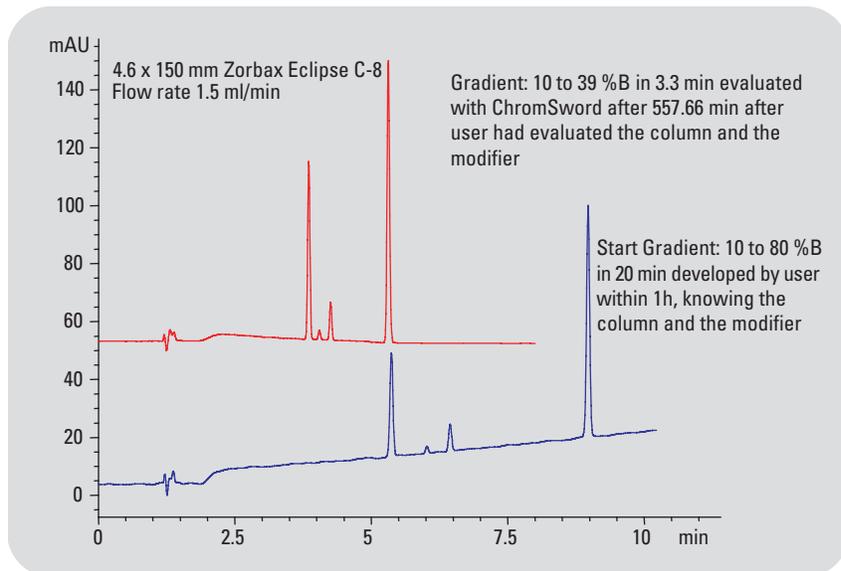


Figure 17
The benefit of the ChromSword software in this case is that the user found the starting conditions and that the software further optimizes the gradient.

Experiment 3: Application development of antiasthmatic Drugs using ChromSword

To test the application development of antiasthmatic drugs with ChromSword already known separation conditions have been used as reference. The reference chromatogram is shown in figure 18.

Water: Acetonitrile 92:8 v/v
 Flow: 1 mL/min
 Temperature: 50 °C
 UV Detection: 270 nm standard cell
 Column: ODS Hypersil, 5 µm, 125 x 4 mm

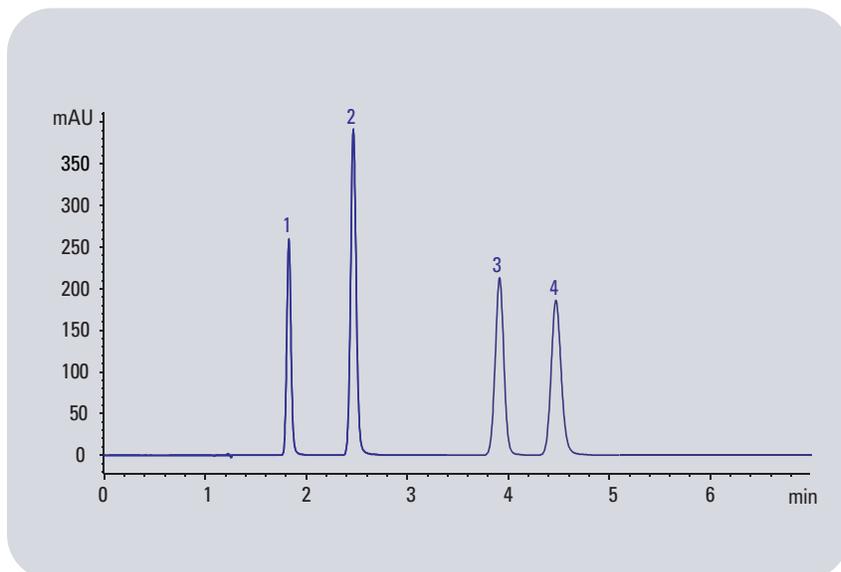


Figure 18
Reference chromatogram: Theobromine (1), Theophylline (2), Enprophylline (3), Caffeine (4)

In contrast to this application, Carbamazepine should have been separated in addition to Theobromine, Theophylline and Caffeine, and because of the different column id the flow rate had to be adjusted to achieve identical linear flow velocity. It was expected to obtain a solution preferably for both isocratic and gradient elution conditions. Further, the influence of temperature change between 40 °C and 60 °C should be investigated based on the obtained optimization conditions.

Optimization of isocratic and gradient elution conditions

- System components: Agilent 1100 Series system
- Sample: Theobromine, Theophylline, Carbamazepine, Caffeine
- Starting conditions: Acetonitrile-Water mobile phase
- Flow rate: 0.8 mL/min
- Injection volume: 1 µL
- Zero time t(0): 0.7 min
- Gradient dwell time: 1.38 min
- Column temperature: 50°C
- UV detection: 270 nm
- Column: ZORBAX SB C18, 5 µm, 150 x 3 mm

Acetonitrile: Water 20:80 v/v
 Flow: 0.8 mL/min
 Temperature: 50°C
 UV Detection: 270 nm standard cell
 Time required for automatic optimization: 9 h overnight

All analytes had been prepared as individual standards and positioned in the sampler at positions 1 through 4. Position 5 was the mixture. Elution Test Conditions:

- Starting concentration: 40 % ACN
- Test time and run time: 3 min
- Equilibration time between each individual run: 8 min
- ChromSword method development program: Isocratic or Gradient

Results

ChromSword delivered final results for both isocratic and gradient elution conditions. Figures 19 and 20 show the final optimized chromatograms as obtained by the method development software for both conditions.

Optimization of best temperature conditions based on both proposals

To optimize further the separation conditions the temperature had been taken into account. Here, the goal was to find out the influence of temperature to further reduce separation time and at the same time to monitor its impact on peak symmetry. ChromSword enables to use the data from previous experiments and add further ChemStation data that had been prepared in two additional runs controlled by the ChemStation only. These two additional runs used the ChromSword gradient condition already obtained except that the temperature set point was changed from 50°C to 40°C and 60°C.

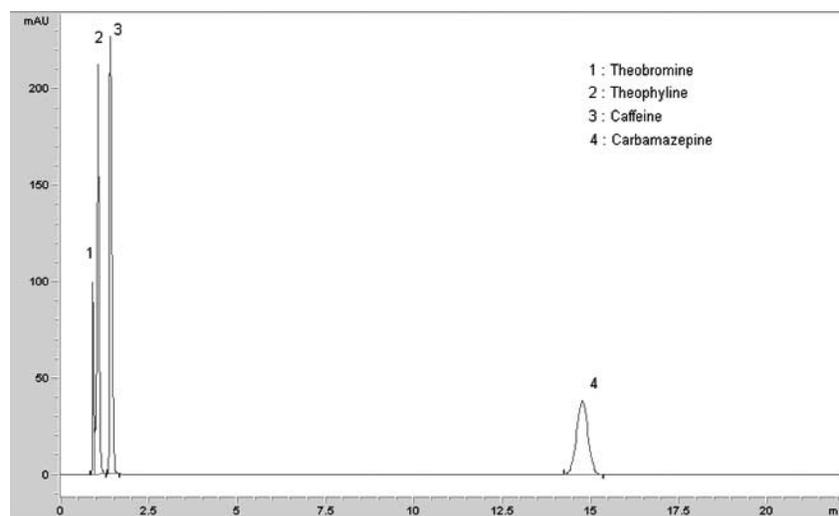


Figure 18
 Optimized chromatogram under isocratic conditions based on ZORBAX StableBond column

Gradient Profile:

(Flow 0.8 mL/min, Solvent A: Water,
Solvent B: Acetonitrile)

Step #	Time (min)	% A	% B
1	0.0	90	10
2	0.3	90	10
3	0.7	83	17
4	1.4	83	17
5	1.8	55	45
6	6.4	55	45
7	11.0	55	45

The new data (based on retention time and peak width) were added to the ChromSword table called Concentration and Temperature. Having entered all additional information, ChromSword was used offline to find out new separation conditions that would give best symmetry at shortest run time. The chromatograms in figure 21 show that temperature has no significant impact on the total separation. Conditions used at 40°C do not differ dramatically whereas 60°C does not yield a significant education in retention times. These results offered the opportunity to look for different decision-making criteria to identify the overall best separation conditions. Eventually the separation conditions at 60°C were found to be optimal, see figure 22, because it yielded the best signal-to-noise ratio in addition to shortest analysis time. When temperature impacts column longevity, lower temperature conditions should be used. In this case high temperature was not an issue, because of the excellent stability of ZORBAX columns at high temperatures.

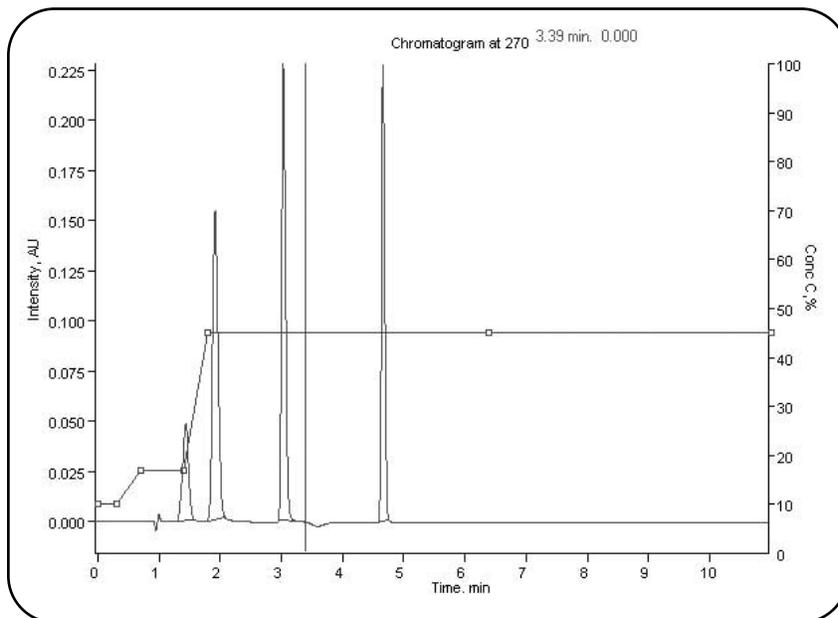


Figure 20
Optimized chromatogram gradient conditions

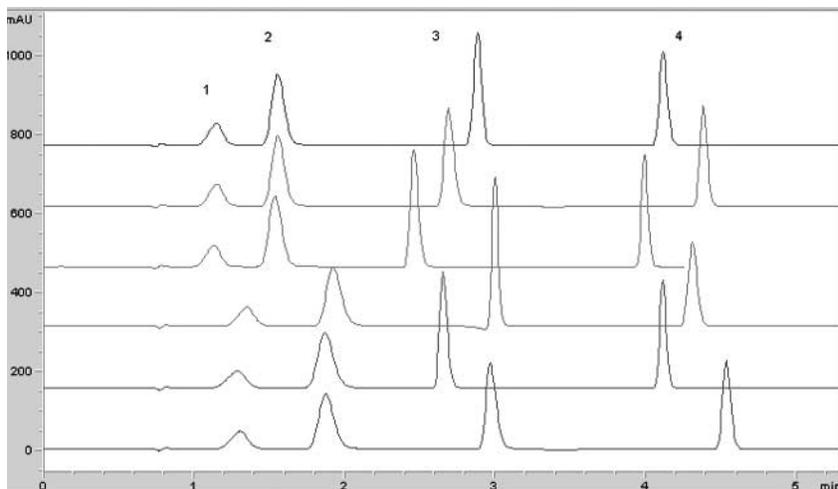


Figure 21
Chromatograms at different temperatures

Acetonitrile-Water 10 to
84 % Acetonitrile in 4.1 min
Flow: 0.8 mL/min
Temperature: 60°C
UV Detection: 270 nm standard cell

Conclusion

The chromatographic method development software ChromSword can be used for unattended optimization of reversed-phase, normal-phase and ion-exchange separations. During the procedure the composition of the mobile phase, its pH and the column temperature can be optimized. This can be done either online in combination with Agilent 1100 Series HPLC systems and Agilent ChemStation software, or alternatively offline. When used in offline mode ChromSword performs virtual chromatography using the structural formulas of the compounds investigated and the software's database of more than 60 reversed-phase columns and several mobile phases. In online mode real experiments are performed whereby the separation is optimized using the resulting chromatographic parameters. As a first step the structures of the different substances have to be determined. The ChromSword software then automatically calculates a first guess for column, mobile phase and temperature.

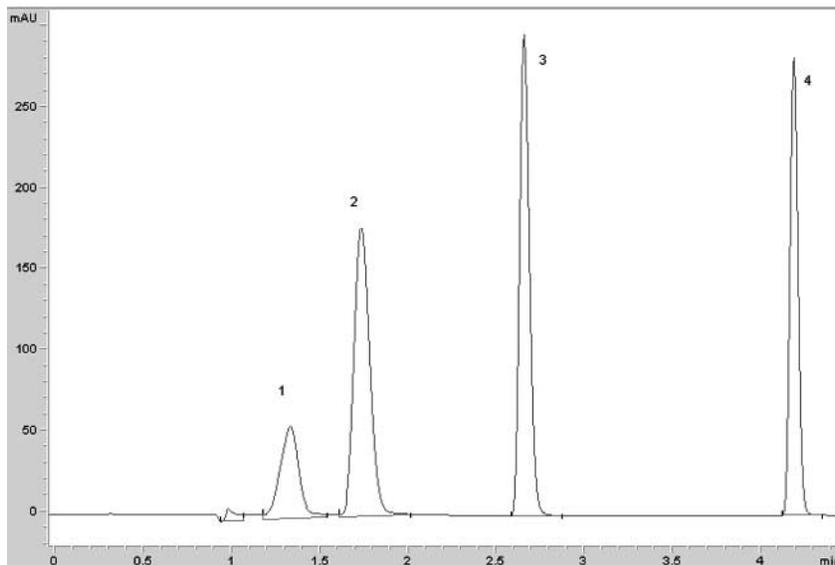


Figure 22
Final chromatogram at 60°

Different optimization experiments follow and finally a report is generated, showing all the results and including the most successful conditions in terms of separation and analysis time. The constructed data set can then be used for further optimization either virtually or by real experiments.

The ChromSword software can achieve fast and unattended optimization of separations in combination with complete control of the important chromatographic parameters and offers the opportunity to simulate chromatograms for further optimization, making it an invaluable tool for both chromatographic beginners and experts.

Contacts and Links

For more information about ChromSword, contact Sergey Galushko by e-Mail at galushko@t-online.de

ChromSword is distributed in Europe by Agilent Technologies Sales & Services GmbH & Co KG Project Services Organization Europe
pso_europe@agilent.com
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*Sergey Galushko is owner of Dr. Galushko Software Entwicklung, Mühlthal Germany
Ralf Honsberg is Requirement Analyst for Data Systems, Angelika Gratzfeld-Huesgen is Application Chemist and Bernd Hoffmann is Research and Development Chemist, all at Agilent Technologies GmbH, Waldbronn, Germany.*

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