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Validation of a Reversed-Phase HPLC Method for Well-Characterized Biopharmaceuticals

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Abstract

Development of analytical methods to quantitate/qualitate biopharmaceutical drug substances and their metabolites in complex matrixes is not a trivial undertaking. The method itself plays an important role in the determination of the bioequivalence, bioavailability and pharmacokinetic attributes of a drug substance. It is, therefore, essential to employ a well-defined, fully validated analytical method that has the ability to yield reliable results.

In this paper, we demonstrate a systematic, straightforward approach to the validation of a Reversed-Phase HPLC method for the analysis of peptides. Some of the validation parameters investigated for the probe (tryptic digested bovine Cytochrome *c*) include robustness, reproducibility, accuracy and precision.

Definition of a Well-Characterized Biopharmaceutical

- "A well-characterized biopharmaceutical is defined as: a chemical entity whose identity, purity, impurities, potency and quantity can be determined and controlled".[1]

1. Characterization of Biotechnology Pharmaceutical Products, December 11-13, 1995.

What is Method Validation?

- Validation of a method can be defined as the authentication of the fact that the said method reliably performs for the intended application.

The Parameters Essential to Ensure the Acceptability of the RP-HPLC Method Performance are:

- stability of the drug in the matrix under storage conditions, accuracy, linearity, selectivity and sensitivity

- **precision and robustness**

- **evaluation and selection of an HPLC column which provides the reproducibility and performance necessary to achieve reliable results**

Intended Application:

- **This method is intended for qualitative purposes only**

- **In this example, we are interested in validating the method precision and robustness of the RP-HPLC method for tryptic digested bovine Cytochrome *c*.**

HPLC Conditions

- Instrument: Waters Alliance 2690 XE, 486 UV Detector, Millennium Data System
- Column: Symmetry300™ C_{18} 5 µm 3.9 x 150 mm
- Mobile Phase: A = 0.05 % TFA in Water
  B = 0.1 % TFA in Acetonitrile
- Gradient: 0 - 28 % B in 45 min, linear
- Flow Rate: 1.0 mL/min
- Temperature: 35ºC
- Detection: Absorbance at 220 nm

Sample Probe: Bovine Heart Cytochrome *c*

T1 T4

Ac GLY ASP VAL GLU LYS/ GLY LYS/ LYS/ ILE PHE VAL GLN LYS/ CYS

T5

ALA GLN CYS HIS THR VAL GLU LYS/ GLY GLY LYS/ HIS LYS/ THR GLY

T8 T9

PRO ASN LEU HIS GLY LEU PHE GLY ARG/ LYS/ THR GLY GLN ALA PRO

T10

GLY PHE SER TYR THR ASP ALA ASN LYS/ ASN LYS/ GLY ILE THR TRP

T12 T13

GLY GLU GLU THR LEU MET GLU TYR LEU GLU ASN PRO LYS/ LYS/ TYR

T14 T15

ILE PRO GLY THR LYS/ MET ILE PHE ALA GLY ILE LYS/ LYS/ LYS/ GLY

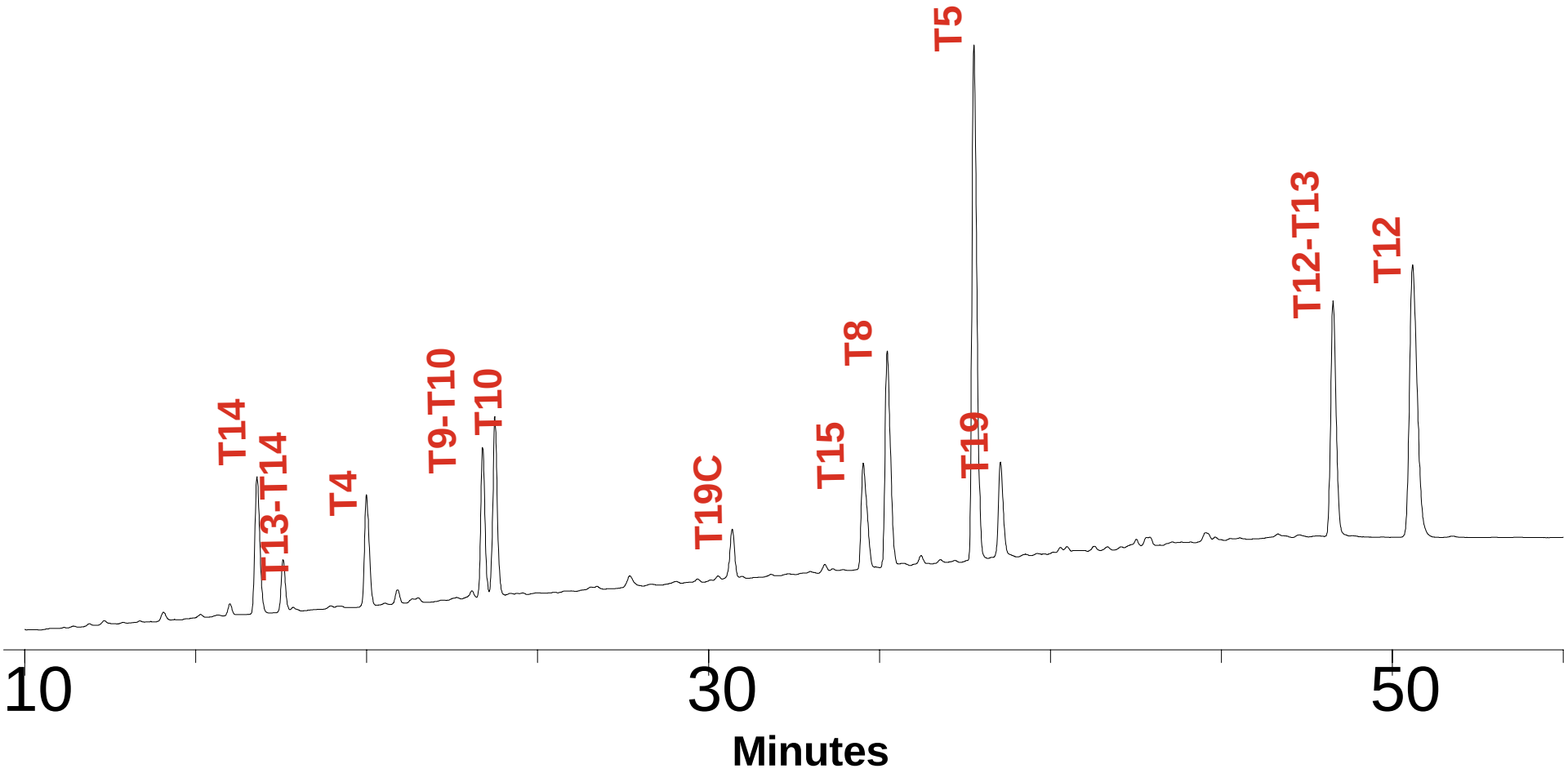
<----------T19---------->

GLU ARG/ GLU ASP LEU ILE ALA TYR/ LEU LYS/ LYS/ ALA THR ASN GLU

<----------T19C---------->

*BONDED TO HEME GROUP

Bovine Cytochrome *c* Tryptic Map



Precision - the degree of reproducibility of test results obtained by the analysis of the same samples under normal test conditions. Intermediate precision expresses within-lab variation, as on different days, or with different analysts or equiptment within the same laboratory.[2]

2. USP23, p. 1982

Method Precision

Conditions:

-Employed 8 different Symmetry300™,C18, 5 µm columns from the same batch (#107)

-Conducted evaluation over a 3 week time period

-Different mobile phase preparations for each run

Table 1:

Peak	Mean	Standard. Deviation	RSD
	Retention Times, Minutes n = 8	minutes	%
T1	9.78	0.03	0.3
T14	16.80	0.05	0.3
T13-T14	17.55	0.05	0.3
T4	20.00	0.05	0.2
T9-T10	23.34	0.04	0.2
T10	23.73	0.03	0.1
T19C	30.73	0.05	0.2
T15	34.49	0.06	0.2
T8	35.17	0.06	0.2
T5	37.85	0.05	0.1
T19	38.52	0.05	0.1
T12-T13	48.26	0.07	0.1
T12	50.65	0.09	0.2

r.t. std. dev. < 0.1;

% RSD < 0.4

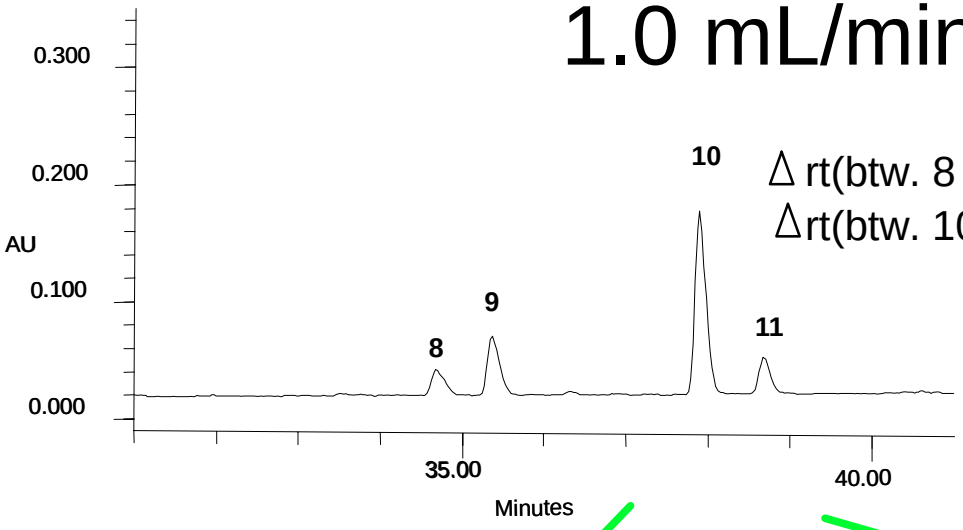
Robustness - a measure of a method's capacity to remain unaffected by small but deliberate variation in method parameters and provides an indication of a method's reliability during normal usage.[2]

Parameters Evaluated for the Determination of Method Robustness:

- Flow Rate
- Temperature
- Gradient Slope
- % Modifier in Solvent A
- % Modifier in Solvent B

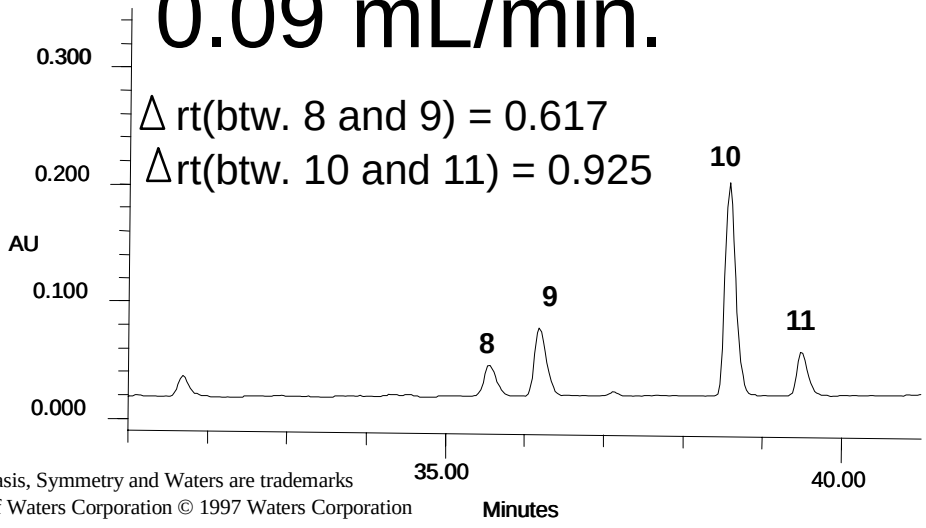
Effect of Slight Changes in Flow Rate

1.0 mL/min.



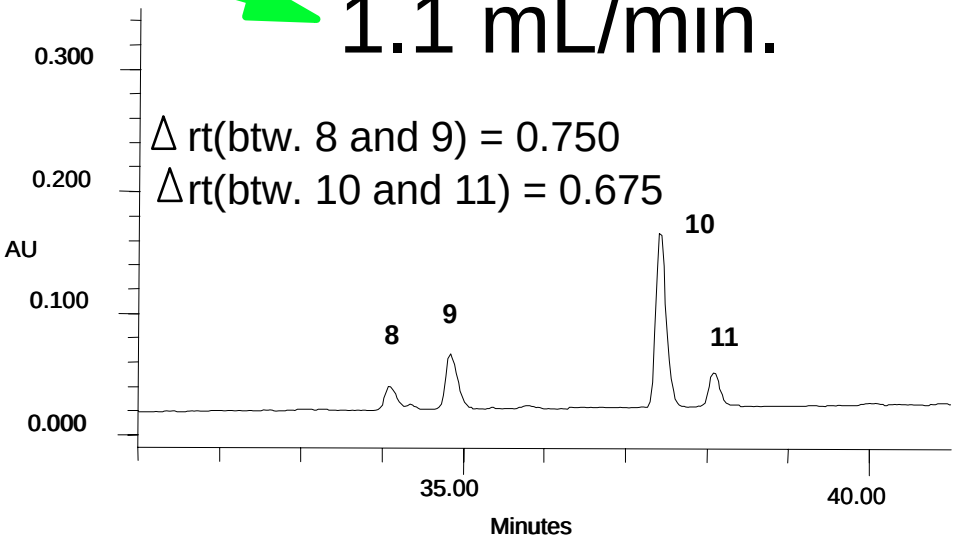
Δ rt(btw. 8 and 9) = 0.692
Δrt(btw. 10 and 11) = 0.791

0.09 mL/min.



Δ rt(btw. 8 and 9) = 0.617
Δrt(btw. 10 and 11) = 0.925

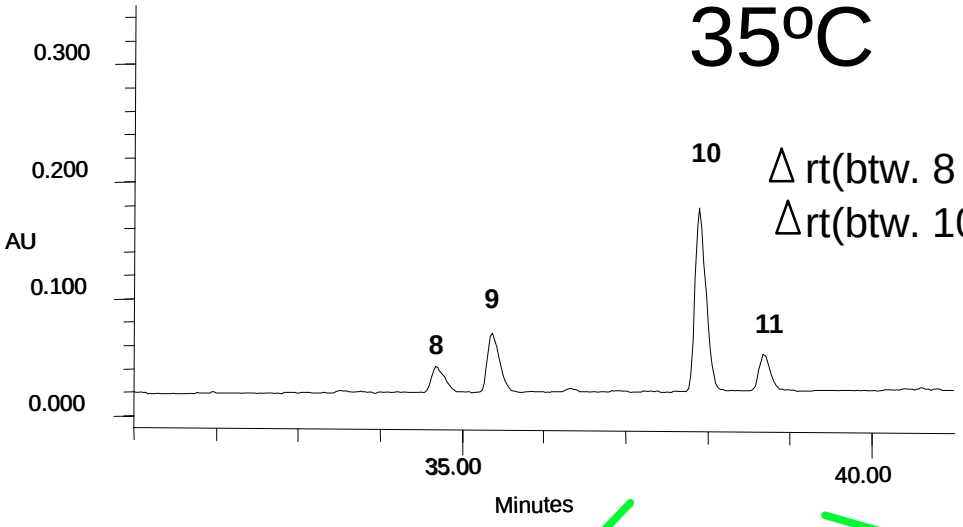
1.1 mL/min.



Δ rt(btw. 8 and 9) = 0.750
Δrt(btw. 10 and 11) = 0.675

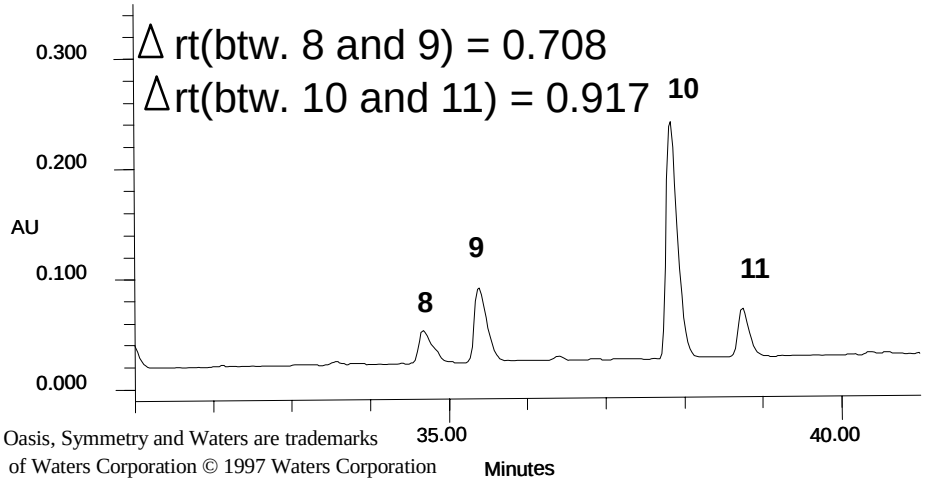
Effect of Slight Changes in Temperature

35ºC



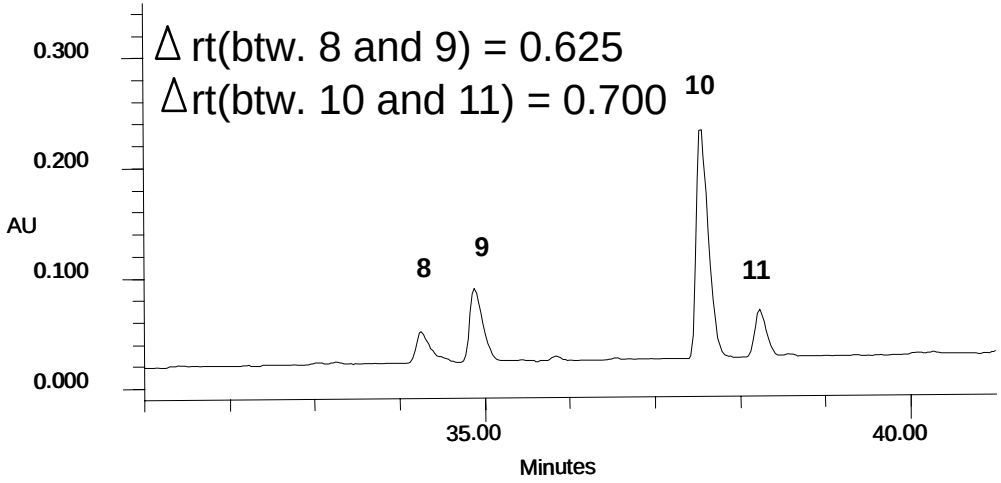
Δ rt(btw. 8 and 9) = 0.692
Δrt(btw. 10 and 11) = 0.791

33ºC



Δ rt(btw. 8 and 9) = 0.708
Δrt(btw. 10 and 11) = 0.917

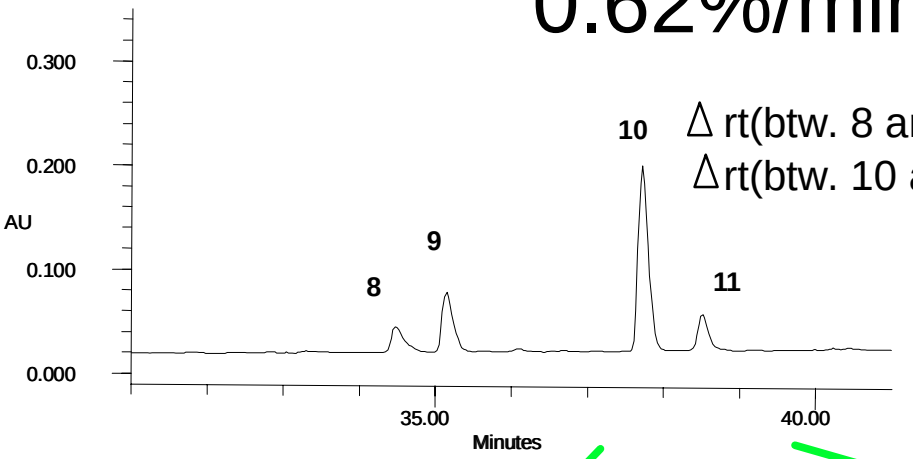
37ºC



Δ rt(btw. 8 and 9) = 0.625
Δrt(btw. 10 and 11) = 0.700

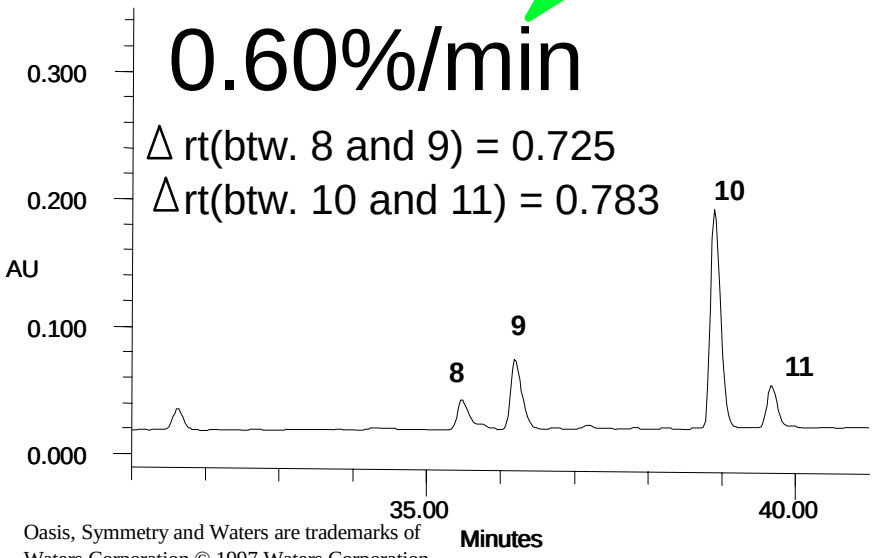
Effect of Slight Changes in Gradient Slope

0.62%/min



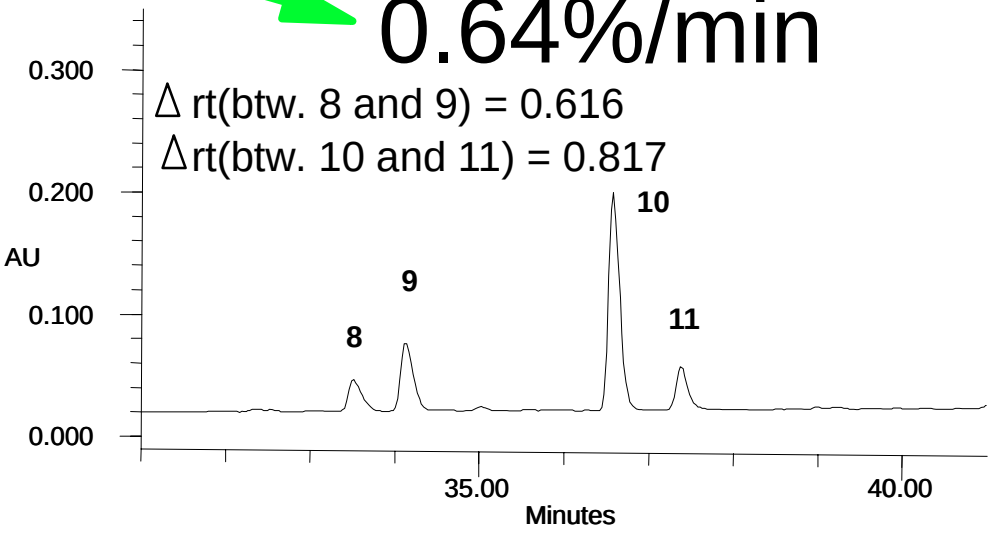
Δ rt(btw. 8 and 9) = 0.667
Δrt(btw. 10 and 11) = 0.800

0.60%/min



Δ rt(btw. 8 and 9) = 0.725
Δrt(btw. 10 and 11) = 0.783

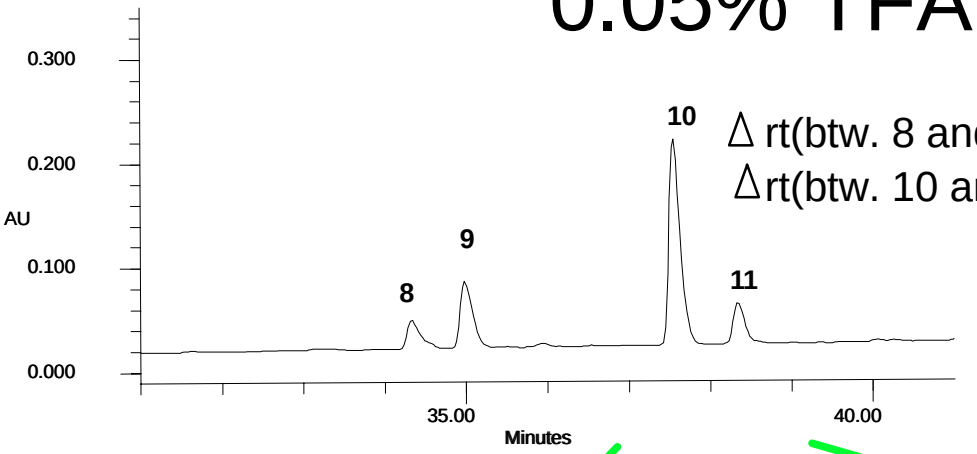
0.64%/min



Δ rt(btw. 8 and 9) = 0.616
Δrt(btw. 10 and 11) = 0.817

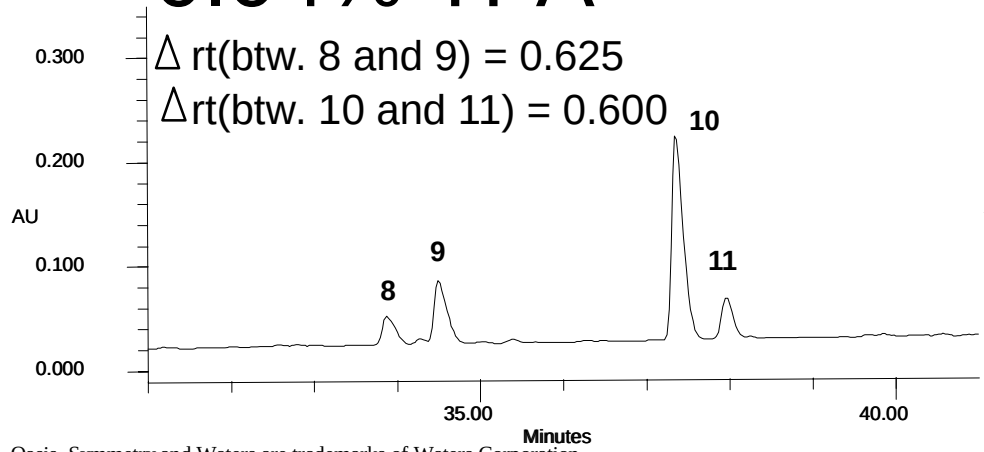
Effect of Slight Changes in TFA Conc. in Solvent A

0.05% TFA in Solvent A



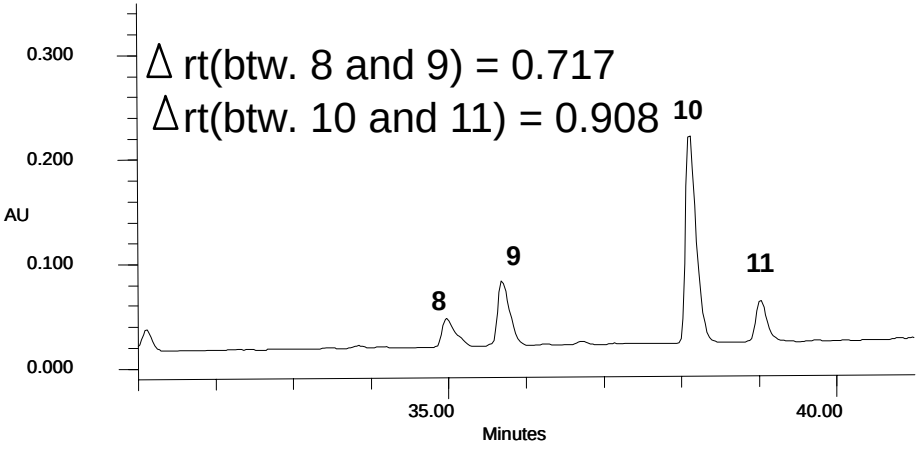
Δ rt(btw. 8 and 9) = 0.658
Δrt(btw. 10 and 11) = 0.791

0.04% TFA



Δ rt(btw. 8 and 9) = 0.625
Δrt(btw. 10 and 11) = 0.600

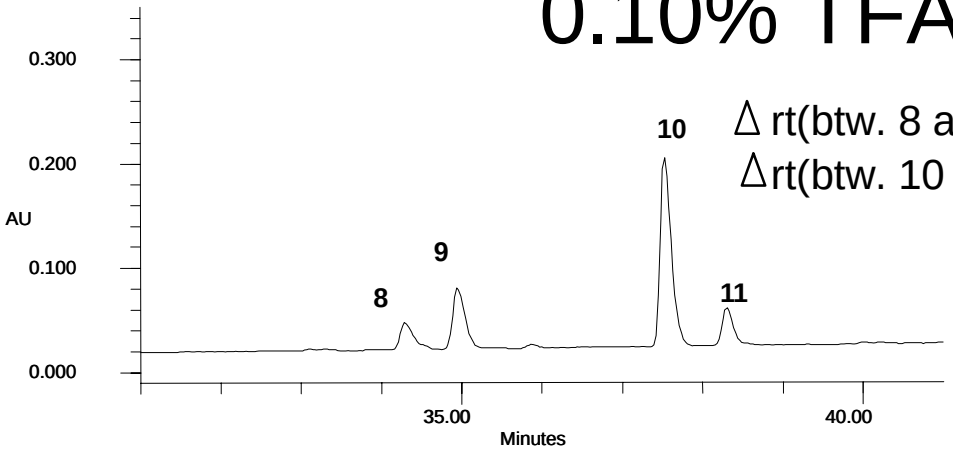
0.06% TFA



Δ rt(btw. 8 and 9) = 0.717
Δrt(btw. 10 and 11) = 0.908

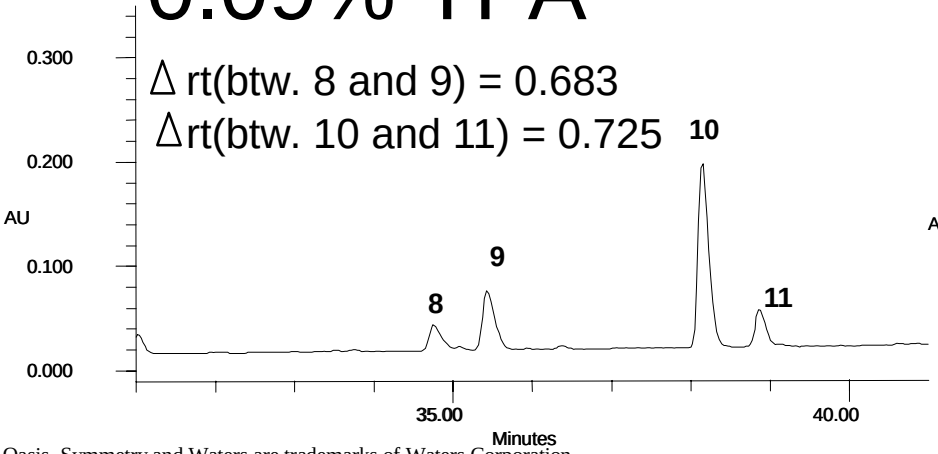
Effect of Slight Changes in TFA Conc. in Solvent B

0.10% TFA in Solvent B



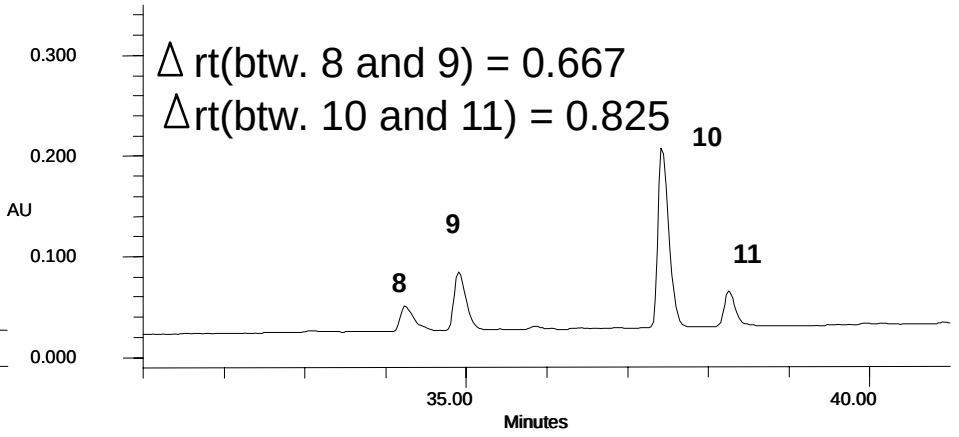
Δ rt(btw. 8 and 9) = 0.658
Δrt(btw. 10 and 11) = 0.775

0.09% TFA



Δ rt(btw. 8 and 9) = 0.683
Δrt(btw. 10 and 11) = 0.725

0.11% TFA



Δ rt(btw. 8 and 9) = 0.667
Δrt(btw. 10 and 11) = 0.825

Summary of Robustness Evaluation

- If the change in retention times of the peptide peaks is closely monitored as parameters are changed, general trends can be observed.

- However, if the change in retention times between certain peptide pairs is monitored, one will observe that certain parameters (flow rate, gradient slope etc.) affect some peptides more than others thereby changing important factors such as resolution between peak pairs.

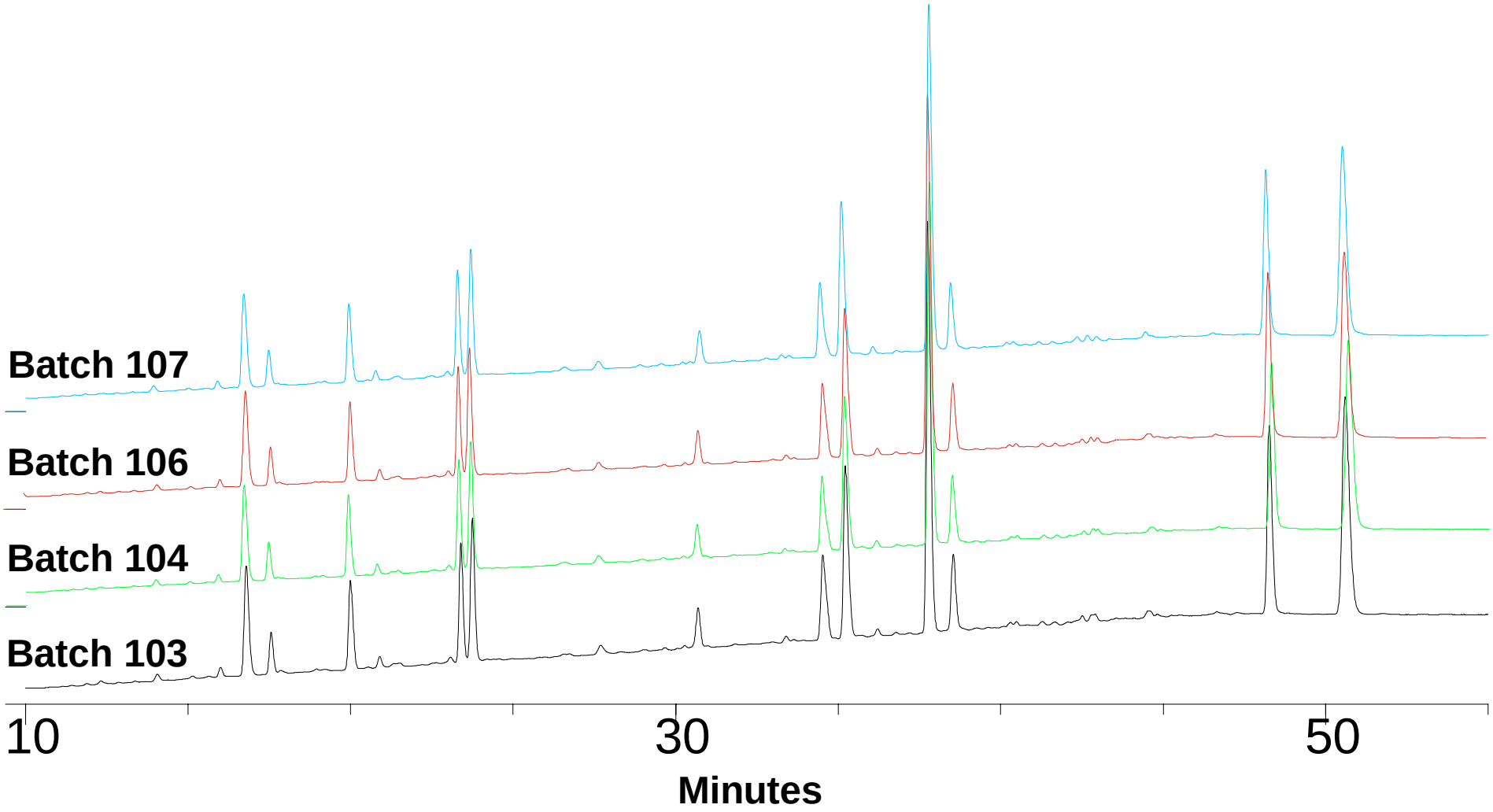
Reproducibility

- **Validated RP-HPLC assays require HPLC columns to be reproducible from column-to-column (shown in Table 1) and batch-to-batch.**

- **Columns which perform reproducibly in terms of selectivity and separation characteristics from batch-to-batch ensures reliable, reproducible and robust assays over the life of the product.**

Batch-to-Batch Reproducibility

Symmetry300™, C_{18}, 5 µm



Conclusion

- There are several factors to assess when validating a RP-HPLC method:
  - The first step is to establish what information you want to obtain from applying said method (quantitative or qualitative)
  - Document the intended application in a validation proposal; include acceptance criteria
  - Thoroughly evaluate those parameters you've identified as being most informative about the variability of the method (i.e. flow rate, gradient Slope etc.)

Conclusion (continued)

- Consider the batch-to-batch reproducibility of the column. It is of the utmost importance to evaluate when developing a validated/transferable method. Symmetry300™ has unsurpassed Batch-to Batch reproducibility.

- Symmetry300™ is the only column in the industry where the batch-to-batch reproducibility is tested by not only a small molecule test but also a protein tryptic digest.