Peptide Mapping

Hardware and Column Optimization

Peptide Mapping Hardware Challenges

Very complex samples require long, shallow gradients

Mobile phase components require mixing, however delay volume within the pump needs to be low to deliver accurate gradients

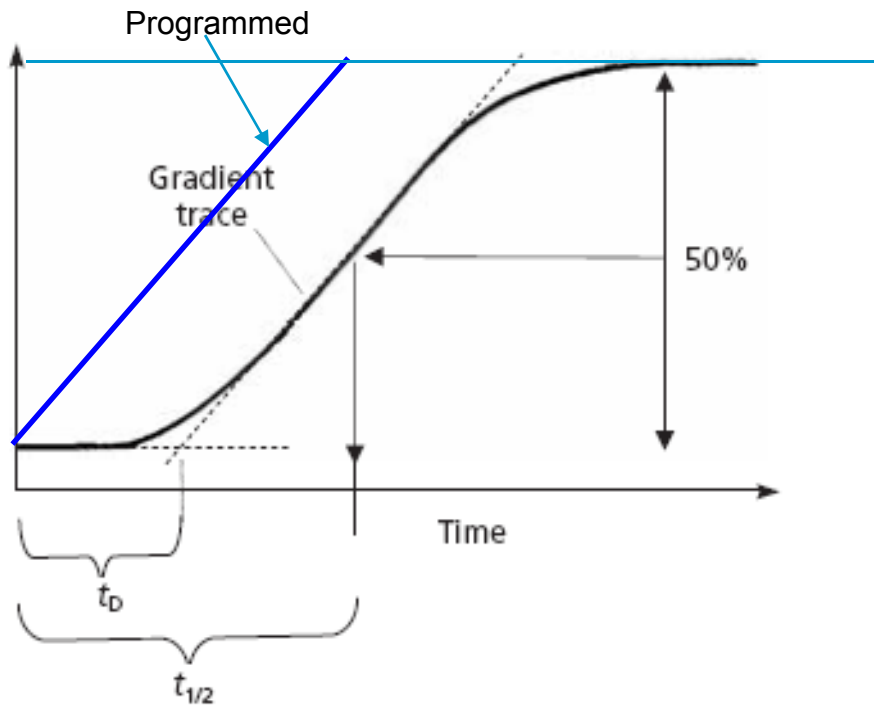
Overall system dispersion needs to be kept to a minimum to keep peaks separated

Compositional and flow accuracy and precision are needed for reproducibility of complex runs

Why is delay/dwell volume important

1. Different dwell volumes result in a RT time shift *(the time for the mobile phase to reach the column head)*
2. Different dwell volume could effect resolution *(peaks spends different time under isocratic/gradient conditions)*
3. additionally, the dwell volume effects the gradient shape *(dispersion effects, flush out behavior => the programmed gradient becomes deteriorated)*
4. Therefore even with the same “geometrical” delay volume the chromatograms could look different on different systems
5. The dwell volume has an big impact for narrow bore applications, especially combined with fast gradient

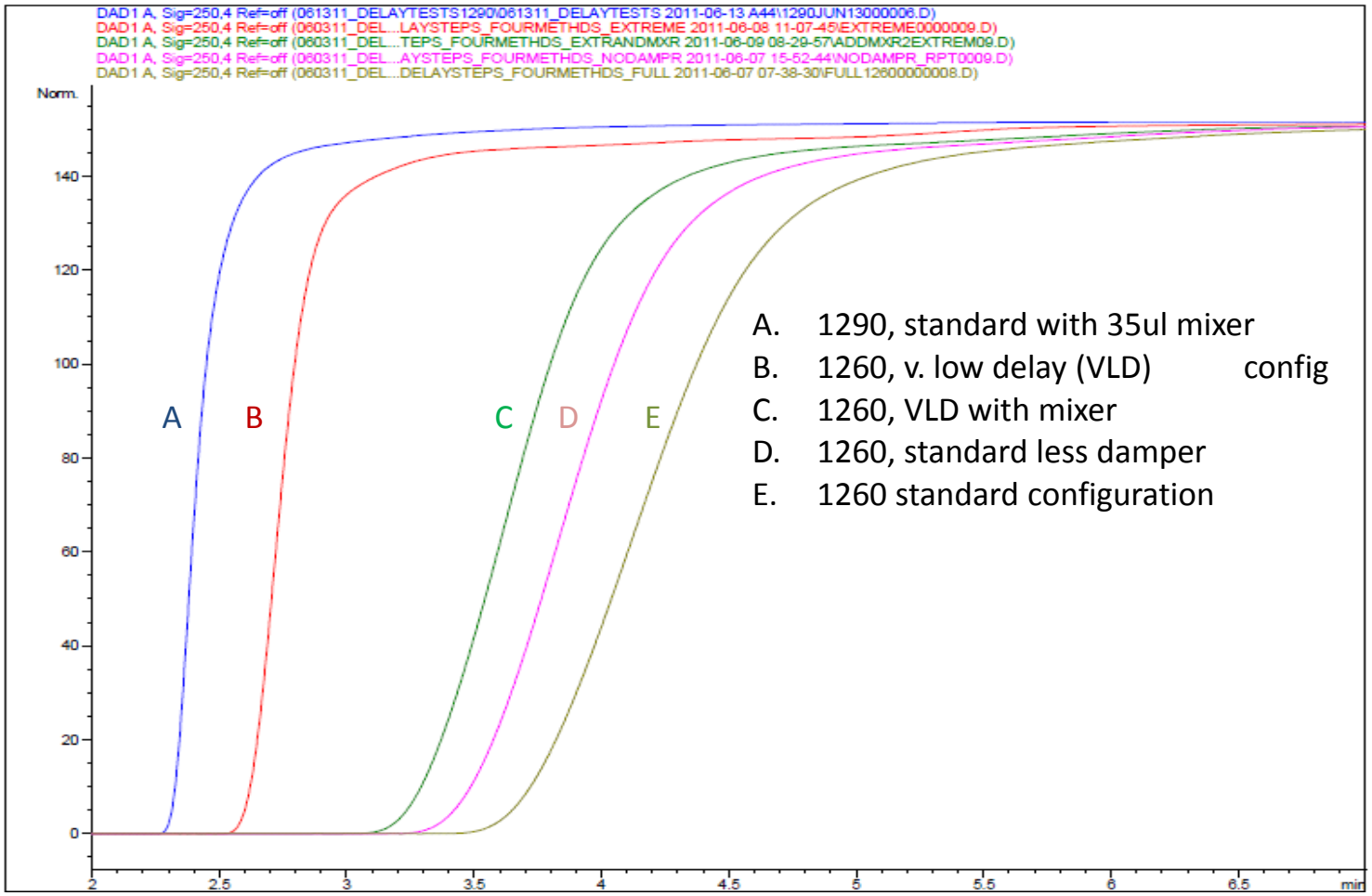
Dwell volume determination



W. Dolan LCGC Vol 24, No 5, 458-466

The system setup is simple. Use water for the A-solvent and water spiked with 0.1% acetone for the B-solvent. Replace the column with ≈ 1 m of 0.005-in. i.d. tubing, set the detector to 265 nm, and set the flow rate so that there is sufficient backpressure for reliable check valve operation (for example, 2 mL/min). Run a 0–100% B gradient in 20 min. The data system output should be a curve similar to the one below. You can measure the dwell time (t_D) by drawing a tangent to the main part of the gradient curve (dashed line in Figure 4) and extend the baseline to intersect this tangent. The time it takes from the start of the program to this intersection is the dwell time. Multiply by the flow rate to get the dwell volume.

Delay Volume Profiles



A. 1290, standard with 35ul mixer
B. 1260, v. low delay (VLD) config
C. 1260, VLD with mixer
D. 1260, standard less damper
E. 1260 standard configuration

Delay Volume Variability within Agilent Systems

Configuration	Delay V* (µl)
1290 Pump	10
1290 Pump + Fixed Loop[1] (for MS)	20
1290 Pump + Jet Weaver + Fixed[1] Loop	55
1290 Pump + ALS (for MS)	75
1290 Pump + Jet Weaver[1] + ALS	110
1200 RRLC (low delay volume)	260
1200 RRLC (standard delay volume)	740-940

Agilent 1290 Infinity Quaternary Pump

Specifications & Benefits

Power Range

- **For any kind of analysis**

Composition Accuracy and Precision

< 0.15 % RSD or 0.02 min SD

± 0.4 % (1-99 % Composition B)

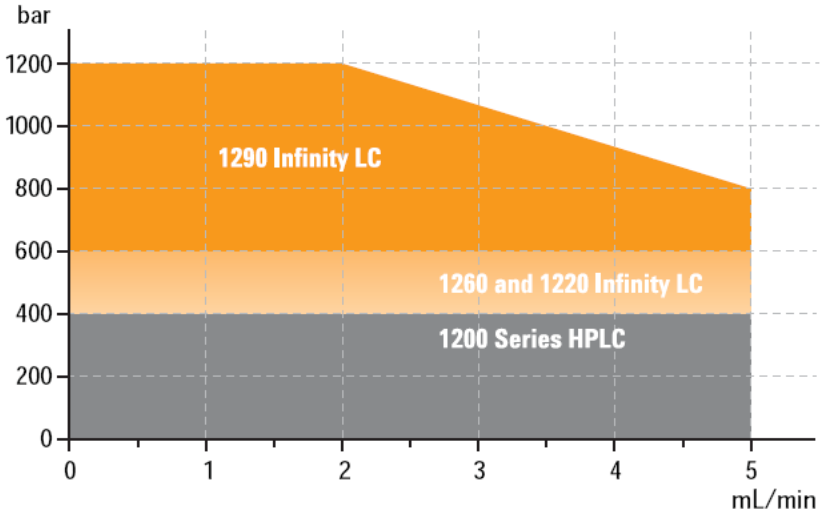
- ***High RT precision in gradient runs***

Flow Accuracy and Precision

< 0.07 % RSD or 0.01 min SD

± 1.0 % or 10 µL

- **High RT precision in isocratic runs**



Composition Range

1-99 %

- **Wide analytical range**

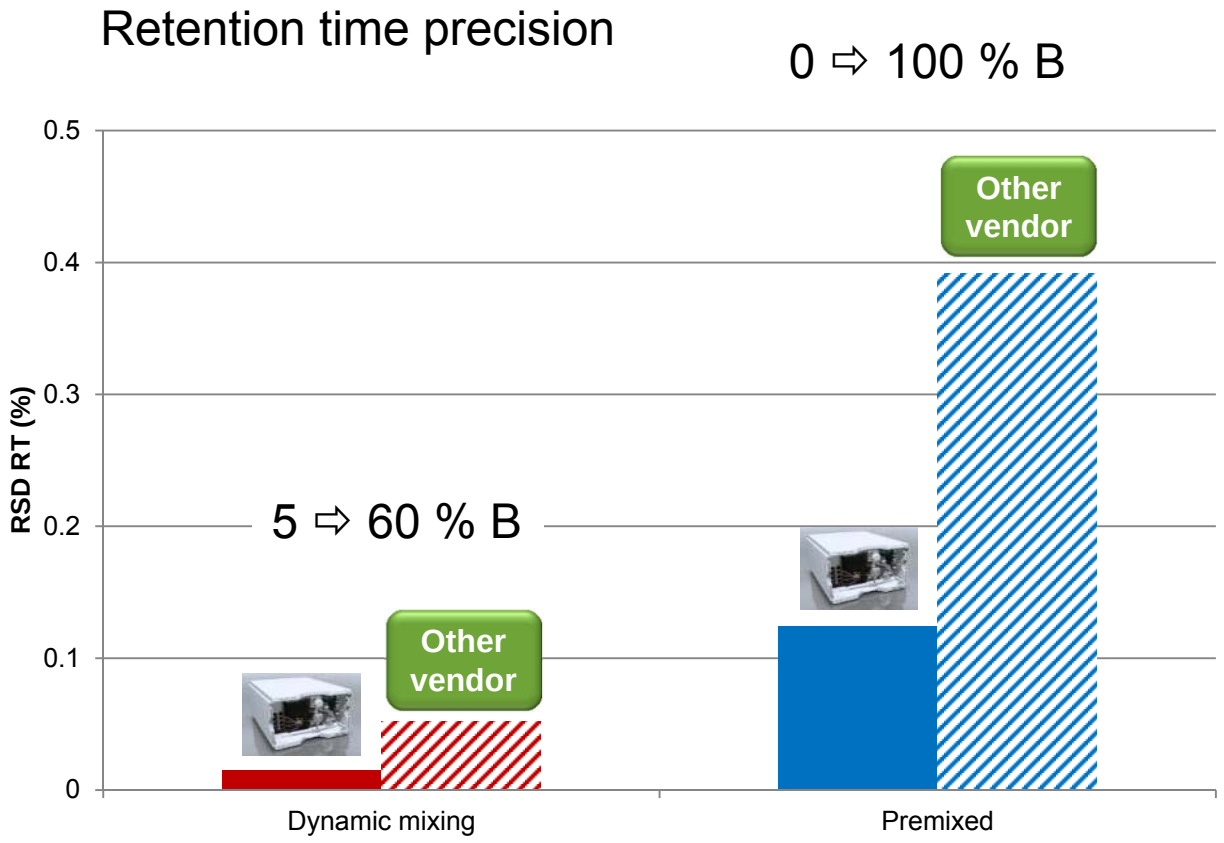
Delay Volume

< 350 µL

- **For fast quaternary gradients**

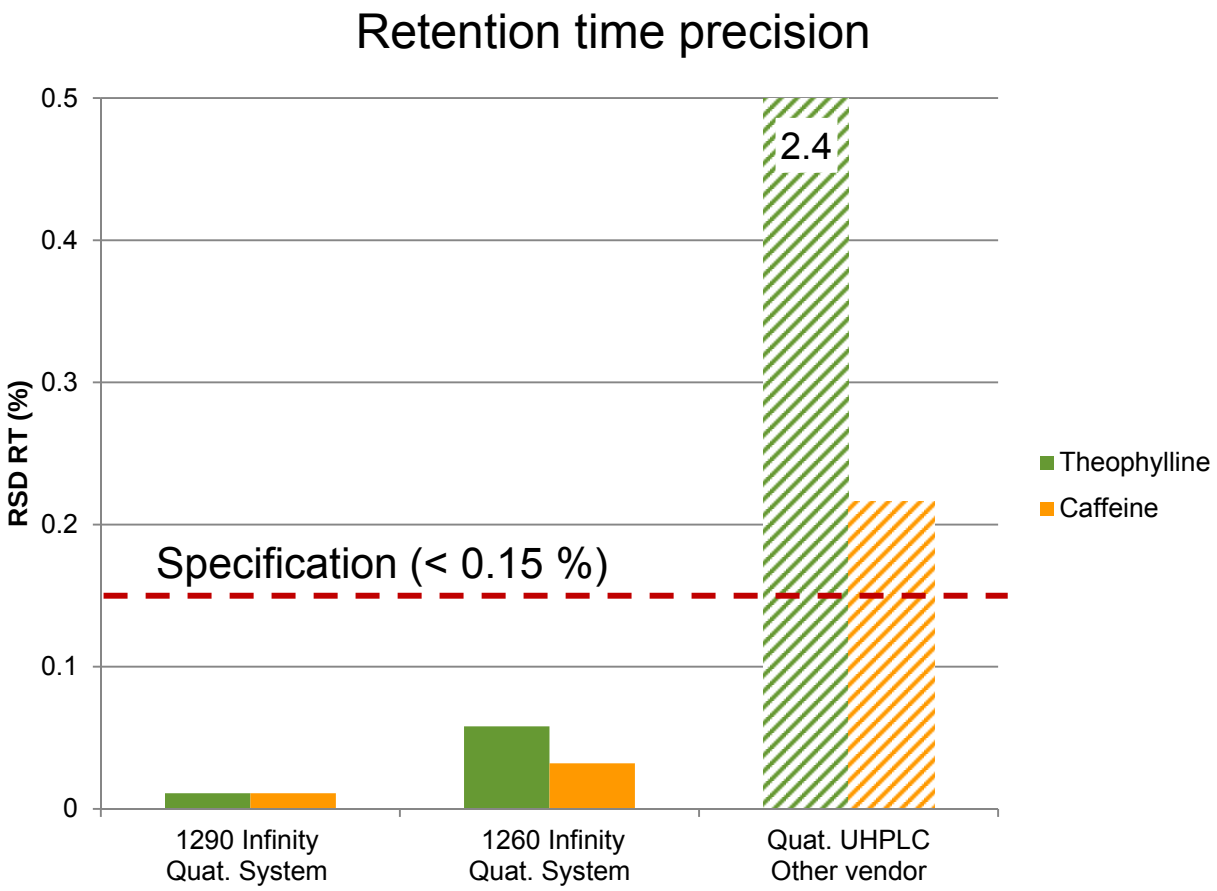
Composition Accuracy and Precision

Comparison with Quaternary UHPLC pump from other vendor



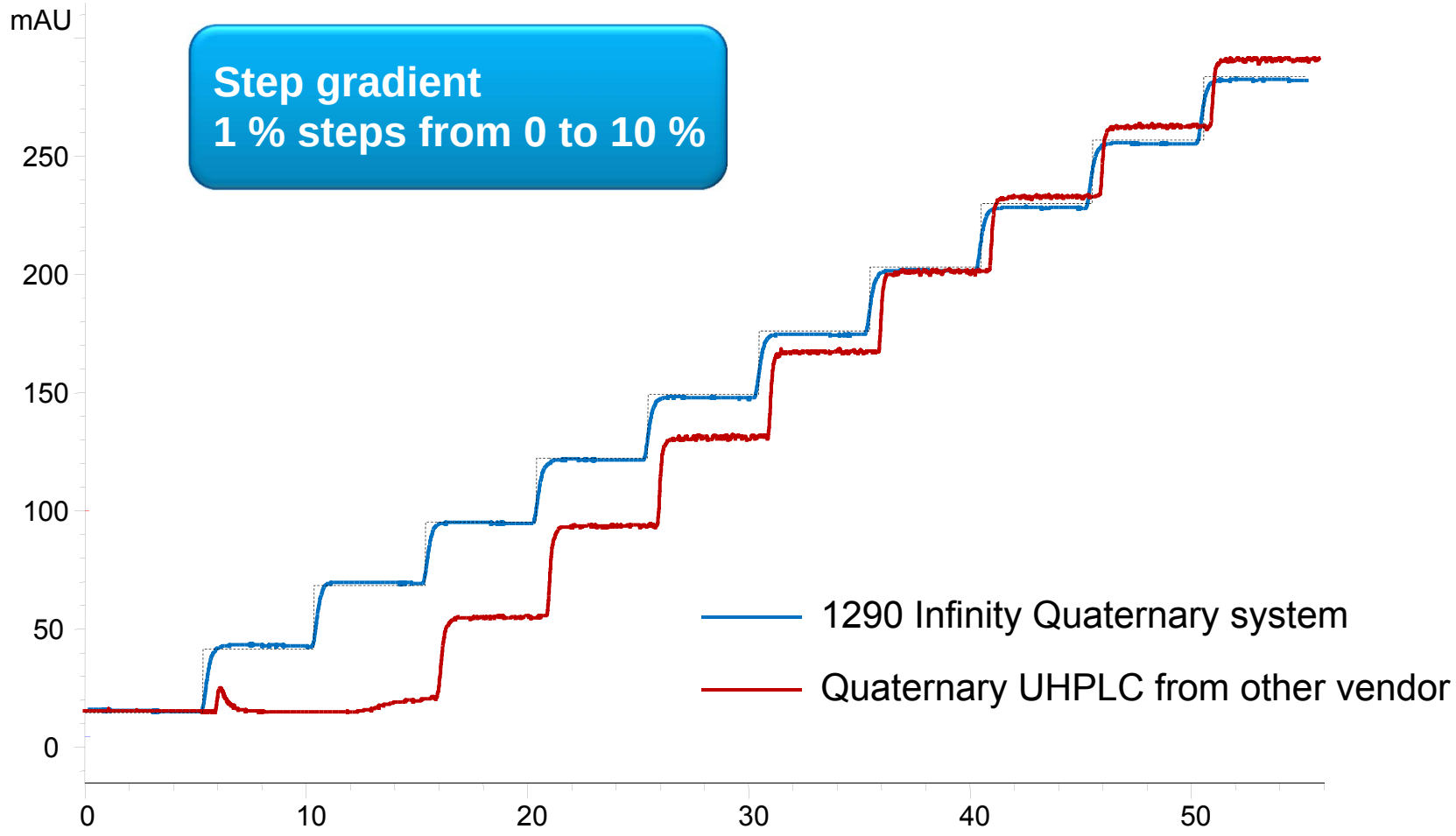
Composition Range

Shallow gradient at low organic concentration



Composition Range

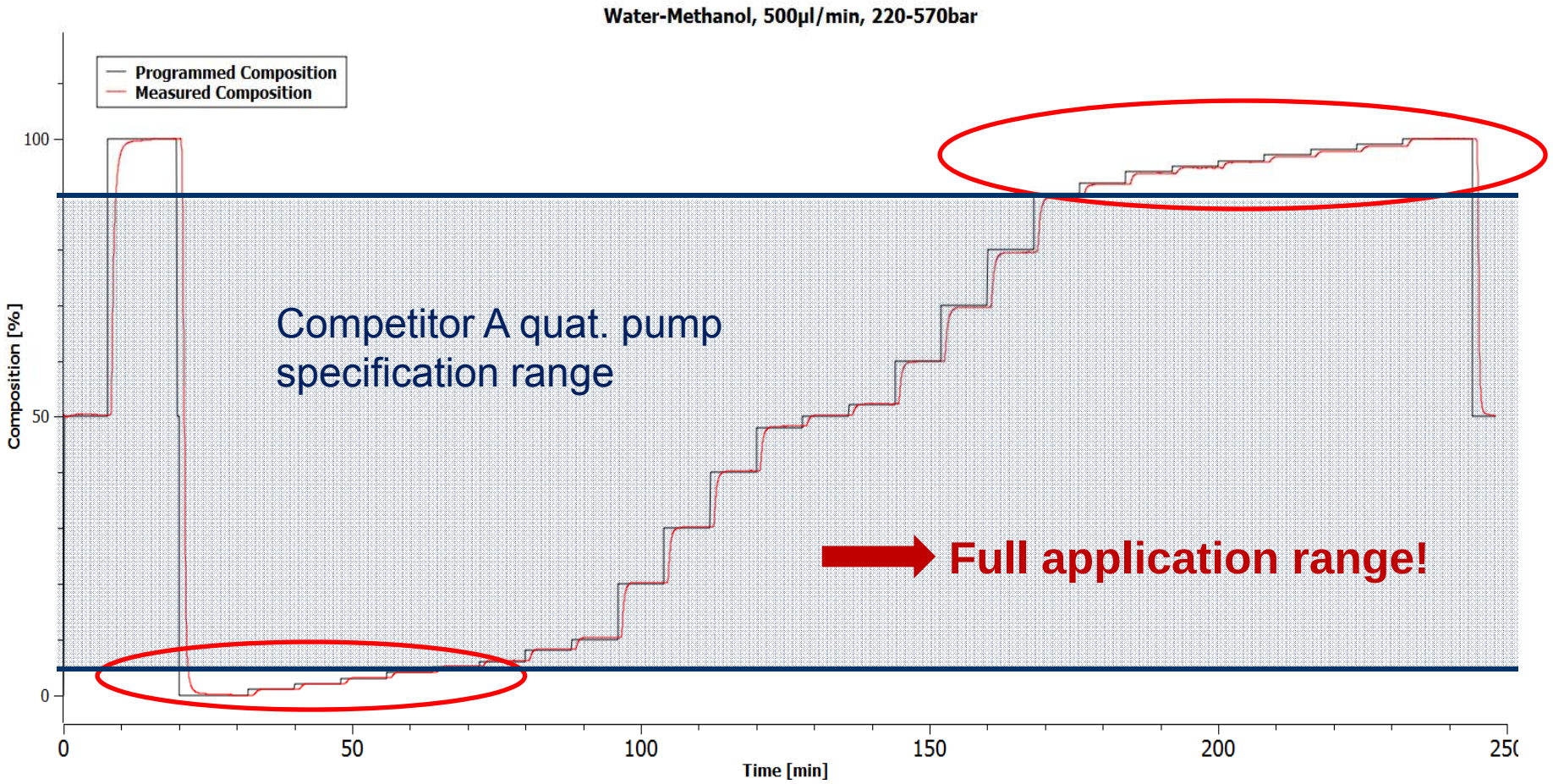
Shallow gradient at low organic concentration



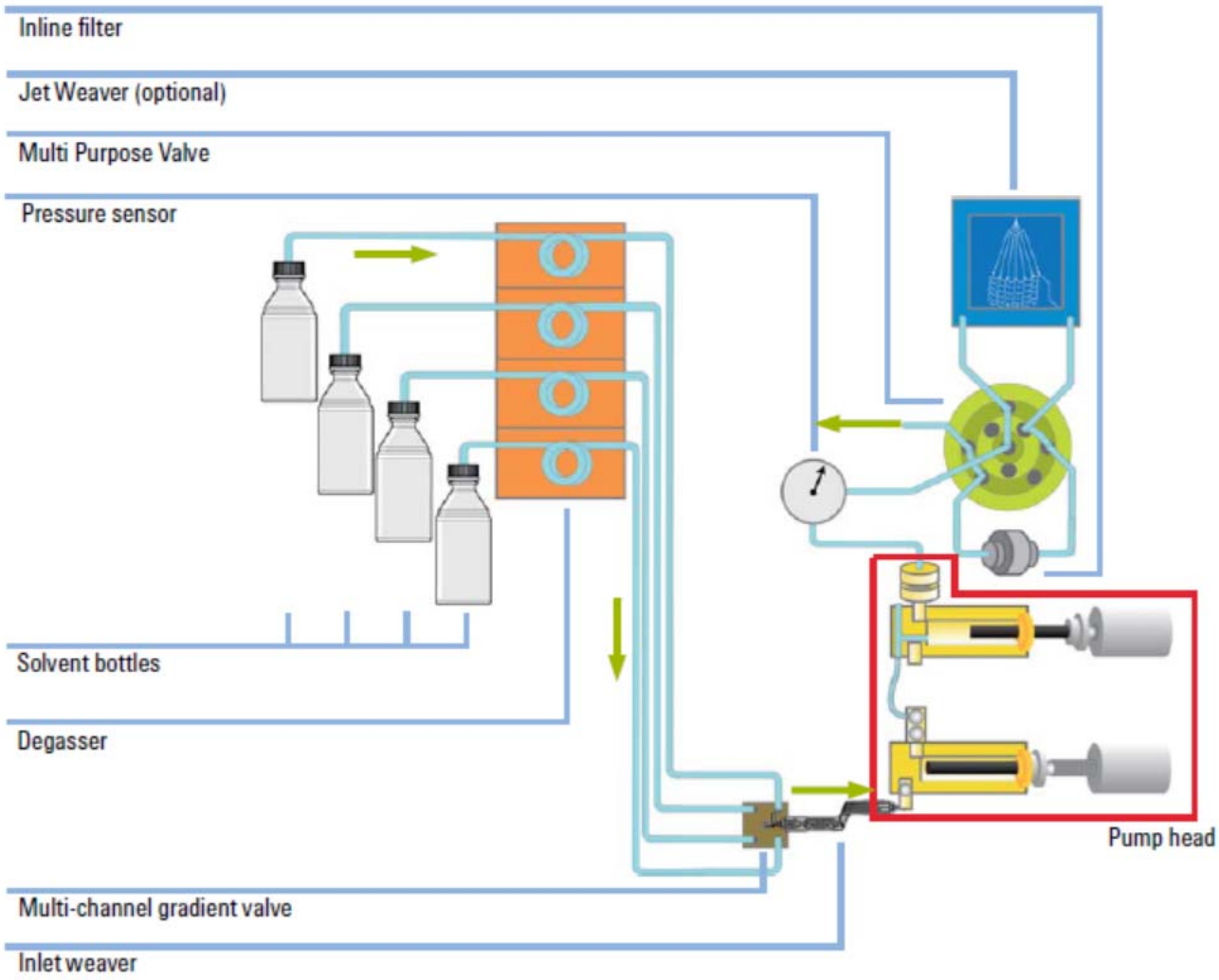
Precision & Accuracy

1st Quaternary pump with Bin-like performance

Different mixtures (H_2O, MeOH, ACN) at different pressures and different flow rates

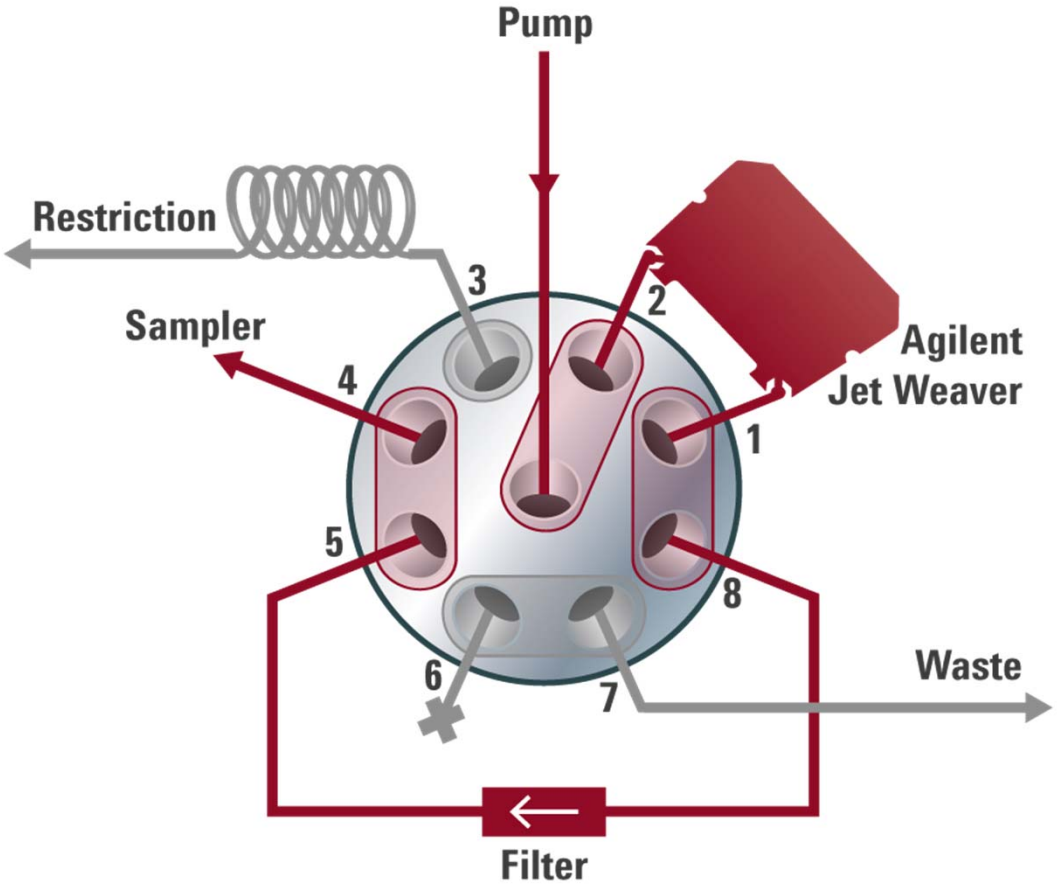


1290 Quaternary Pump Flow Diagram

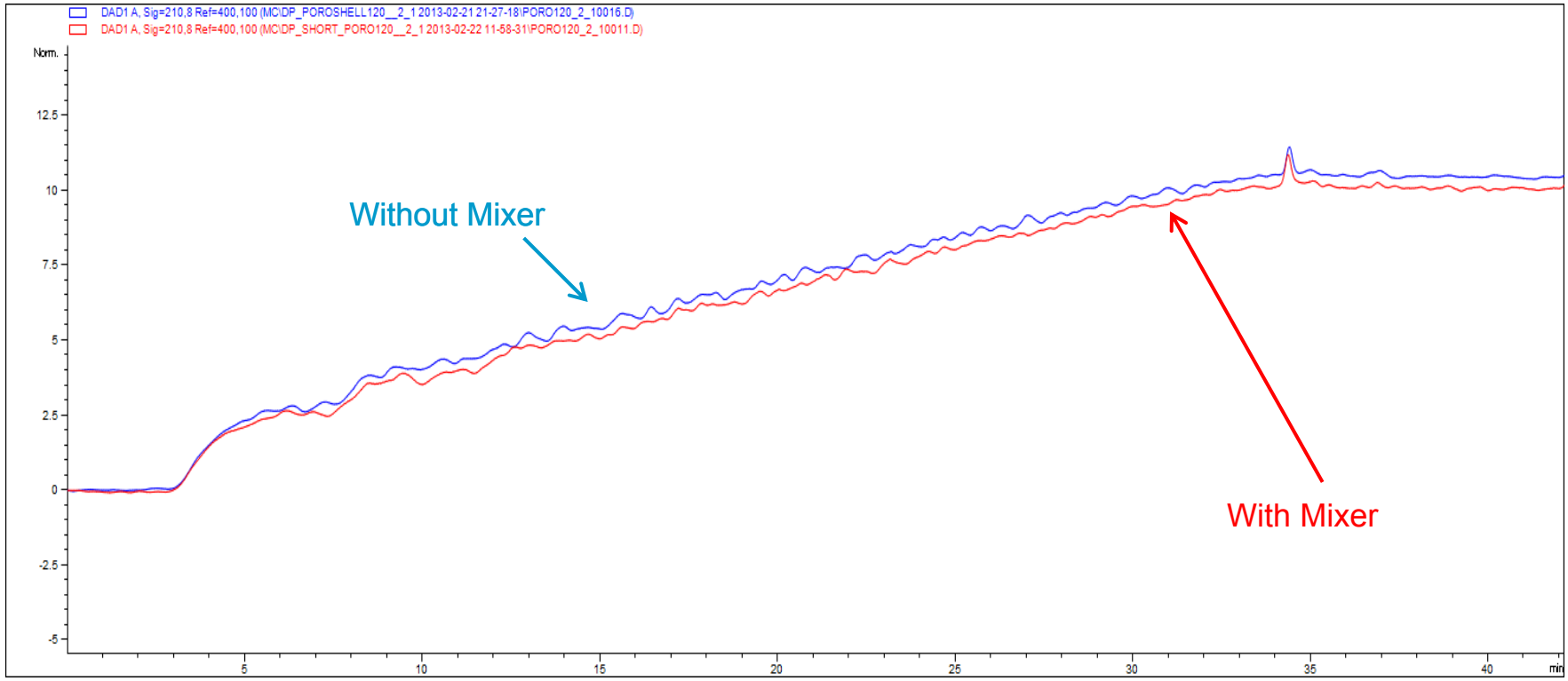


Multipurpose Valve

Optional Extra Mixing Volume for lowest baseline ripple (TFA applications)



TFA Mixing Noise:
Effect of JetWeaver Mixer: Lower Short Term Noise



Innovation: 1290 Infinity Quaternary Pump

Quaternary Pump with Binary Pump-like Performance

1290 Infinity Quaternary Pump

- **Boost Performance**

  Highest accuracy and precision for composition and flow, with exceptionally low delay volume

- **Save Time**

  Accelerates transfer of existing HPLC methods to UHPLC

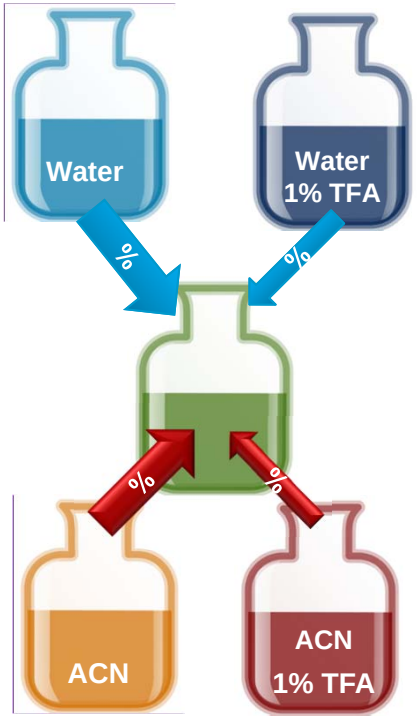
- **Reduce Costs**

  Outstanding UHPLC performance at HPLC-like costs of ownership

1290 Infinity Quaternary Pump - BlendAssist

Simple tool for online-dilution of modifiers and gradient set-up

You need different concentrations of modifiers in your analysis, would like to have just one stock-solution and do online dilution to profite from the quaternary mixing capability of your pump? Here is a simple tool – ***BlendAssist***!



Desired method conditions - example:

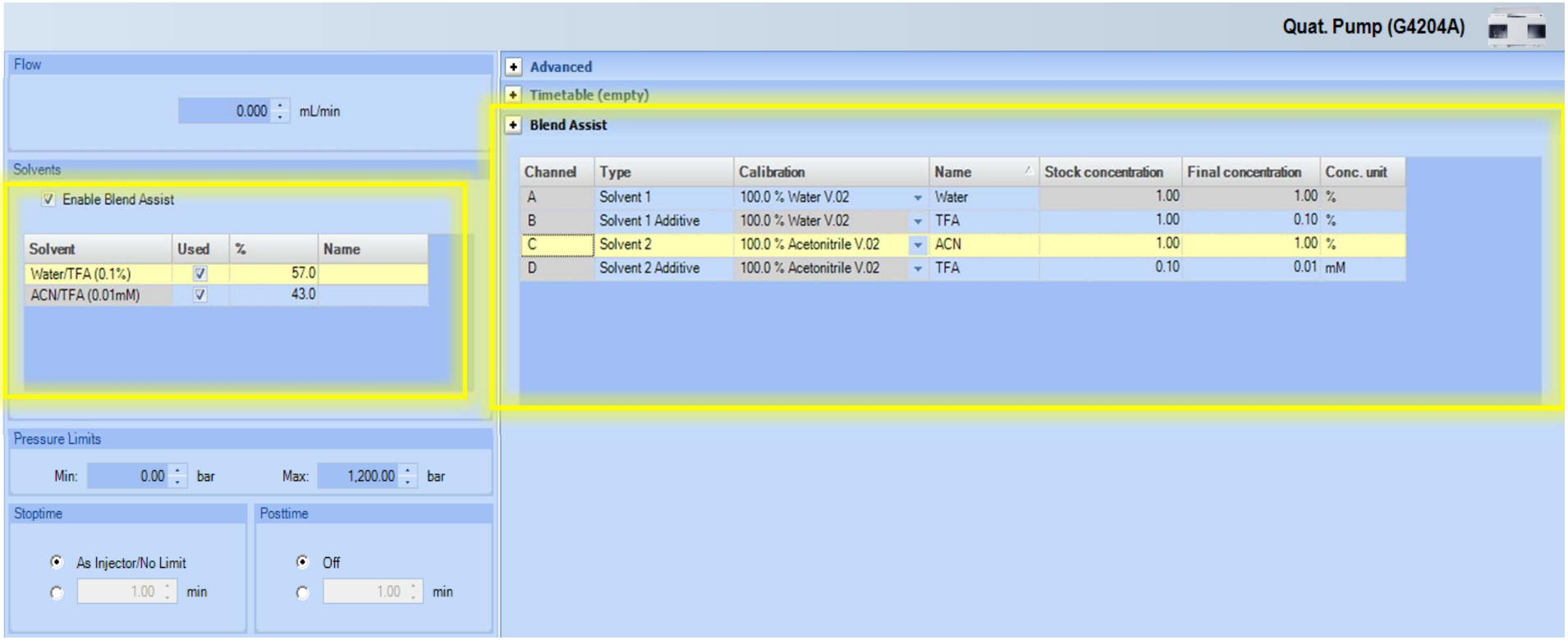
1. 5 to 95% gradient of ACN with 0.1% TFA in Water and 0.08% TFA in ACN
2. 20 – 80% gradient of ACN with 0.5% TFA in Water and 0.4% TFA in ACN

Without BlendAssist you need to either pre-mix the required solvents or by using stock-solutions of TFA in Water and ACN to program complex gradients (%A, B, C, D).

With BlendAssist: just program your binary organic/aqueous gradient and define the dilution factor!

1290 Infinity Quaternary Pump - BlendAssist

Simple tool for online-dilution of modifiers and gradient set-up



Peak Dispersion in HPLC

- Dispersion is the sample peak broadening or dilution which occurs in connecting tubing, sample valves, flow cells and in column end-fittings. It begins with the injector and ends at the **last detector** in the system
- As column internal diameter and length decrease the potential peak broadening in a non-optimized LC system increases.
- Higher efficiency in the column can only be realized if the system dispersion does not substantially degrade the column performance.
- As particle size decreases, resolution increases as a result of narrower peak widths.
- Narrower peaks are more susceptible to extra-column dispersion.
- The smaller the column dimension, too, the smaller the expected peak volume. Thus the small particle size columns used in low volume configurations require the greatest attention to plumbing in the LC system.

How to optimize your LC-system

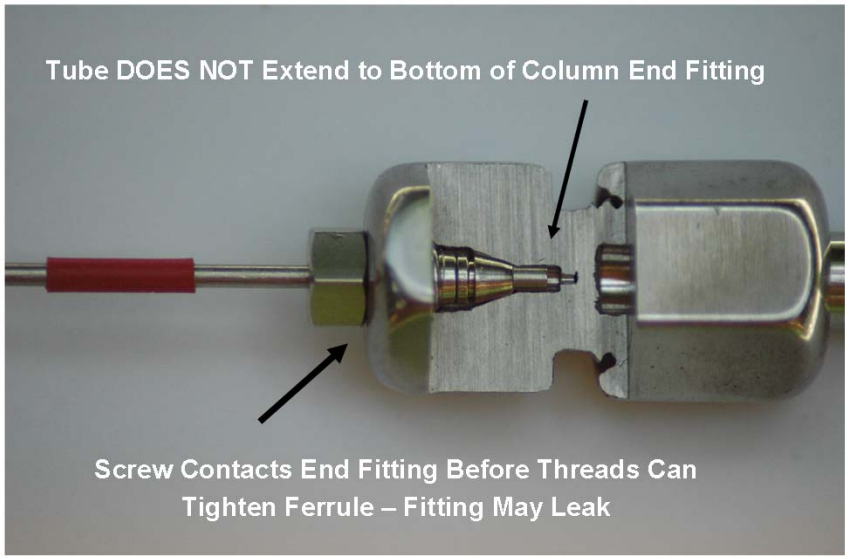
Non-column sources of peak broadening:

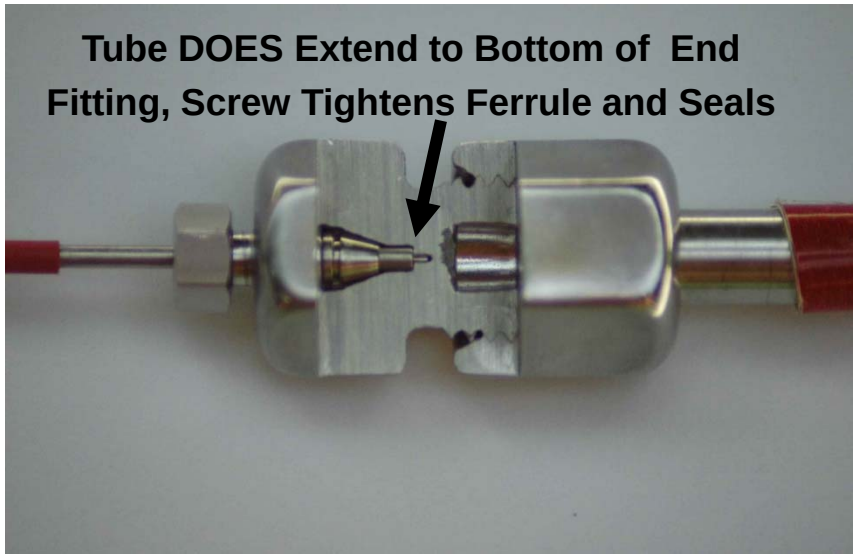
- **General**
  - Connecting tubing (internal diameter too big, tubing too long)
  - Connectors (unions, tees, bulkhead fittings)
  - Switching valves for automated SPE or alternating column regeneration
- **Sampler**
  - Sample aspirating needle and loading/transfer port
  - Sampler switching valve(s) contacting sample
- **Detection**
  - Inlet heat exchangers, flow cell volume and geometry
  - Incorrect data rate selection and data filtering effects in high speed applications

Tips for minimizing dispersion in LC systems

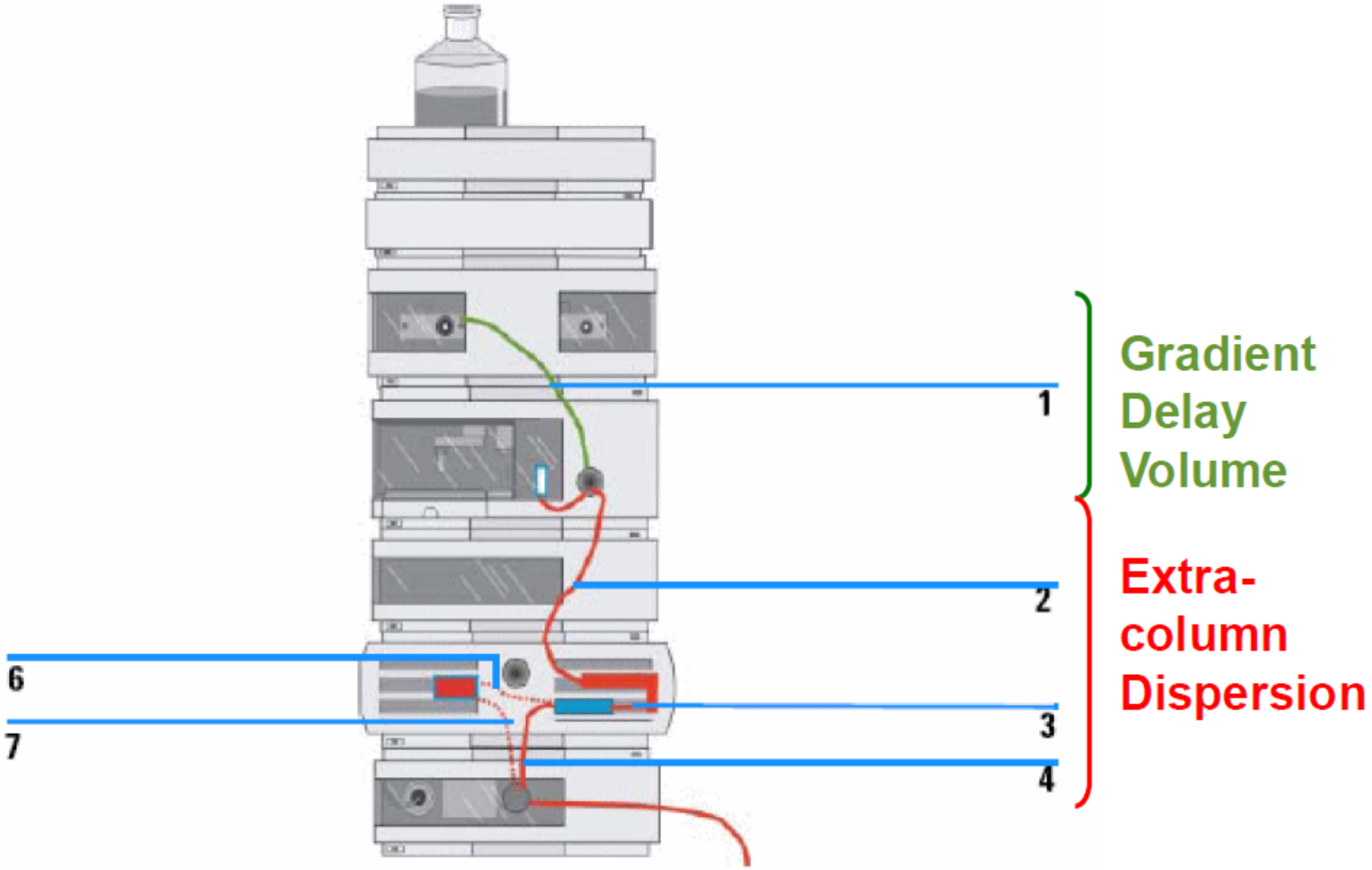
Minimize interconnection volume from the injector to detector, with minimal junctions and smallest i.d. and length tubing

Make fittings carefully, using appropriate connectors, and do not re-use seated stainless steel ferrules in different locations, including different ports on switching valves

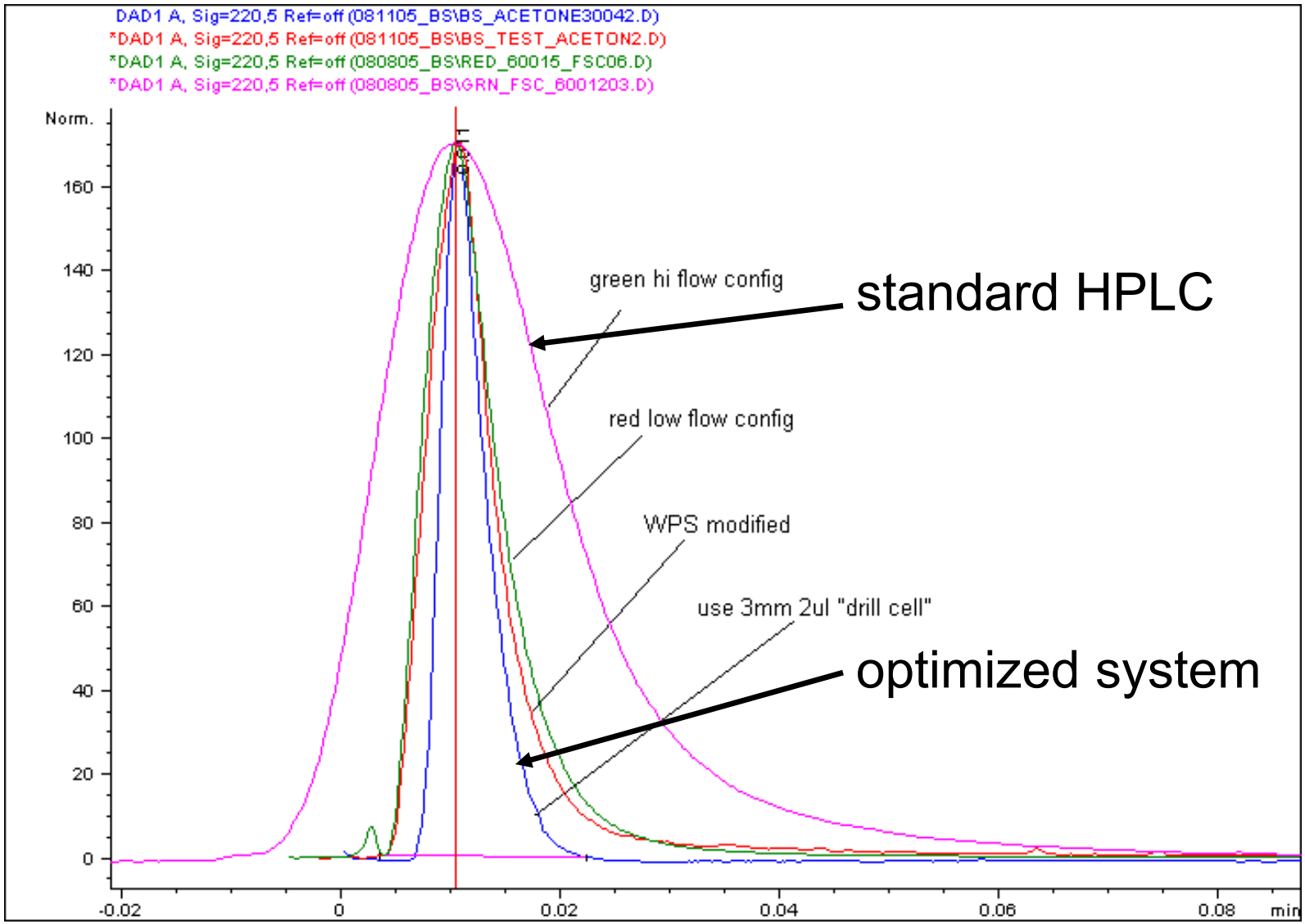




LC System -- Variable Configurations for Dispersion Volume and Delay/Dwell Volume



Time-aligned overlay of HPLC dispersion tests



Dispersion Volume within Flow Cell

Simple example: dispersion in a straight tube

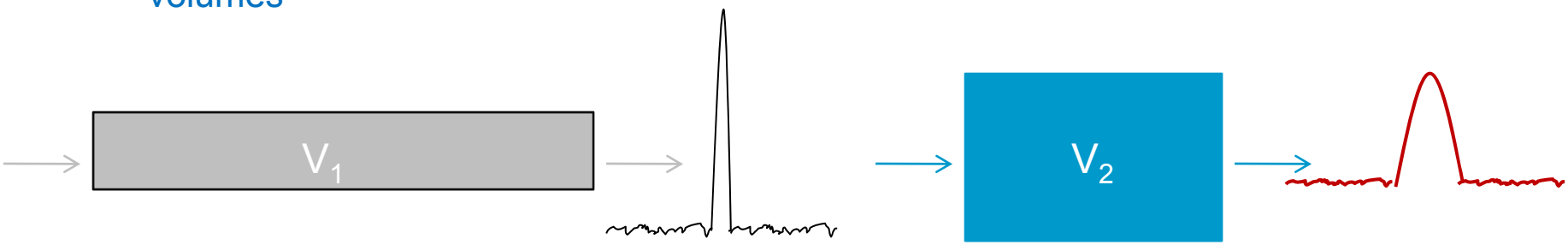
Dispersion:

$$\sigma^2 = \frac{\pi \cdot r^4 \cdot F \cdot L}{24 \cdot D_m}$$

Volumetric peak variance
F: Flow rate
D_m: Molecular diffusion coefficient
L: Capillary length

Derived from Aris, Taylor and Golay equation

Same geometrical volume ($V_1 = V_2$), but totally different dispersion volumes



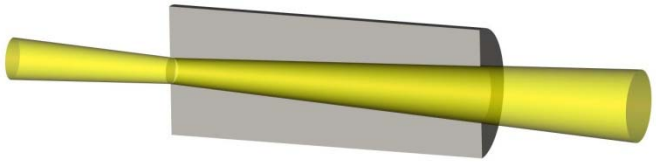
R. Tijssen, "Mechanism and Importance of Zone-Spreading, in Handbook of HPLC, Vol 78, 1998, Marcel Dekker, NY

Why do we need a new cell technology?

Short 2.1 mm ID column

+

10 mm pathlength
13 µl volume



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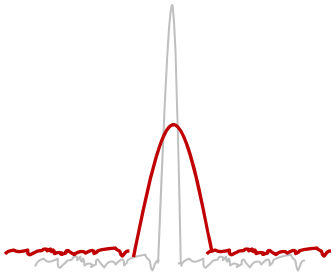
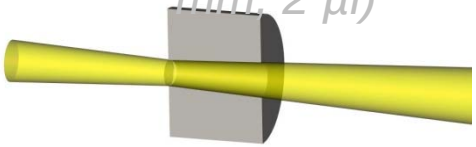
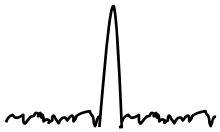


How to achieve smaller cell volume?

short path-length (3 mm, 2 µl)

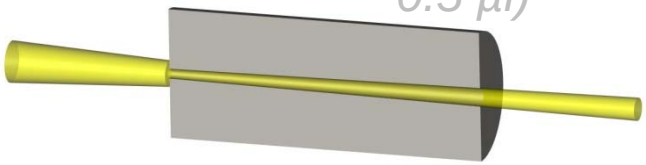


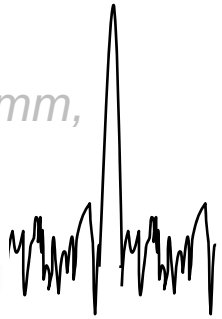


Low S/N

High light transmission => low noise

long path-length (10 mm, 0.5 µl)



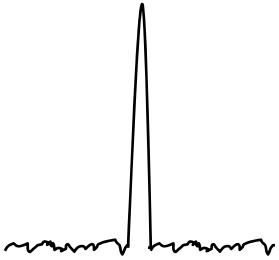


Low S/N

Low light transmission => high noise

Solution:

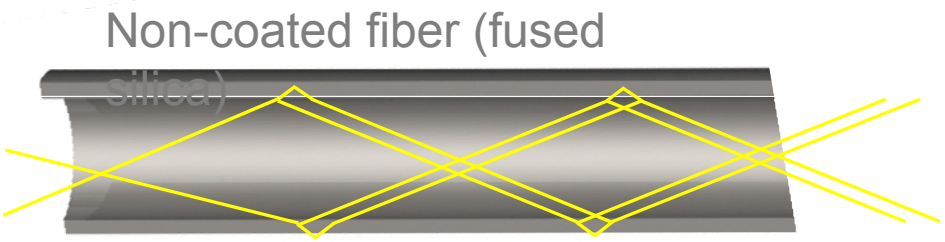
Optofluidic Waveguides
- Long path length
- Small cell volume
- High light transmission

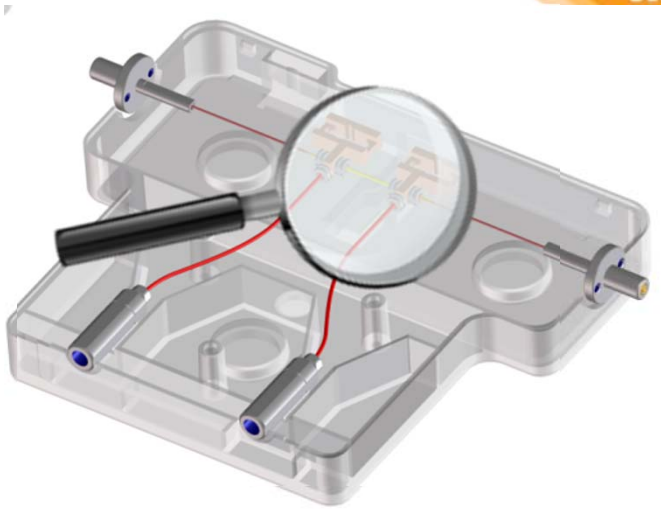


Highest S/N

Max-Light Cartridge Cell

- Optofluidic waveguides





High Light Transmission due to Total-Internal Reflection (TIR) principle
(~ 100 % Light efficiency)

Benefits:

- *Highest Sensitivity (S/N) with small cell volumes (dispersion effects)*
- *More reliable and robust peak integration (automated) due to nearly no Refractive Index and thermal effects (solvent temperature)*
- *Coating free fused silica (no special care instructions or smiling baseline effects)*
- *Easy cell selection (one cell for all major applications)*
- *Cartridge design for ease of use*

Materials Needed to Optimize a 1290 Infinity LC for Ultra-Low Dispersion

Ultra-Low Dispersion Kit for 1290 Infinity LC System

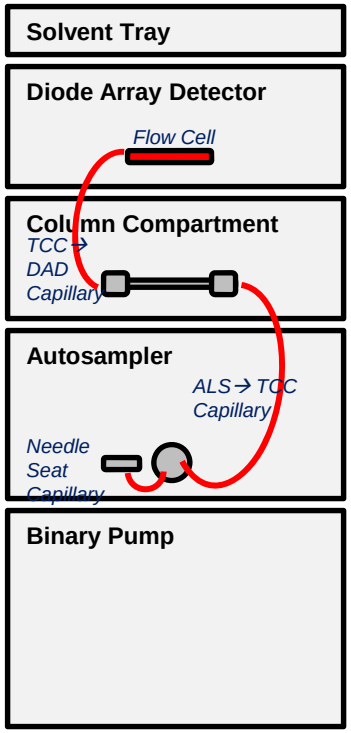
Includes:

- 0.075 x 220 mm capillary
- 0.075 x 340 mm capillary
- 1.0 uL heat exchanger
- 0.075 mm id needle seat

Ultra-Low Dispersion Max-Light Cartridge Flow Cell

1290 Infinity LC System Set-Up

Default 1290



Needle Seat Capillary: 0.12 x 100 mm = 1.1 µL
ALS→ TCC Capillary: 0.12 x 340 mm = 3.8 µL
TCC→ DAD Capillary: 0.12 x 220 mm = 2.5 µL
Flow Cell V(σ)1.0 µL = 2.3 µL
Total Extra-column Volume = 9.7 µL

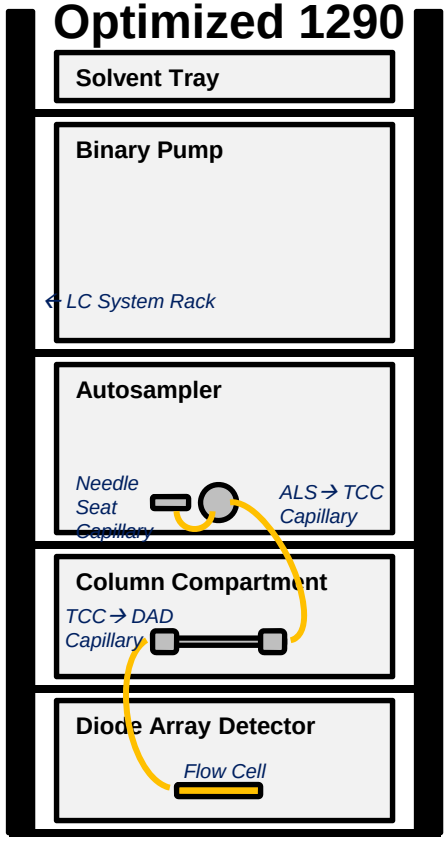
Volume of 2.1 x 50 mm column = 172.3 µL
Void Volume of Column = 103.9 µL

Percent Extra-column Volume = 9.3 %

→

60% Reduction in Extra-Column Volume

Optimized 1290

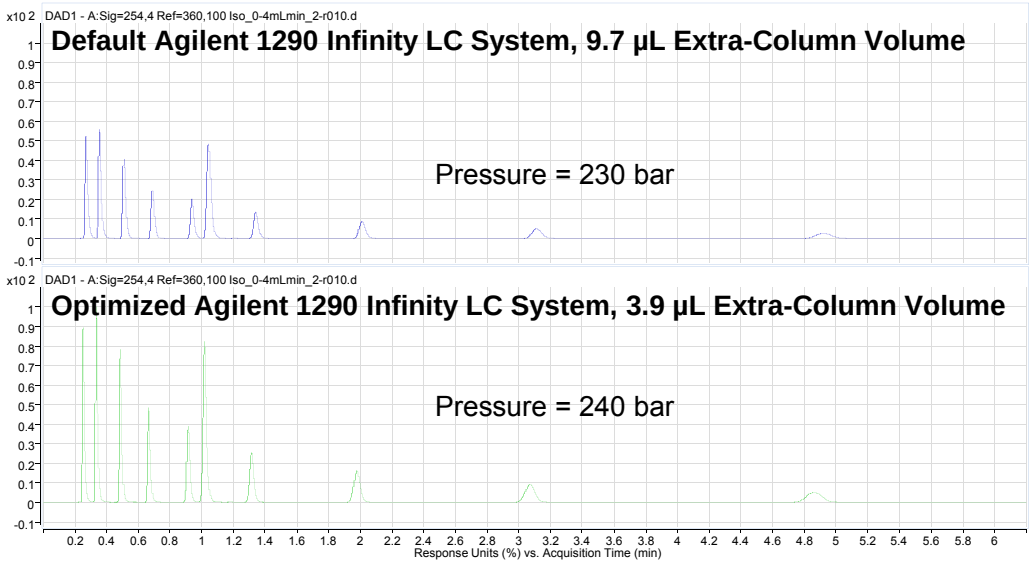


Needle Seat Capillary: 0.11 x 100 mm = 0.9 µL
ALS→ TCC Capillary: 0.08 x 220 mm = 1.1 µL
TCC→ DAD Capillary: 0.08 x 220 mm = 1.1 µL
Flow Cell V(σ)0.6 µL = 0.8 µL
Total Extra-column Volume = 3.9 µL

Volume of 2.1 x 50 mm column = 172.3 µL
Void Volume of Column = 103.9 µL

Percent Extra-column Volume = 3.7 %

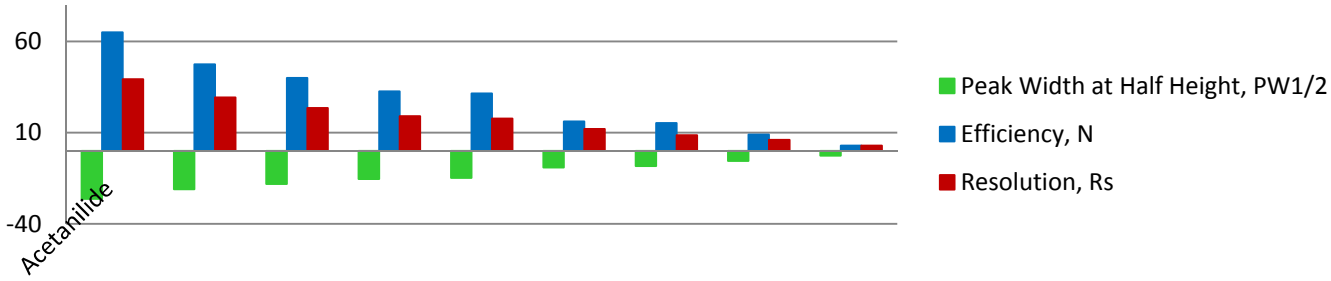
Isocratic Analyses of Alkylphenones



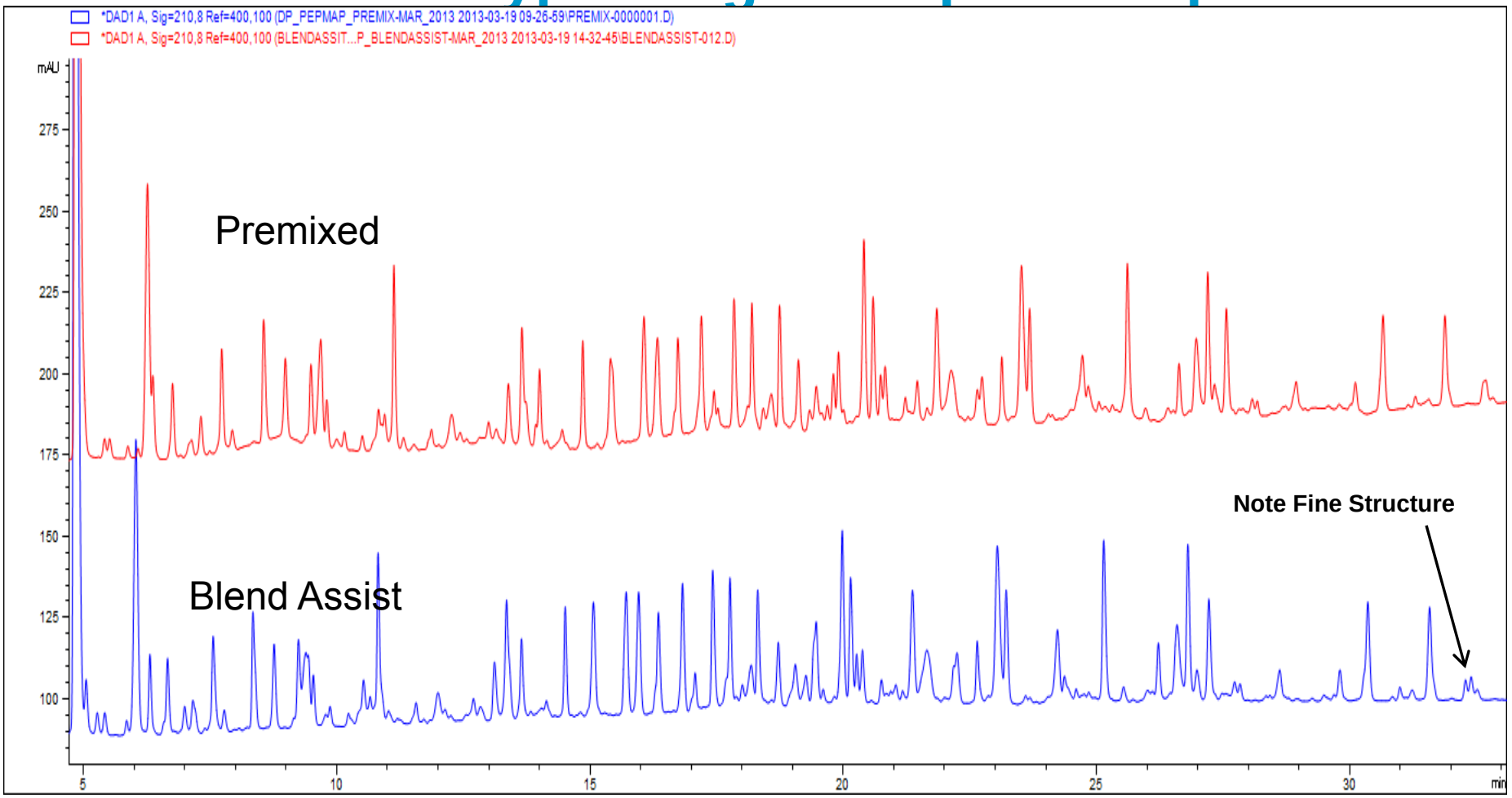
A: H_2O; B: CH_3CN; 0.4 mL/min Isocratic, 60% B
1 µL injection of RRLC Checkout Sample (PN 5188-6529) spiked w/ 50 µL 2 mg/mL thiourea in water/acetonitrile
TCC: 26 °C
DAD: Sig = 254, 4 nm; Ref = Off
Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 mm x 50 mm, 1.8 µm
Sample:

1. Thiourea (v_0 marker)
2. Acetanilide
3. Acetophenone
4. Propiophenone
5. Butyrophenone
6. Benzophenone
7. Valerophenone
8. Hexanophenone
9. Heptanophenone
10. Octanophenone

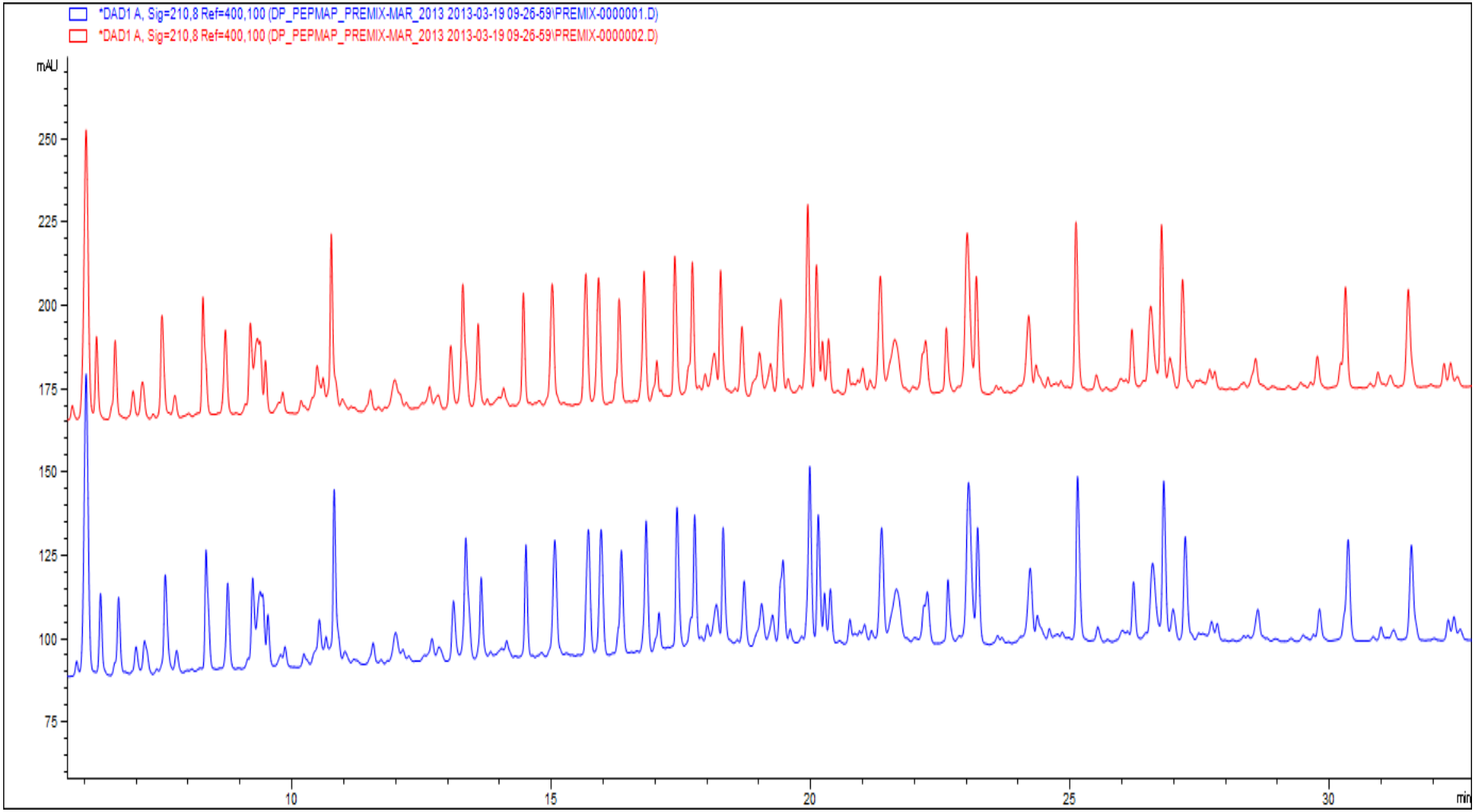
% Improvement from Default to Optimized Agilent 1290 Infinity LC System with an Isocratic Analysis



Premixed versus Blend Assist; 0.05%TFA-Water/0.04%TFA ACN BSA Tryptic Digest Peptide Map

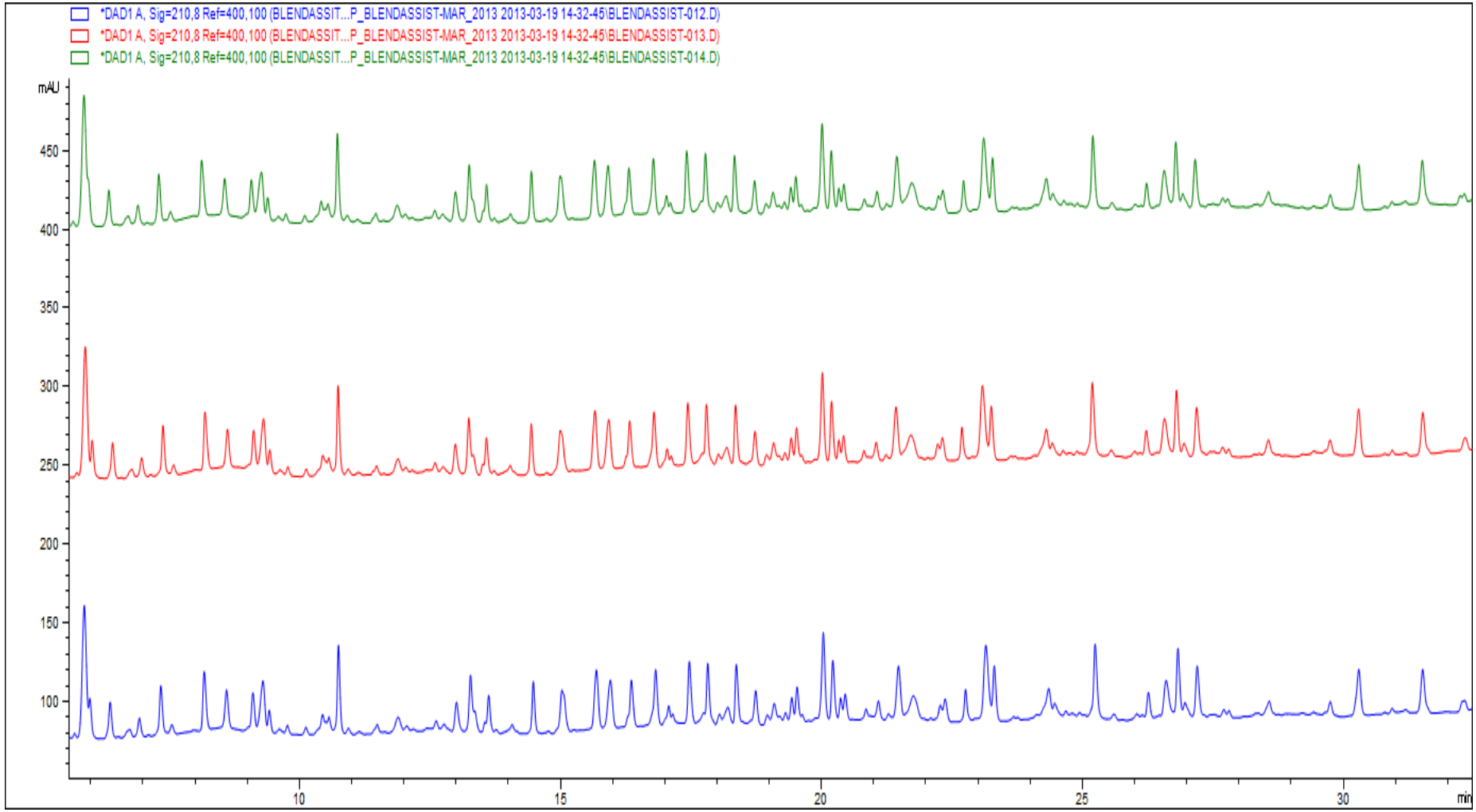


Duplicate Runs Premixed 0.05%TFA-Water/0.04%TFA ACN BSA Tryptic Digest Peptide Map

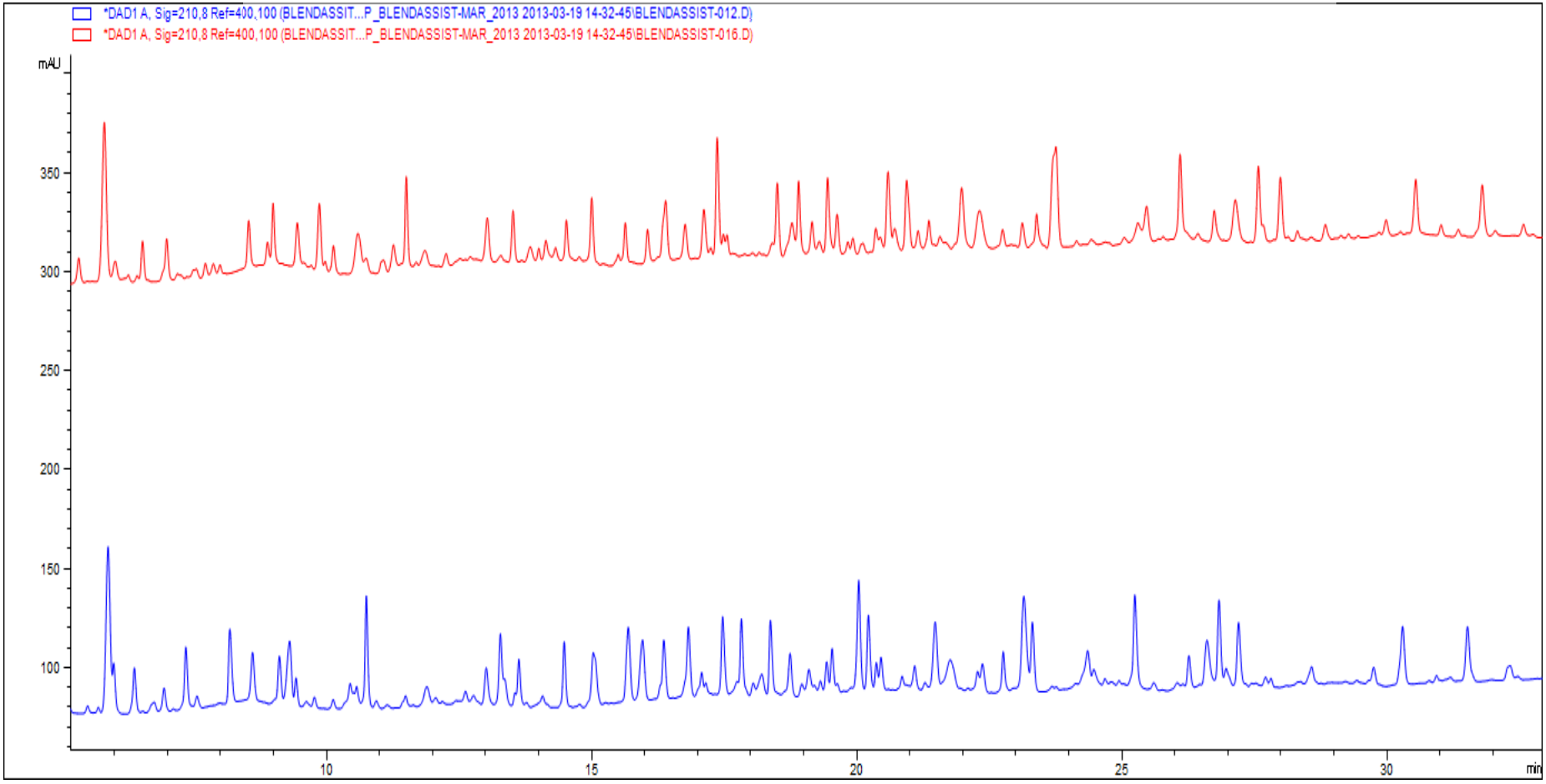


Triplicate Runs Blend Assist: Stock 1%TFA; 0.05%TFA Water/0.04%TFA ACN BSA Tryptic Digest Peptide Map

1200 Infinity Series



Overlay of Blend Assist TFA percent Differences:
Blue: 0.05%TFA-Water/0.04%TFA-ACN
Red: 0.1%TFA-Water/0.08%TFA-ACN
BSA Tryptic Digest Peptide Map



OpenLAB CDS MatchCompare

Comparison tool for complex Chromatograms

Problem / Solution

Problem

I need to compare a chromatogram against a reference and obtain ***objective*** data

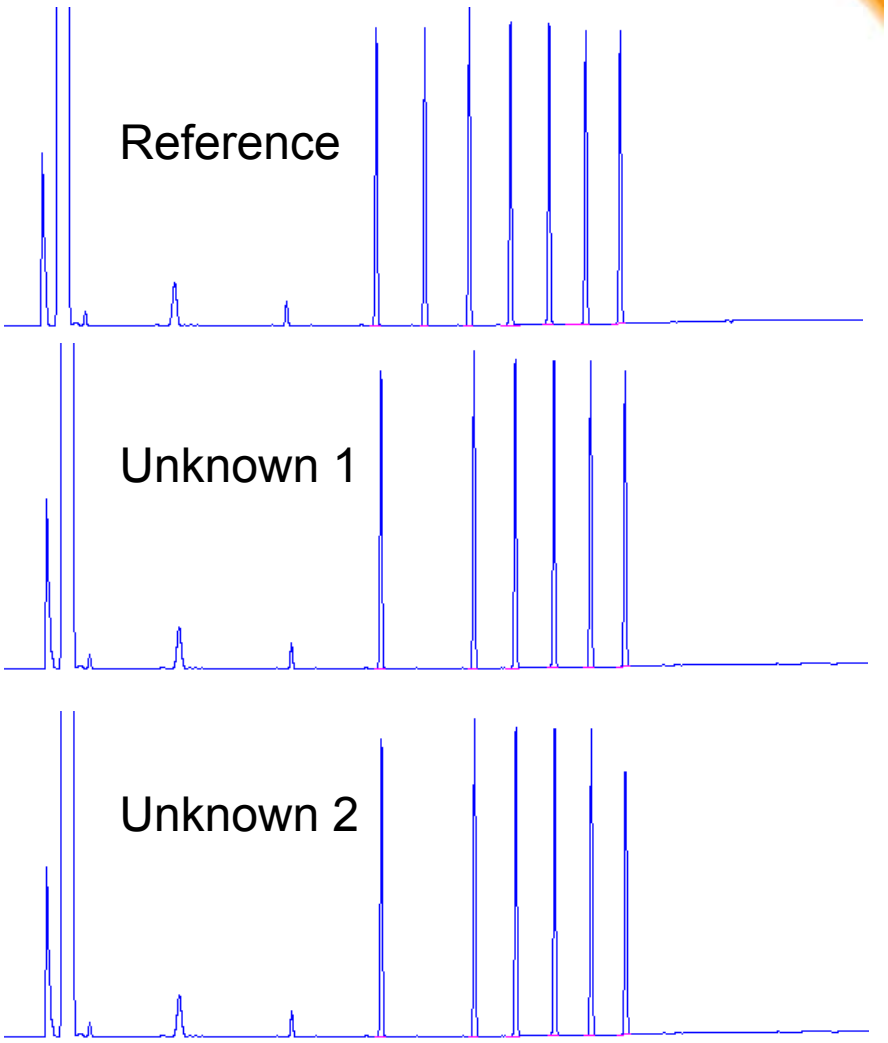
Solution

OpenLAB CDS Match Compare Add-on Software

Matching Chromatograms

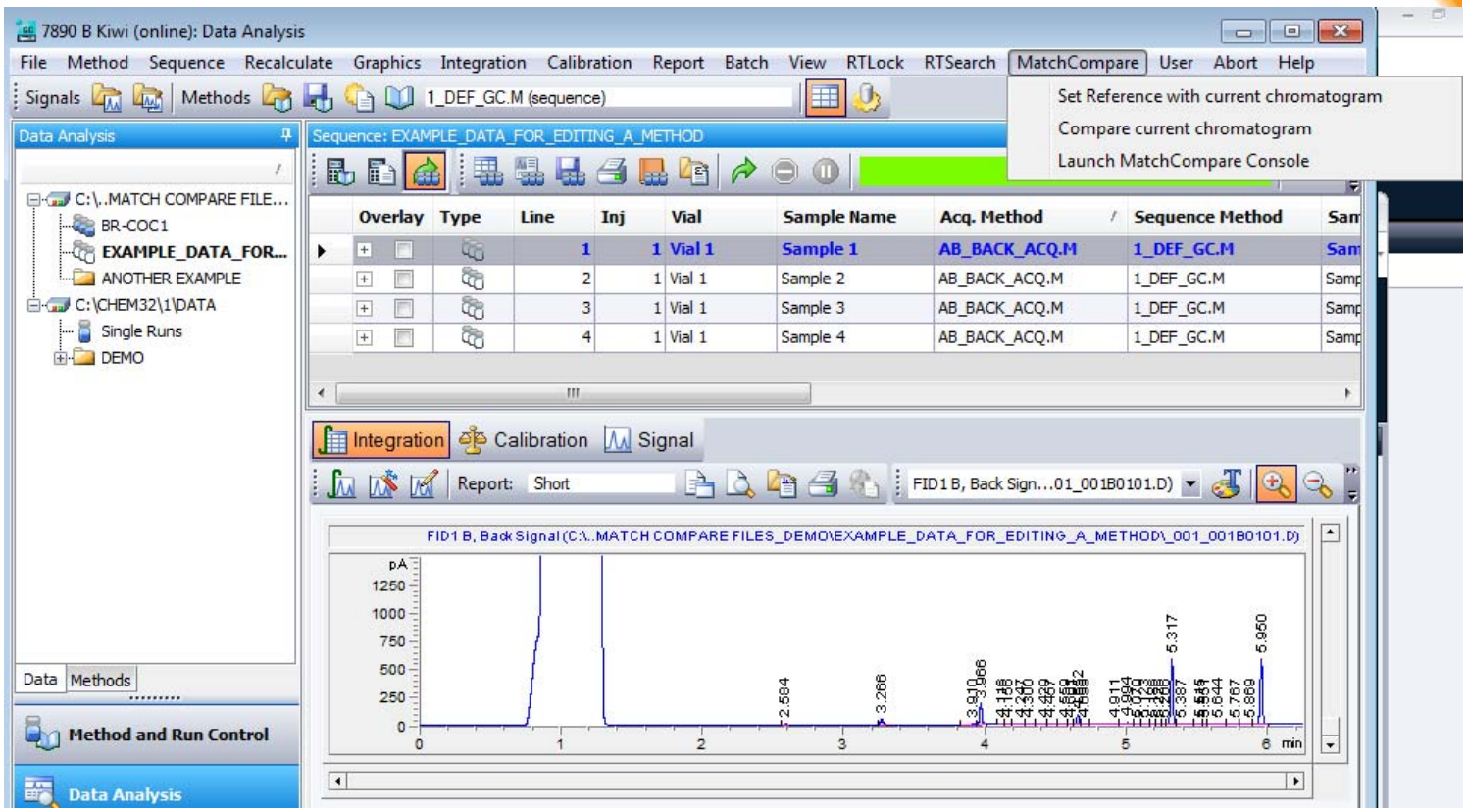
Reference defined

- Area percent tolerance defined by peak
- RT (RI) tolerance defined in time (Index)
- Initial shift allows for injection delays



Run the comparison

From the “Files” tab, select the sample chromatogram and click “Compare”



Matching Chromatograms

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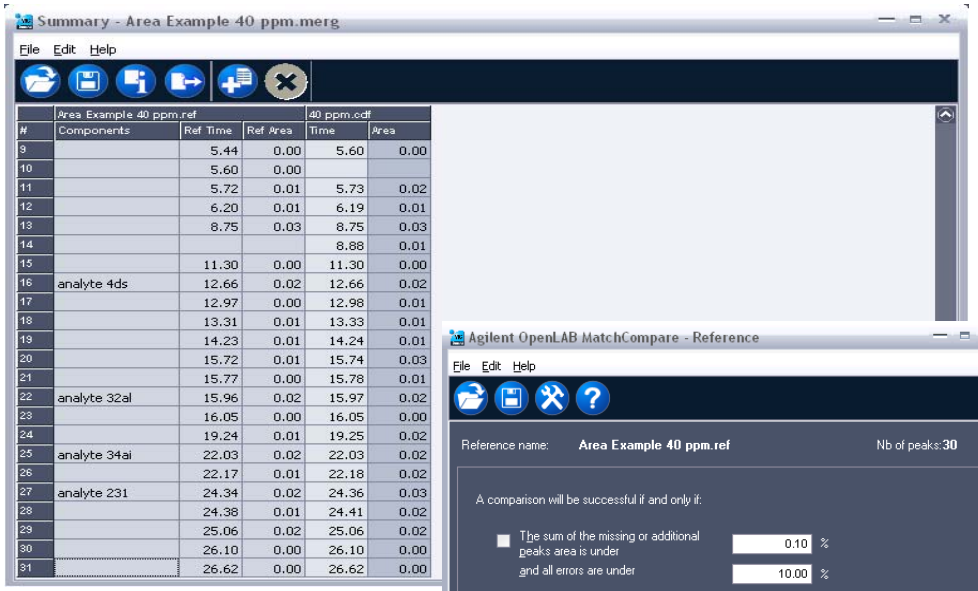
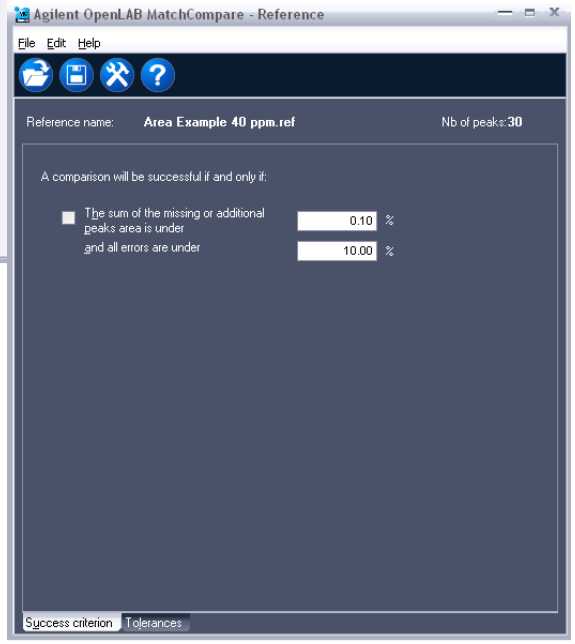
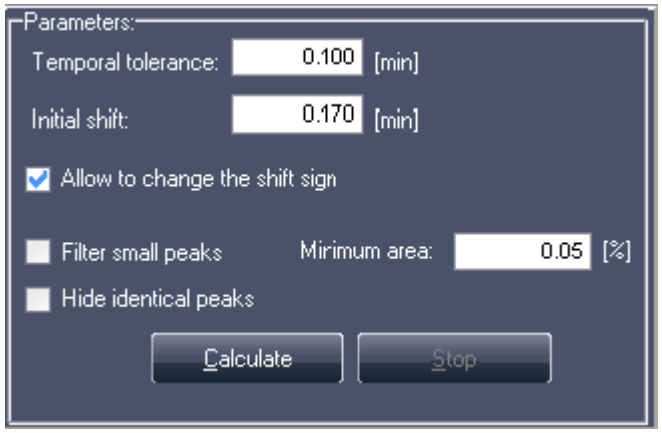
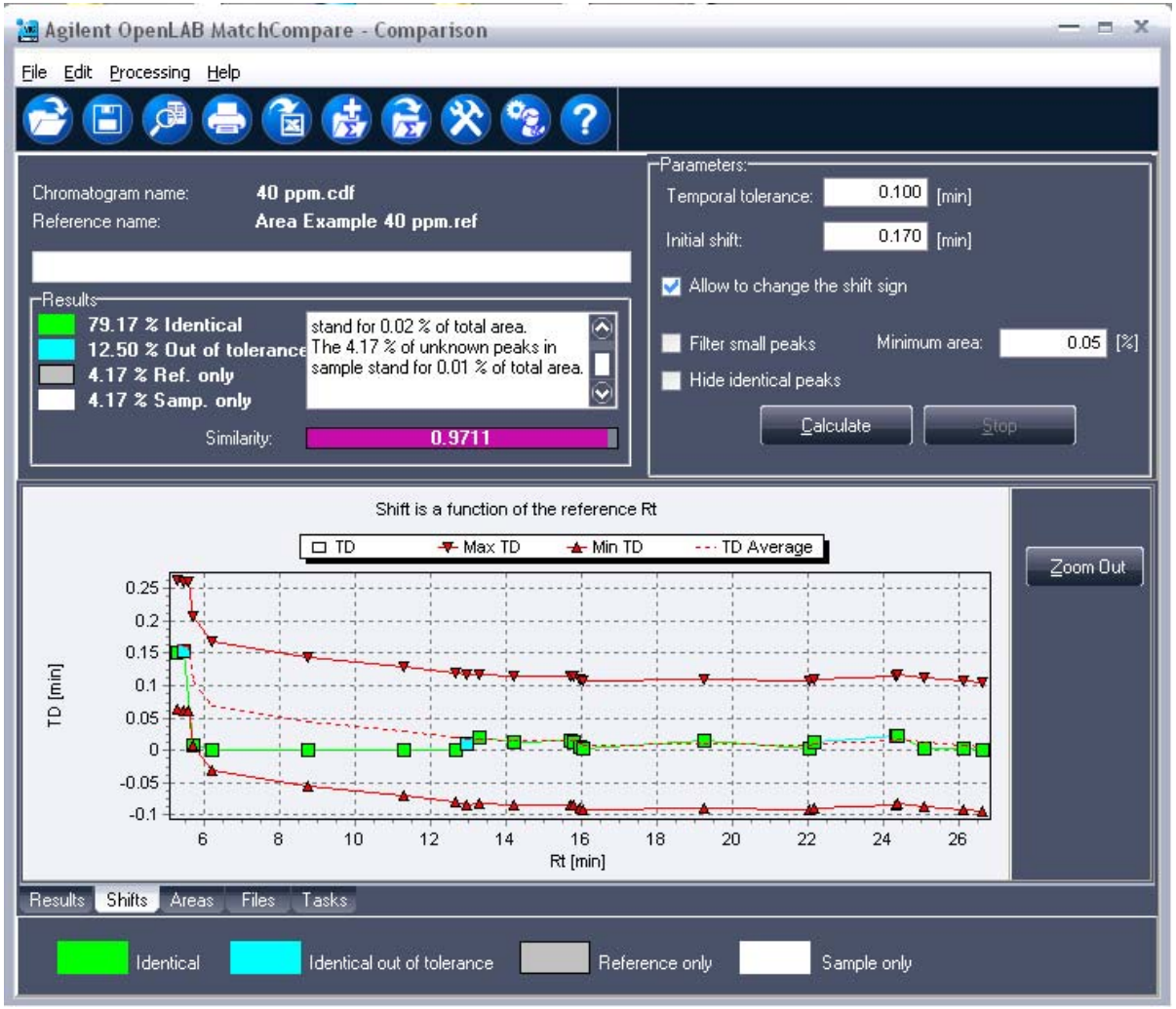




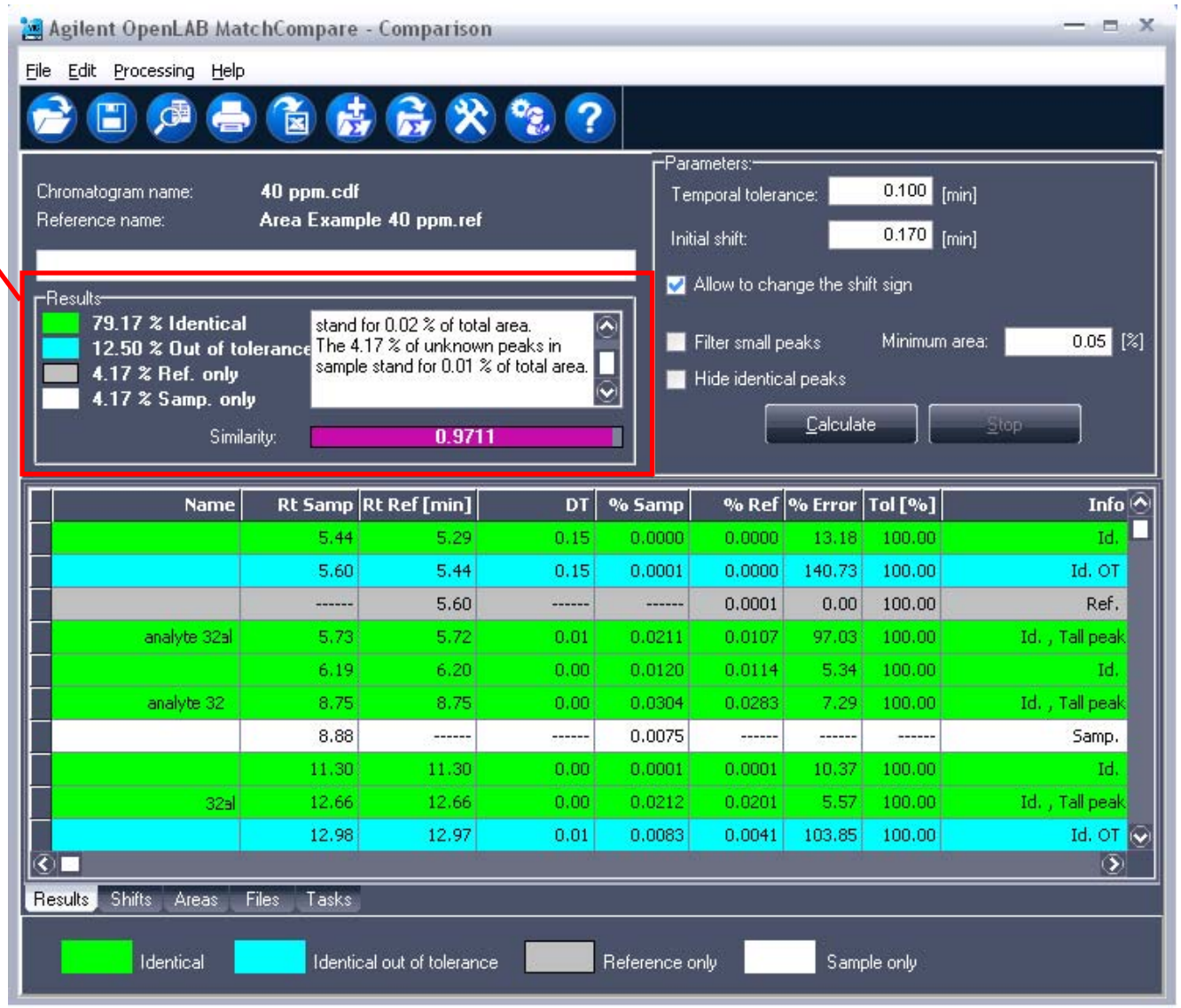


Time shifts


Agilent OpenLAB MatchCompare - Comparison
File Edit Processing Help
Chromatogram name: 40 ppm.cdf
Reference name: Area Example 40 ppm.ref
Results
79.17 % Identical
12.50 % Out of tolerance
4.17 % Ref. only
4.17 % Samp. only
stand for 0.02 % of total area.
The 4.17 % of unknown peaks in sample stand for 0.01 % of total area.
Similarity: 0.9711
Parameters:
Temporal tolerance: 0.100 [min]
Initial shift: 0.170 [min]
Allow to change the shift sign
Filter small peaks
Minimum area: 0.05 [%]
Hide identical peaks
Calculate
Stop
Shift is a function of the reference Rt
TD
Max TD
Min TD
TD Average
TD [min]
Rt [min]
Zoom Out
Results
Shifts
Areas
Files
Tasks
Identical
Identical out of tolerance
Reference only
Sample only

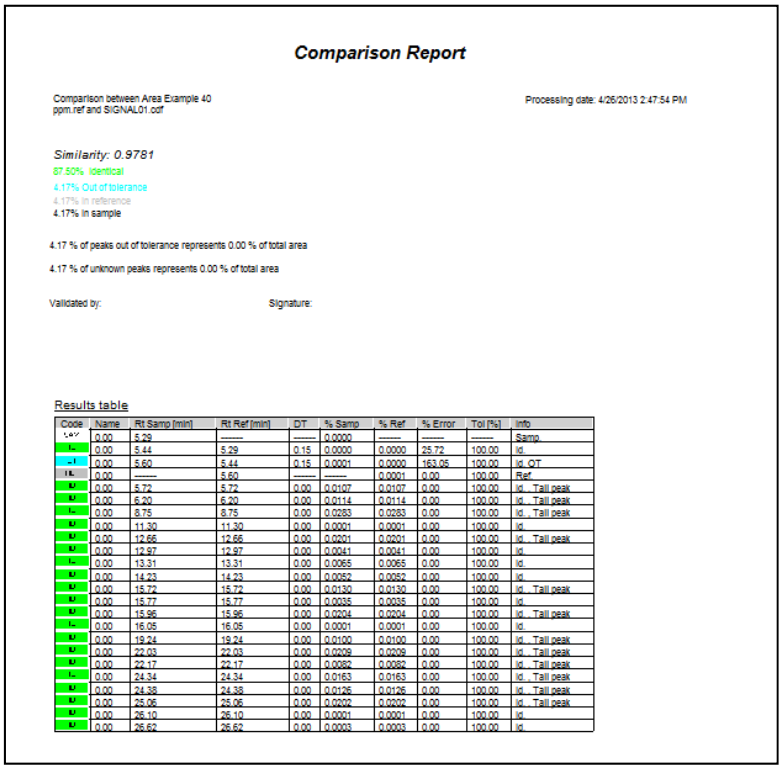
Peak results

Comparison Summary



Area comparison

OpenLAB CDS Match Compare Reports



Comparison Report

Comparison between Area Example 40 ppm.ref and SIGNAL01.cdf

Processing date: 4/26/2013 2:47:54 PM

Similarity: 0.9781

87.50% Identical

4.17% Out of tolerance

4.17% In reference

4.17% In sample

4.17 % of peaks out of tolerance represents 0.00 % of total area

4.17 % of unknown peaks represents 0.00 % of total area

Validated by: Signature:

Results table

Code	Name	Rt Samp [min]	Rt Ref [min]	DT	% Samp	% Ref	% Error	Tol [%]	Info
----	0.00	5.29	------	------	0.0000	------	------	------	Samp
I...	0.00	5.44	5.29	0.15	0.0000	0.0000	25.72	100.00	Id.
...I	0.00	5.60	5.44	0.15	0.0001	0.0000	163.05	100.00	Id. OT
IR.	0.00	------	5.60	------	------	0.0001	0.00	100.00	Ref
IJ	0.00	5.72	5.72	0.00	0.0107	0.0107	0.00	100.00	Id. Tall peak
IJ	0.00	6.20	6.20	0.00	0.0114	0.0114	0.00	100.00	Id. Tall peak
I...	0.00	8.75	8.75	0.00	0.0283	0.0283	0.00	100.00	Id. Tall peak
IJ	0.00	11.30	11.30	0.00	0.0001	0.0001	0.00	100.00	Id.
IJ	0.00	12.66	12.66	0.00	0.0201	0.0201	0.00	100.00	Id. Tall peak
IJ	0.00	12.97	12.97	0.00	0.0041	0.0041	0.00	100.00	Id.
I...	0.00	13.31	13.31	0.00	0.0065	0.0065	0.00	100.00	Id.
IJ	0.00	14.23	14.23	0.00	0.0052	0.0052	0.00	100.00	Id.
IJ	0.00	15.72	15.72	0.00	0.0130	0.0130	0.00	100.00	Id. Tall peak
IJ	0.00	15.77	15.77	0.00	0.0035	0.0035	0.00	100.00	Id.
IJ	0.00	15.96	15.96	0.00	0.0204	0.0204	0.00	100.00	Id. Tall peak
I...	0.00	16.05	16.05	0.00	0.0001	0.0001	0.00	100.00	Id.
IJ	0.00	19.24	19.24	0.00	0.0100	0.0100	0.00	100.00	Id. Tall peak
IJ	0.00	22.03	22.03	0.00	0.0209	0.0209	0.00	100.00	Id. Tall peak
IJ	0.00	22.17	22.17	0.00	0.0082	0.0082	0.00	100.00	Id. Tall peak
I...	0.00	24.34	24.34	0.00	0.0163	0.0163	0.00	100.00	Id. Tall peak
IJ	0.00	24.38	24.38	0.00	0.0126	0.0126	0.00	100.00	Id. Tall peak
IJ	0.00	25.06	25.06	0.00	0.0202	0.0202	0.00	100.00	Id. Tall peak
IJ	0.00	26.10	26.10	0.00	0.0001	0.0001	0.00	100.00	Id.
IJ	0.00	26.62	26.62	0.00	0.0003	0.0003	0.00	100.00	Id.

Summary - Area Example 40 ppm.merg

File Edit Help

	Area Example 40 ppm.ref			40 ppm.cdf	
#	Components	Ref Time	Ref Area	Time	Area
9		5.44	0.00	5.60	0.00
10		5.60	0.00		
11		5.72	0.01	5.73	0.02
12		6.20	0.01	6.19	0.01
13		8.75	0.03	8.75	0.03
14				8.88	0.01
15		11.30	0.00	11.30	0.00
16	analyte 4ds	12.66	0.02	12.66	0.02
17		12.97	0.00	12.98	0.01
18		13.31	0.01	13.33	0.01
19		14.23	0.01	14.24	0.01
20		15.72	0.01	15.74	0.03
21		15.77	0.00	15.78	0.01
22	analyte 32al	15.96	0.02	15.97	0.02
23		16.05	0.00	16.05	0.00
24		19.24	0.01	19.25	0.02
25	analyte 34ai	22.03	0.02	22.03	0.02
26		22.17	0.01	22.18	0.02
27	analyte 231	24.34	0.02	24.36	0.03
28		24.38	0.01	24.41	0.02
29		25.06	0.02	25.06	0.02
30		26.10	0.00	26.10	0.00
31		26.62	0.00	26.62	0.00

Overview of the principals of Match Compare

- Comparison of two chromatograms
  - Pattern matching, not pattern recognition
  - Matching based on retention time (or retention index) and area percent
- Comparison parameters can be individually tailored
- Report can be on all peaks or just those falling outside the limits

Live Demonstration of MatchCompare Using Peptide Maps

Appendix

Analyzing protein drug by RP-HPLC peptide map - pyroglutamate formation

Reverse-Phase Chromatography/ Mass Spectrometry Analysis of Reduced Monoclonal Antibodies in Pharmaceuticals

Douglas Rehder, Thomas Dillon, Gary Pipes, and Pavel Bondarenko, Journal of ChromatographyA, 1102 (2006), p.164-175

Analyzing protein drug by RP-HPLC Peptide map 1- amino acid substitution

Identification of a Glu > Lys substitution in the activation segment of human pepsinogen A-3 and -5 isozymogens by peptide mapping using endoproteinase Lys-C

Ruud A. Bank, Bart C. Crusius, Toon Zwiers, Stephan G.M. Meuwissen*, Fre Arwert
and Jan C. Pronk Institute of Human Genetics and *Department of Gastroenterology, Free University, Amsterdam. The Netherlands, Volume 238, number 1, 105-108 FEB 06339 September 1988

Analyzing protein drug by RP-HPLC Peptide map-mirror image

LC/ESI-MS/MS analysis of recombinant IgG2 mAb after Lys-C digest

Wypych J et al. J. Biol. Chem. 2008;283:16194-16205

Analyzing protein drug by RP-HPLC Peptide map-deamidation

Quantification and characterization of antibody deamidation by peptide mapping with mass spectrometry

Weijie Wang, Andrea R. Meeler, Luke T. Bergerud, Mark Hesselberg, Michael Byrne, Zhuchun Wu, Analytical Sciences Department, Human Genome Sciences, Inc., 14200 Shady Grove Road, Rockville, MD 20850, United States

Analyzing protein drug by RP-HPLC Peptide map - disulfide bonds

Disulfide Linkage Analysis of IgG1 using an Agilent 1260 infinity Bio-inert LC System with an Agilent Zorbax RRHD Diphenyl sub-2um Column

M. Sundaram Palaniswamy, Agilent Technologies, Inc., Bangalore, India

Analyzing protein drug by RP-HPLC Peptide map - PEGylation

Toward Top-Down Determination of PEGylation Site Using MALDI In-Source Decay MS Analysis

Chul Yoo, Detlev Suckau,Volker Sauerland,Michael Ronk, and Minhui Maa, Analytical R&D, Amgen Inc., Thousand Oaks, California, USA

Questions