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Software-Assisted Method Development for SFC and UHPLC on the Agilent 1260 Infinity II SFC/UHPLC Hybrid System

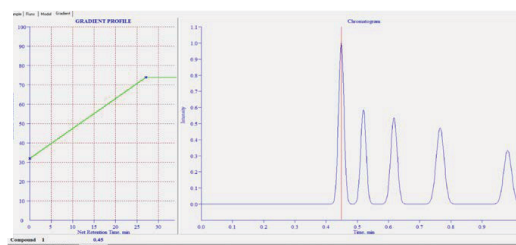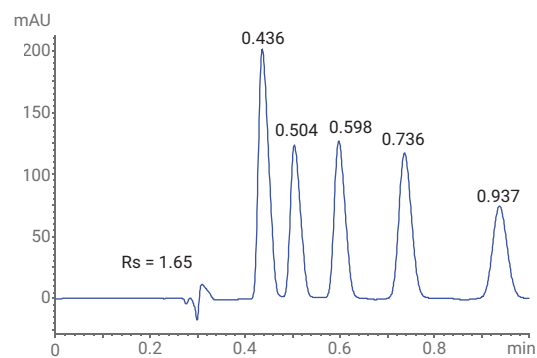## Separation of Illicit Synthetic Colorants Using Supercritical Fluid Chromatography

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Abstract

This Application Note demonstrates the approach of software-assisted method development to shorten the development time for the SFC and UHPLC parts of a method for the same sample using an Agilent 1260 Infinity II SFC/UHPLC Hybrid System.

Introduction

Method development in supercritical fluid chromatography (SFC) presents special challenges because the setting of the initial conditions involves the selection from a wide variety of stationary and mobile phases. All stationary phases available for HPLC, and any solvent miscible with carbon dioxide (and compatible with the stationary phase), may be selected for the method development process in SFC.

Knowledge of the retention behavior in the liquid phase is often of no help because the absence of water in the mobile phase causes drastic differences in behavior when comparing reversed-phase UHPLC and SFC[1]. Thus, only SFC studies are helpful when developing an SFC method.

This Application Note describes an efficient way for SFC method development using software that also supports UHPLC method development. The most popular illicit synthetic colorants, Sudan Red I–IV and Para Red, were chosen as samples for the evaluation (Figure 1).
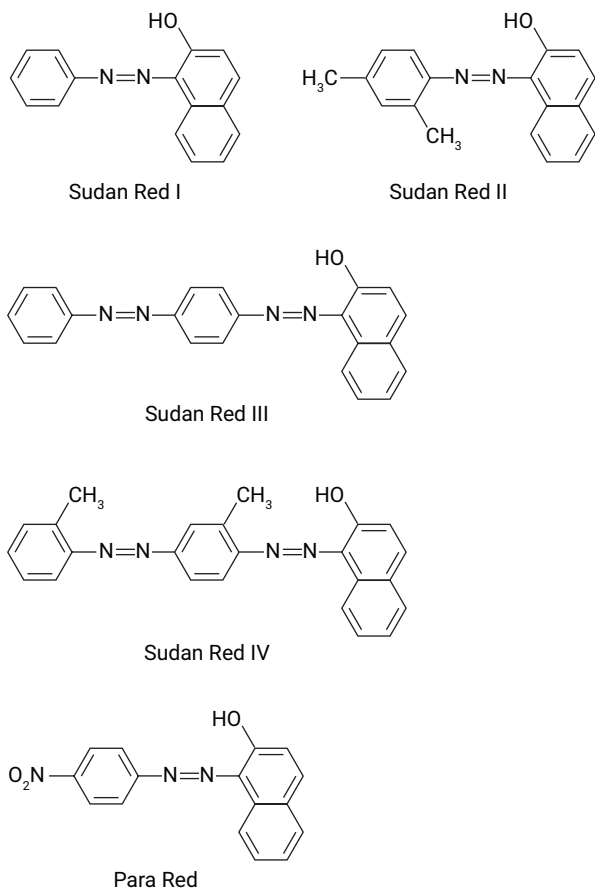

Figure 1. Chemical structures of illicit synthetic colorants.

Experimental

Instrumentation

An Agilent Infinity II SFC/UHPLC Hybrid System comprising the following modules:

- Agilent 1260 Infinity II SFC Control Module (G4301A)
- Agilent 1260 Infinity II SFC Binary Pump (G4782A)
- Agilent 1260 Infinity II SFC Multisampler (G4767A)
- Agilent 1260 Infinity II LC Quaternary Pump (G1311B)
- Agilent 1260 Infinity II Diode Array Detector (DAD) (G7115A) with a high-pressure SFC flow cell
- Agilent 1290 Infinity Valve Drive (G1170A) with a 2-position/10-port valve (G4232B)
- Agilent 1260 Infinity II Column compartment (G1316A)

The setup of the Agilent 1260 Infinity II SFC/UHPLC Hybrid System was described earlier in more detail[2].

Column

Agilent ZORBAX SB C-18 (4.6 × 150 mm, 5 µm)

Software

- Agilent OpenLAB Chromatography Data System, ChemStation Edition (C.01.07) for data acquisition
- ChromSword Auto 4.0 software for method optimization

Instrument Conditions

UHPLC Mode	
Flow rate	1 mL/min
Mobile phase A	Water
Mobile phase B	Acetonitrile, methanol, or isopropanol (IPA)
Compositions	80, 85, 90, 95, or 100 %B (isocratic)
Column temperature	40 °C
DAD	Wavelength 480 nm, data rate 20 Hz
Multisampler	Flowthrough injection Needle wash: 3 seconds with methanol
SFC Mode	
Flow rate	5 mL/min
Mobile phase A	CO_2
Mobile phase B	Acetonitrile, methanol, or IPA
Compositions	2, 5, 10, 20, or 40 %B (isocratic)
BPR	160 bar
BPR temperature	60 °C
Column temperature	40 °C.
DAD	Wavelength 480 nm, data rate 20 Hz
Multisampler	Feed injection Feed speed: 400 µL/min Overfeed volume: 4 µL Needle wash: 3 seconds with methanol

Chemicals

- Sudan I: Wako Chemical, 193-14131
- Sudan II: Wako Chemical, 190-14141
- Sudan III: Wako Chemical, 197-14151
- Sudan IV: Wako Chemical, 194-14161
- Para Red: Wako Chemical, 160-22171

Solvents

All solvents were purchased from Merck, Germany. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with an LC-Pak Polisher and a 0.22-μm membrane point-of-use cartridge (Millipak).

Results and Discussion

SFC separation

To test the SFC separation mode of the Agilent 1260 Infinity II SFC/UHPLC Hybrid System, the modifier solvent was first optimized. Figure 2 shows typical chromatograms of the illegal synthetic colorants for the different tested modifiers. The retention time (RT) of the compounds were shifted to earlier elution by a stepwise increase of the content of modifier (the start and end of the optimization is shown in Figure 2, and the method parameters are described in the Experimental section). The column used for all tested conditions was an Agilent ZORBAX SB C-18 column, typically used for reversed-phase separations. The results of the optimization showed that methanol at 20 % is the most suitable modifier for CO_2 to separate these dyes using a ZORBAX SB C-18 column.
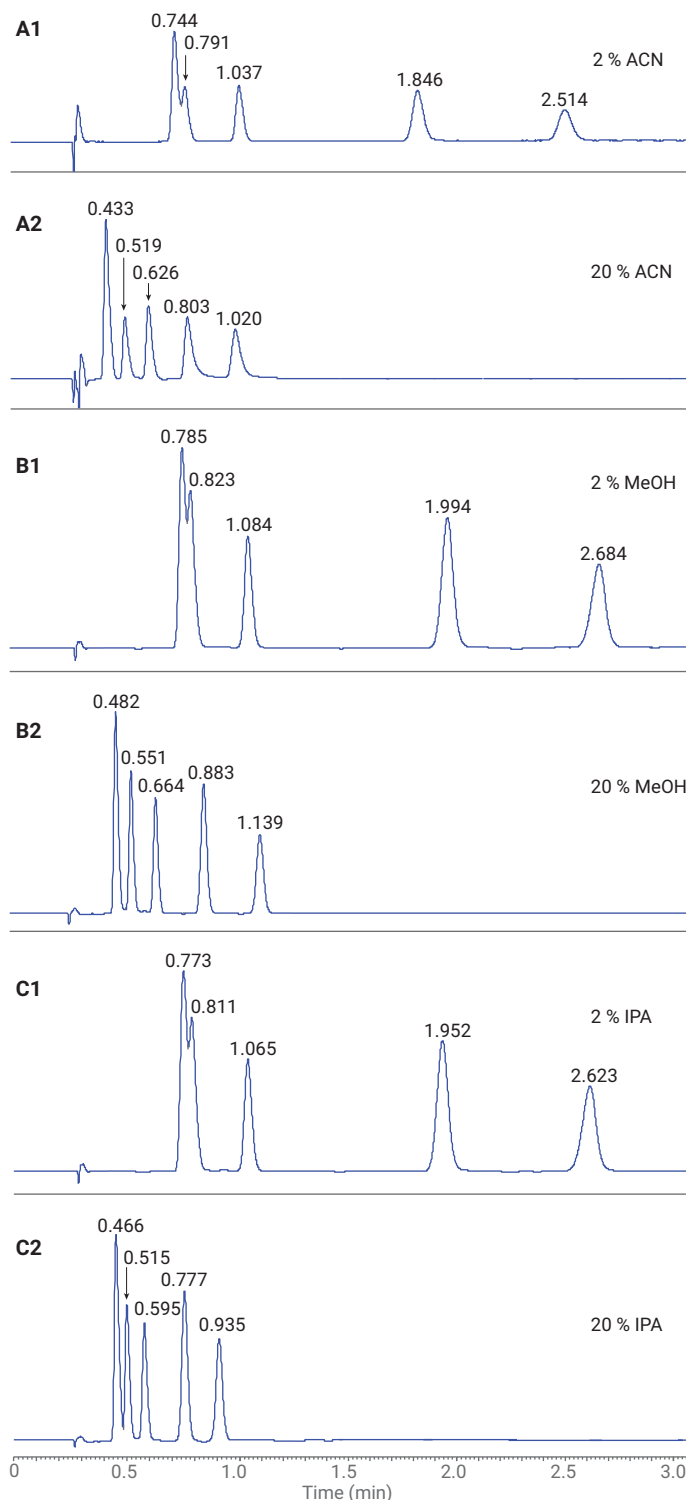

Figure 2. SFC chromatograms for illicit synthetic dyes. The SFC conditions were: (A1) 2 % ACN, (A2) 20 % ACN, (B1) 2 % MeOH, (B2) 20 % MeOH, (C1) 2 % IPA, and (C2) 20 % IPA.

UHPLC Separation

Figure 3 shows the chromatograms of the illegal synthetic colorants separated on the 1260 Infinity II SFC/UHPLC Hybrid System in UHPLC mode. The RT of the compounds increased when the content of organic solvent in the composition was decreased (the method parameters are described in the Experimental section). In this case, the separation was done in reversed-phase UHPLC mode on the 1260 Infinity II SFC/UHPLC Hybrid System with a ZORBAX SB C-18 column.

Figures 2 and 3 show that the SFC and UHPLC modes separated the same sample using the same ZORBAX SB C-18 column. The compound elution order was the same for both UHPLC and SFC modes, but with the burden of an increased effort in method development for SFC mode. To overcome this, we tried to optimize and speed up the development of the SFC separation method using ChromSword Auto simulation software.

ChromSword Auto Simulation for SFC Optimization

To optimize the method, step and linear gradient chromatography were simulated *in silico* with ChromSword Auto simulation software to obtain a peak resolution (Rs) of more than 1.5 for the Para Red and Sudan Red I peaks. Figure 4 shows some of the SFC conditions indicated for sufficient resolution for these two substances. The calculated steep step gradient (Figure 4A1) and the real application of this gradient (Figure 4B1) showed sufficient calculated resolution of >1.5, and a measured resolution of 1.73 using methanol as a modifier. Another *in silico* simulation was done for a linear gradient separation. The simulated results calculated a resolution >1.5 (Figure 4A2), and showed that the measured results gave a resolution of 1.65 using methanol as a modifier (Figure 4B2). All the simulated results matched the measured results.
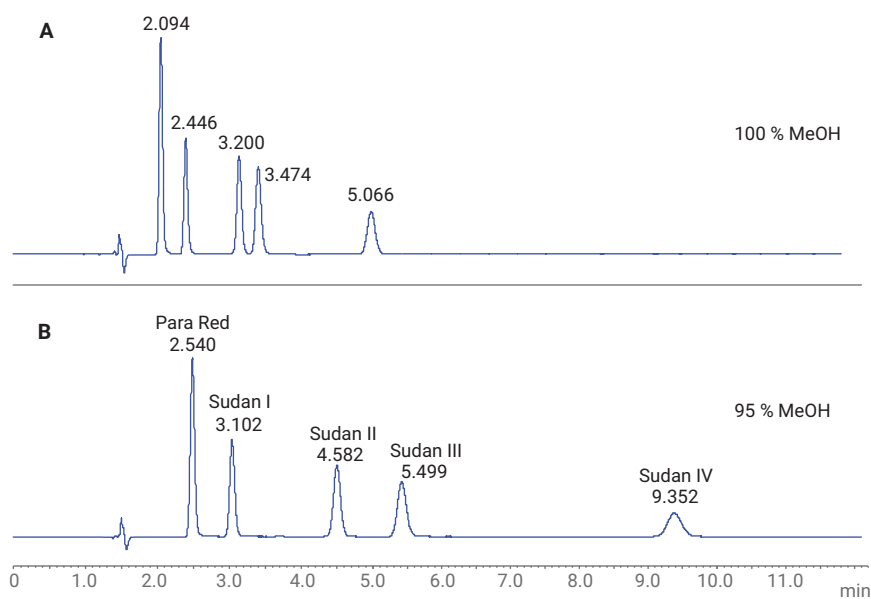

Figure 3. UHPLC chromatograms for illicit synthetic dyes. The UHPLC conditions were: (A) 100% MeOH, and (B) 95 % MeOH.
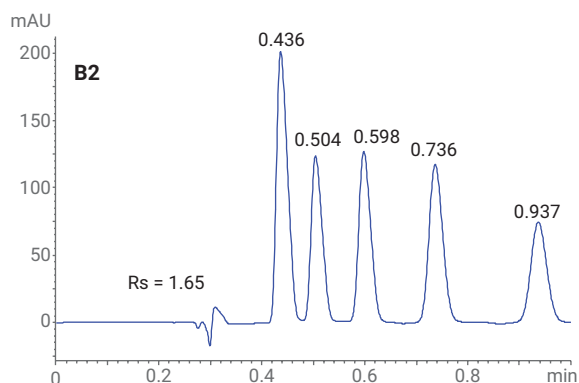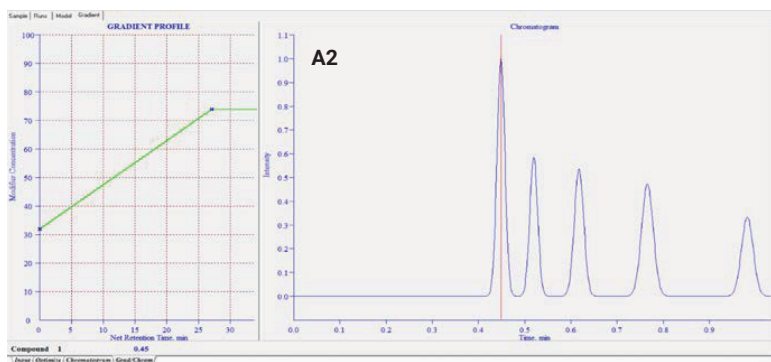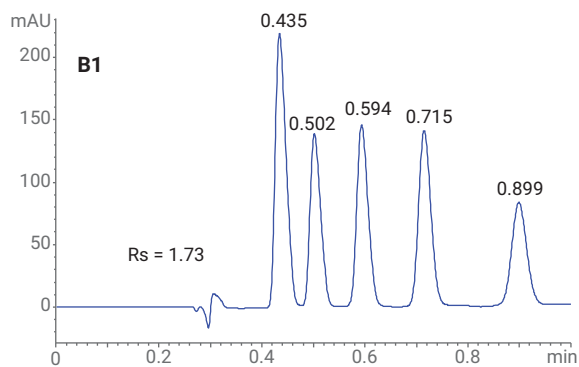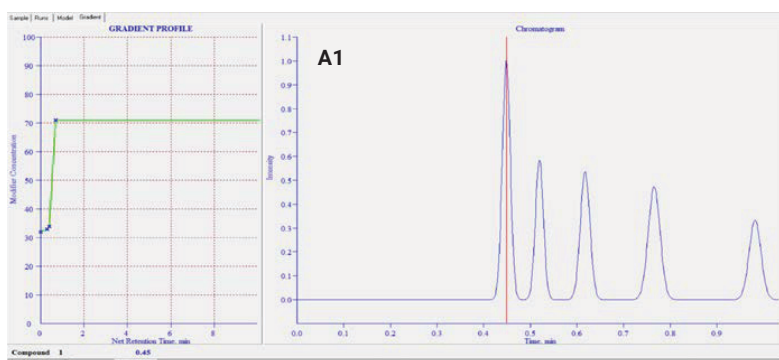
Figure 4. Comparisons of simulated results (A1 and A2) and measured results (B1 and B2) for SFC separation using step and linear gradients with methanol as modifier. The gradient conditions were: (A1) 32 % at 0 minutes, 32 % at 0.4 minutes, 71 % at 0.7 minutes, 71 % at 1.5 minutes, and (A2) 32 % at 0 minutes, 40 % at 5 minutes. The Rs values were calculated with Para Red (B1: 0.435 minutes, B2: 0.436 minutes) and Sudan Red I (B1: 0.502 minutes, B2: 0.504 minutes).

Figure 5 shows the comparisons of simulated and measured results using IPA as a modifier. Sufficient simulated resolution (>1.5) was not obtained using IPA as a modifier. The measured resolution between the Para Red and Sudan Red I peaks was approximately 1.2, and it did not reach 1.5. The measurement showed the same result as the simulation.
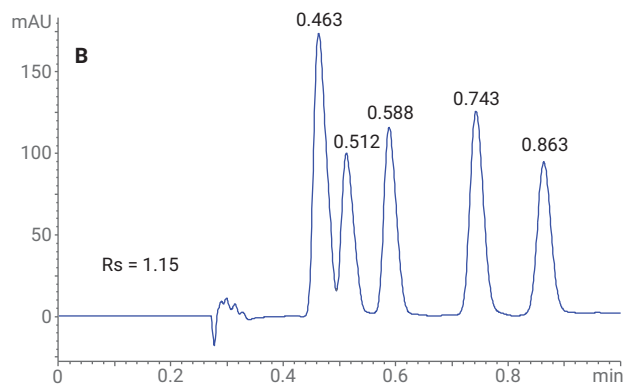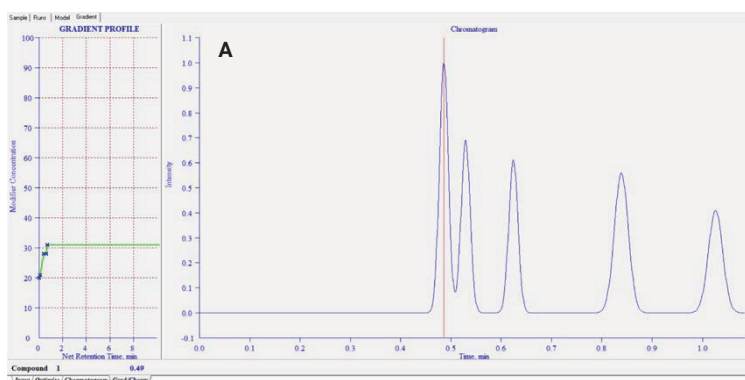

Figure 5. Comparison of simulated results (A) and measured results (B) for the SFC separation using IPA. The gradient conditions were: 20 % at 0 minutes, 31 % at 0.7 minutes, and 31 % at 2.0 minutes. The Rs value was calculated with Para Red (0.463 minutes) and Sudan Red I (0.512 minutes).

To test the method robustness, the simulation was examined under isocratic conditions to confirm its tolerances. Figure 6 shows the simulated isocratic conditions and the real measurements. The calculated results indicated that compositions of 15 to 45 % methanol modifier could separate Para Red and Sudan Red I with a resolution of >1.5 (Figure 6A). The results were reproduced with measurements for modifier concentration of 5 to 40 %, which show a maximum resolution for Para Red and Sudan Red I at modifier concentration of 25 and 30 % (Figure 6B).

This leads to the conclusion that ChromSword Auto software provides a powerful tool for method development in SFC analysis.
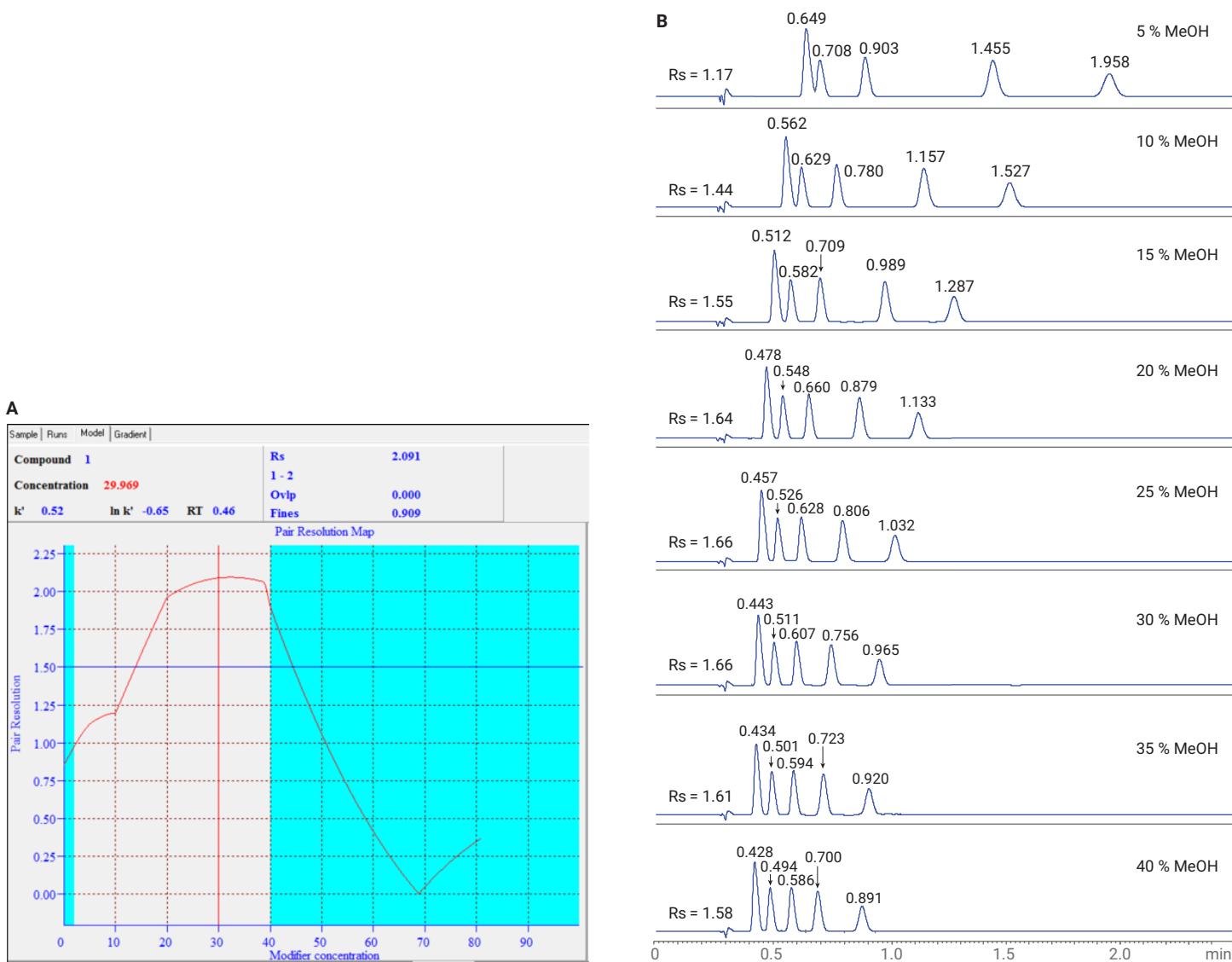

Figure 6. Comparison of simulated results (A) and measured results (B) for SFC separation with methanol under isocratic conditions. The Rs values were calculated for Para Red and Sudan Red I.

Conclusion

Developing SFC methods often requires extra effort to find the best separation conditions. It was demonstrated that method development could be improved dramatically using the Agilent 1260 Infinity II SFC/UHPLC Hybrid System with ChromSword Auto software.

The 1260 Infinity II SFC/UHPLC Hybrid System was used to perform both SFC and HPLC method development. Using this unique hybrid solution, the need for an investment in two stand-alone instruments has been eliminated, which saves significant costs and conserves laboratory space.

References

1. Lesellier, E. Retention mechanisms in super/subcritical fluid chromatography on packed columns, *J. Chromatog. A* **2009**, *1216*, 1881–1890.

2. Naegele, E. Orthogonal Chromatographic Separations using the Agilent 1260 Infinity II SFC/UHPLC Hybrid System, *Agilent Technologies Technical Overview*, publication number 5991-8276EN, **2017**.