Peptide Mapping

1200 Infinity

Hardware and Column Optimization

1



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Peptide Mapping Hardware Challenges

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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)
- 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 $\approx 1 \text{ m of}$ 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.

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Delay Volume Profiles

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1200 Infinity **Delay Volume Variability within Agilent Systems**

Series

| 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
 - \pm 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







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Composition Range

Shallow gradient at low organic concentration



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Composition Range

Shallow gradient at low organic concentration





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Precision & Accuracy

1st Quaternary pump with Bin-like performance

Different mixtures (H₂O, MeOH, ACN) at different pressures and different flow rates



Water-Methanol, 500µl/min, 220-570bar

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1290 Quaternary Pump Flow Diagram

1200 Infinity Series



metweav





Multipurpose Valve

1200 Infinity

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





The Measure of Confidence

TFA Mixing Noise:

Effect of JetWeaver Mixer: Lower Short Term Noise





Innovation: 1290 Infinity Quaternary Pump *Quaternary Pump with Binary Pump-like Performance*

1200 Infinity Series

<image>



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

15



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 stocksolution 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!

16



1290 Infinity Quaternary Pump - BlendAssist

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

| | | | | | | | | | | Qua | t. Pump (G | 4204A) |
|--|---------|------------|----------------|----------|--------------------|---------------------------|---|--------|---------------------|---------------------|------------|--------|
| Flow | | | | + Adva | nced | | | | | | | |
| | | | | + Time | table (empty) | | | | | | | |
| | | 0.000 ; mL | min | + Blend | Assist | | | | | | | |
| Solvents | | | | Chann | el Type | Calibration | | Name 🛆 | Stock concentration | Final concentration | Conc. unit | |
| C Enable Blend Ass | ist | | | A | Solvent 1 | 100.0 % Water V.02 | Ŧ | Water | 1.00 | 1.00 | % | |
| | | | | В | Solvent 1 Additive | 100.0 % Water V.02 | - | TFA | 1.00 | 0.10 | % | |
| Solvent | Used | % | Name | С | Solvent 2 | 100.0 % Acetonitrile V.02 | - | ACN | 1.00 | 1.00 | % | |
| Water/TFA (0.1%) | V | 57.0 | | D | Solvent 2 Additive | 100.0 % Acetonitrile V.02 | - | TFA | 0.10 | 0.01 | mМ | |
| ACN/TFA (0.01mM) | V | 43.0 | | | | | | | | | | |
| Pressure Limits | | | | <u>.</u> | | | | | | | | |
| Min: 0.0 | 0 🕂 bar | Max: | 1,200.00 🗼 bar | | | | | | | | | |
| Stoptime | | Posttime | | | | | | | | | | |
| As Injector/No 1.00 | Limit | • | Off | | | | | | | | | |



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

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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



The Measure of Confidence Page 20 of 31



Optimize LC,

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





Time-aligned overlay of HPLC dispersion tests





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Dispersion:



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

Derived from Aris, Taylor and Golay equation

Same geometrical volume (V₁ = V₂), but totally different dispersion volumes $> \underbrace{V_1} \longrightarrow \underbrace{V_2} \longrightarrow$

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



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Max-Light Cartridge Cell - Optofluidic waveguides

Non-coated fiber (fused





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 <u>no Refractive Index</u> 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 **Optimized 1290 Solvent Tray** Solvent Tray Needle Seat Capillary: 0.12 Needle Seat Capillary: 0.11 **Binary Pump** $x 100 \text{ mm} = 1.1 \mu \text{L}$ $x 100 \text{ mm} = 0.9 \mu \text{L}$ ALS→ TCC Capillary: 0.12 x ALS→ TCC Capillary: 0.08 x **Diode Array Detector** 340 mm = 3.8 µL 220 mm = 1.1 µL TCC→ DAD Capillary: 0.12 TCC→ DAD Capillary: 0.08 Flow Cell x 220 mm = 2.5 µL $x 220 \text{ mm} = 1.1 \mu \text{L}$ Flow Cell V(σ)1.0 μ L = 2.3 Flow Cell V(σ)0.6 µL = 0.8 LC System Rack μL μL Column Compartment Total Extra-column **Total Extra-column** TCC Volume = 9.7 µL Volume = 3.9 µL DAD Autosampler Capillar Volume of 2.1 x 50 mm Volume of 2.1 x 50 mm $column = 172.3 \mu L$ $column = 172.3 \mu L$ Void Volume of Column = Void Volume of Column = Autosampler Needle 103.9 uL 103.9 uL $ALS \rightarrow TCC$ ALS → TCC Seat Capillary Capillary Percent Extra-column Percent Extra-column Needle Volume = Volume = -Seat Column Compartment 3.7 % 9.3 % $TCC \rightarrow DAD$ **Binary Pump** Capillary \rightarrow 60% Reduction Diode Array Detector in Extra-Column Flow Cell Volume



Isocratic Analyses of Alkylphenones



% 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

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The Measure of Confidence



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

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30



Triplicate Runs Blend Assist: Stock 1%TFA; 1200 Infinity 0.05%TFA Water/0.04%TFA ACN BSA Tryptic Digest Peptide Map



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Overlay of Blend Assist TFA percent Differences: 1200 Infinity Blue: 0.05%TFA-Water/0.04%TFA-ACN Red: 0.1%TFA-Water/0.08%TFA-ACN BSA Tryptic Digest Peptide Map



32



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





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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"



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The Measure of Confidence



Matching Chromatograms

Reference defined

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

| Parameters: Temporal tolerance: | 0.100 [min] | |
|------------------------------------|---------------|----------|
| Initial shift: | 0.170 [min] | |
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Series

37



Time shifts

😹 Agilent OpenLAB MatchCompare - Comparison - - X File Edit Processing Help (d) X ? 00 -121 -Parameters:-Chromatogram name: 40 ppm.cdf 0.100 [min] Temporal tolerance: Reference name: Area Example 40 ppm.ref 0.170 [min] Initial shift: 🛃 Allow to change the shift sign -Results \bigcirc 79.17 % Identical stand for 0.02 % of total area. 🔝 Filter small peaks Minimum area: 0.05 [%] 12.50 % Out of tolerance The 4.17 % of unknown peaks in sample stand for 0.01 % of total area. 4.17 % Ref. only Hide identical peaks 4.17 % Samp. only Calculate Similarity: 0.9711 Shift is a function of the reference Rt D TD 🖛 Max TD 📥 Min TD --- TD Average Zoom Out 0.25 0.2 0.15 TD [min] 0.1 0.05 . ----0. -0.05 444 -0.1 12 14 18 20 22 24 26 6 8 10 16 Rt [min] Results Shifts Areas Files Tasks Identical out of tolerance Identical Reference only Sample only





Peak results

1200 Infinity Series

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Comparison Summary

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| analyt | te 32al | 5.73 | 5.72 | 0.01 | 0.02 | 11 0.0107 | 97.03 | 100.00 | Id. , Tall peak |
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| analy | te 32 | 8.75 | 8.75 | 0.00 | 0.03 | 04 0.0283 | 7.29 | 100.00 | Id. , Tall peak |
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Area comparison



The Measure of Confidence



OpenLAB CDS Match Compare Reports

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| Resul Core La L L U U U U U U U U U U U U U U U U | ts table Name 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0. | Rt Samp (min) 5.24 5.64 5.60 5.72 5.72 5.72 13.0 12.67 13.31 14.23 15.77 15.60 15.72 15.72 15.60 19.24 22.03 | Rt.Ref fmin1 5.39 5.44 5.60 5.72 6.20 1.266 1.267 1.266 1.267 1.3.31 1.423 1.577 1.566 1.577 1.591 1.591 1.605 1.924 1.924 1.924 | DT 0.15 0.15 0.00 0.00 0.00 0.00 0.00 0.00 | 3% Samp 0.0000 0.0000 0.0001 0.0107 0.0107 0.0283 0.0001 0.0028 0.0001 0.0005 0.0005 0.0005 0.0005 0.0005 0.0005 0.0005 0.0005 0.0005 0.0005 0.0005 0.0005 0.0005 0.0005 0.0005 0.00000 0.0000 0.0000 0.00000 0.00000 0.00000 0.00000 0.00000 0.000000 | % Ref 0.0000 0.0001 0.0107 0.0114 0.0201 0.0001 0.0001 0.0001 0.0001 0.0001 0.0001 0.0001 0.00052 0.0032 0.0001 0.0001 0.0001 0.0001 0.0001 0.0001 | % Error 25.72 163.05 0.00 0.00 0.00 0.00 0.00 0.00 0.00 | Tol [%] 100.00 100.00 100.00 100.00 100.00 100.00 100.00 100.00 100.00 100.00 100.00 100.00 100.00 100.00 | into Samo, Id. J. Ref. Id. Tall peak Id. Tall peak | |
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| | Area Example 40 p | nm ref | | 40 ppm ed | f |
| # | Components | Ref Time | Ref Area | Time | Area |
| 9 | | 5 44 | 0.00 | 5.60 | 0.00 |
| 10 | | 5.60 | 0.00 | 0.00 | 0.00 |
| 11 | | 5.72 | 0.00 | 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 |

1200 Infinity

The Measure of Confidence



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

42



1200 Infinity Live Demonstration of MatchCompare Using **Peptide Maps**

Series



Appendix

1200 Infinity Series



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



The Measure of Confidence



Agilent Technologies