Bioanalysis

Stereospecific Drug Metabolite Analysis using 2D LC/MS Q-TOF

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Today's Presenters:

Murali Subramanian
Senior Principal Investigator
Biocon Bristol-Myers Squibb Research Centre
(Syngene International)

Smriti Khera
Global Pharmaceutical
Segment Manager
Agilent Technologies, Inc.

Syed Salman Lateef
Lead Applications Scientist, Life Science
Center India
Agilent Technologies India Pvt Ltd.

Jens Trafkowski
Product Manager Analytical HPLC
Agilent Technologies, Inc.

Today's Host:

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Production Editor
FSG publishers of *Bioanalysis*

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Role of 2D LC in Chiral Drug Metabolite Analysis

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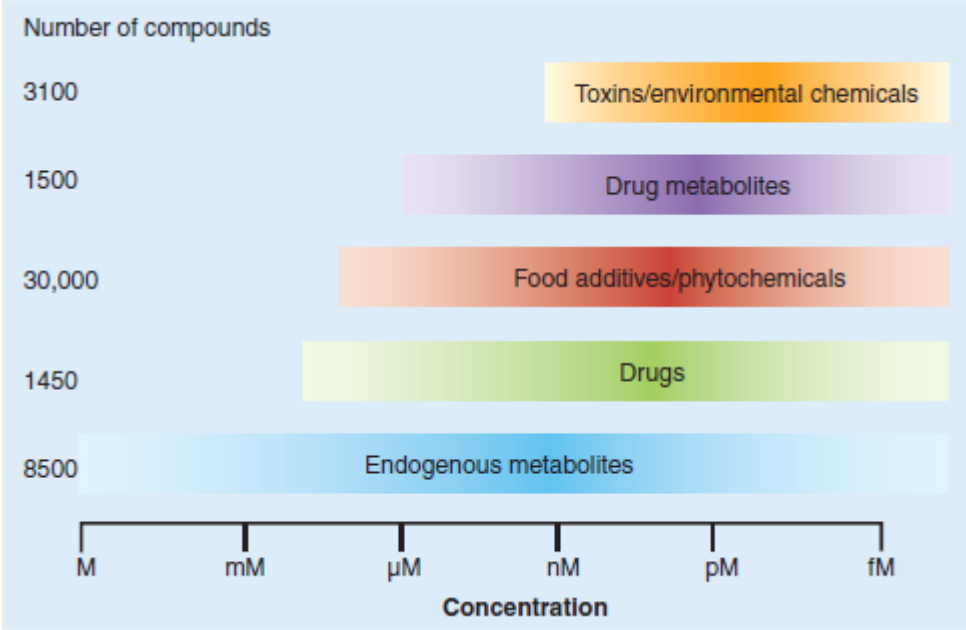
Analytical Challenges Encountered During Drug Metabolite Analysis

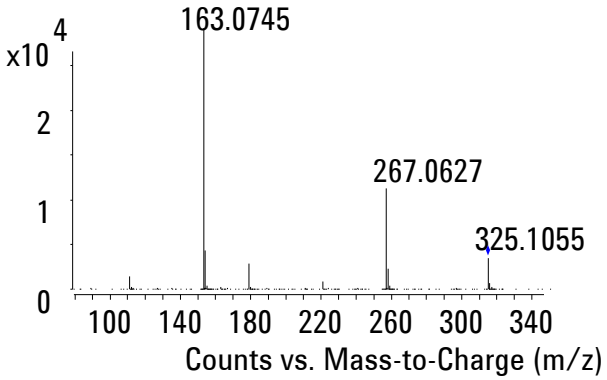
- Complex matrix, matrix removal issues, matrix ion suppression
- Dynamic range issues – Parent vs. Metabolite
  - Need to monitor parent disappearance and metabolite formation
- Dilute analyte (metabolites) and limited sample
  - High sensitivity (nM to fM) is necessary
- Similar physicochemical properties of oxidative metabolites resulting in co-elution
- Isobaric metabolites and subtle or no differences in fragmentation by MS



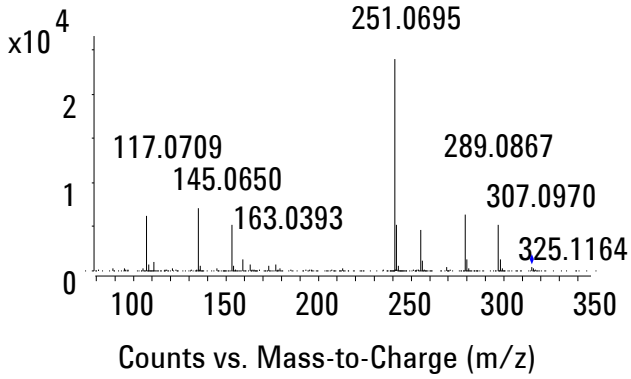
E.g. Warfarin metabolites: Isobaric and with Similar Fragmentation

4-OH and 10-OH has unique fragmentation pattern.
6-, 7- and 8-Hydroxy warfarin affords a similar fragmentation pattern

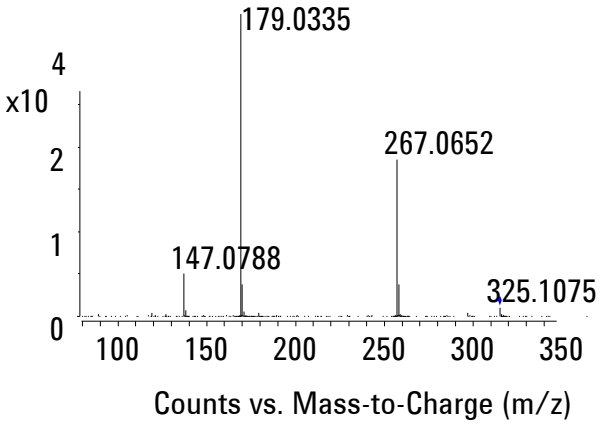
4-Hydroxy warfarin (4-OH)



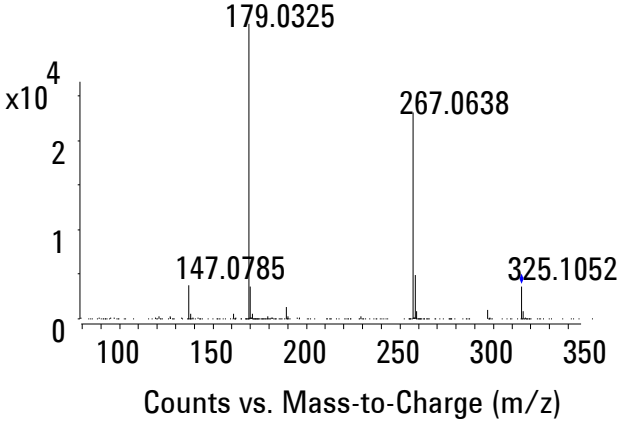
10-Hydroxy warfarin (10-OH)



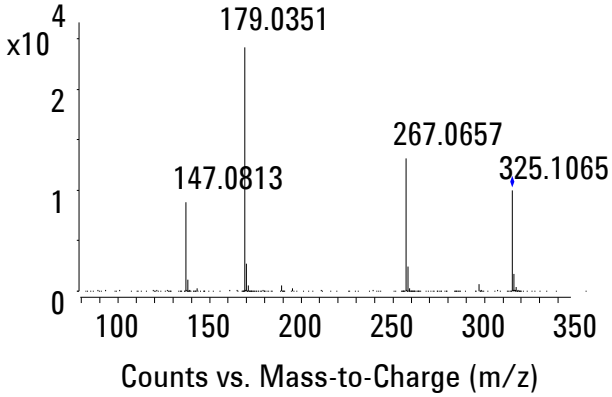
6-Hydroxy warfarin (6-OH)



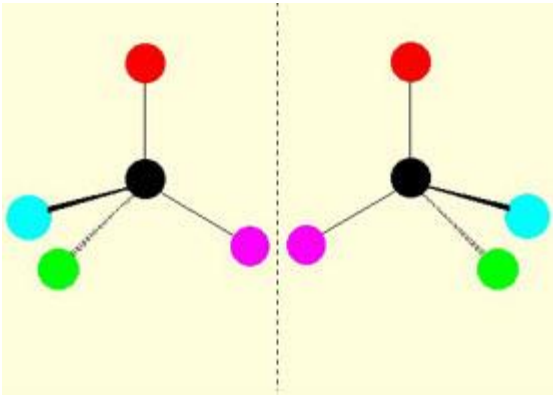
7-Hydroxy warfarin (7-OH)



8-Hydroxy warfarin (8-OH)



Importance of Chiral Methods of Analysis



- ~50% of all marketed SM drugs are chiral
  - 1980s study, Hutt and O'Grady,

  Journal of Antimicrobial Chemotherapy (1996) 37, 7-32
- PK/PD studies needed on the active enantiomer
- In vivo chiral inversion also a concern
- FDA regulatory guidance for stereoisomers/racemic drugs (1992)
- "To evaluate the pharmacokinetics of a single enantiomer or mixture of enantiomers, manufacturers should develop ***quantitative assays for individual enantiomers in in vivo samples early in drug development.*** This will allow assessment of the potential for interconversion and the absorption, distribution, biotransformation, and excretion (ADBE) profile of the individual isomers. When the drug product is a racemate and the pharmacokinetic profiles of the isomers are different, ***manufacturers should monitor the enantiomers individually*** to determine such properties as dose linearity and the effects of altered metabolic or excretory function and drug-drug interactions. If the pharmacokinetic profile is the same for both isomers or a fixed ratio between the plasma levels of enantiomers is demonstrated in the target population, an achiral assay or an assay that monitors one of the stereoisomers should suffice for later evaluation. ***In vivo measurement of individual enantiomers should be available to help assess toxicologic findings***, but if this cannot be achieved, it would be sufficient in some cases to establish the kinetics of the isomers in humans.
  - http://www.fda.gov/drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm122883.htm

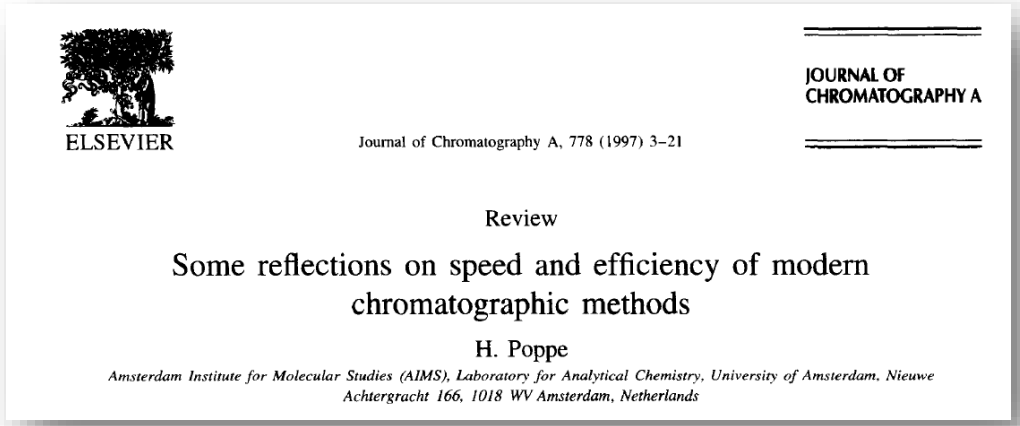
Potential Separation Solutions for the Chiral Resolution Challenge

- Ion mobility mass spectrometry
- SFC: orthogonal selectivity and different elution order, short run times
  - Optimize MS sensitivity for SFC compared to UHPLC
  - Use of very high flow rates, typically split flow prior to MS
  - Normal phase separation, limitation on use of water in injection and mobile phase.
  - Interest in SFC and ongoing evaluations for bioanalytical applications

- Need for peak resolution - complex matrix, dilute analytes, limited sample
- **2D LC (LC-LC) or (LCXLC):**
  - Takes the proven power of RPLC in DMPK studies and boosts resolving power
  - Heart cutting ideal for matrix removal
  - Simple set up for any UHPLC user!
  - In the past, data analysis and system set-up was a challenge limiting its wide spread use

Eric Lesellier, ***Bioanalysis*** (2011) 3(2)

General Thoughts in Separation Science



ELSEVIER

Journal of Chromatography A, 778 (1997) 3–21

JOURNAL OF CHROMATOGRAPHY A

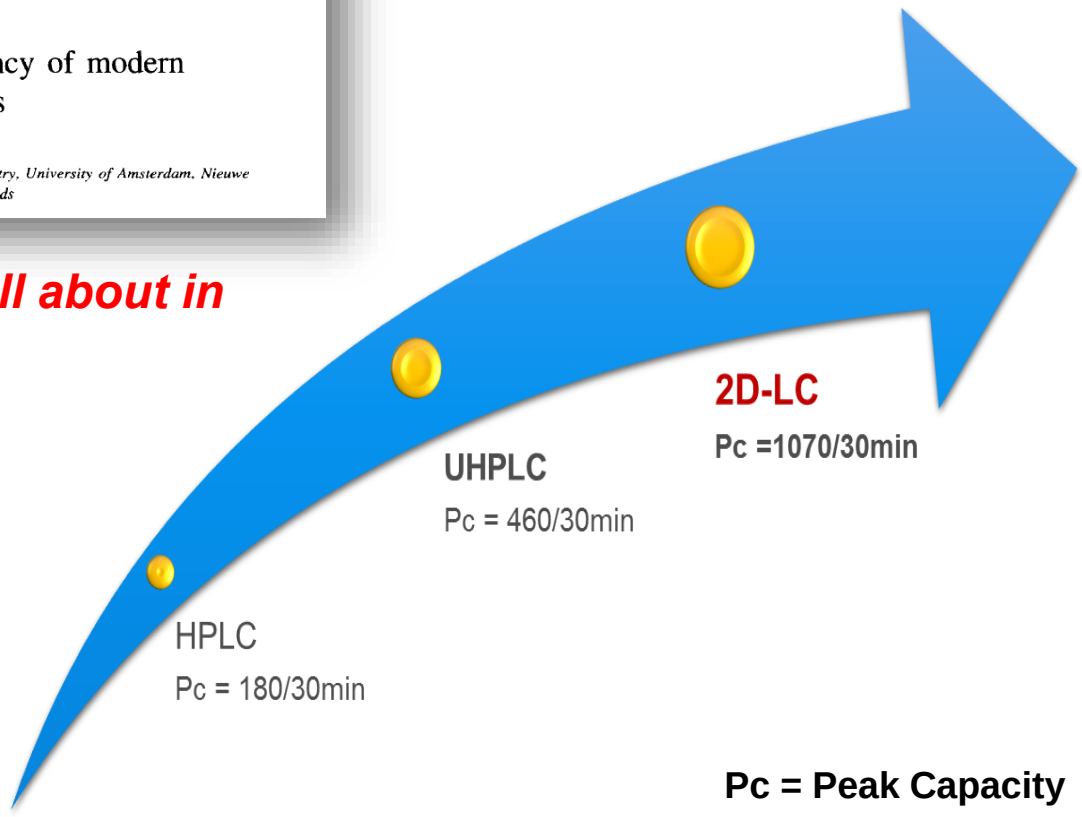
Review

Some reflections on speed and efficiency of modern chromatographic methods

H. Poppe

Amsterdam Institute for Molecular Studies (AIMS), Laboratory for Analytical Chemistry, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, Netherlands

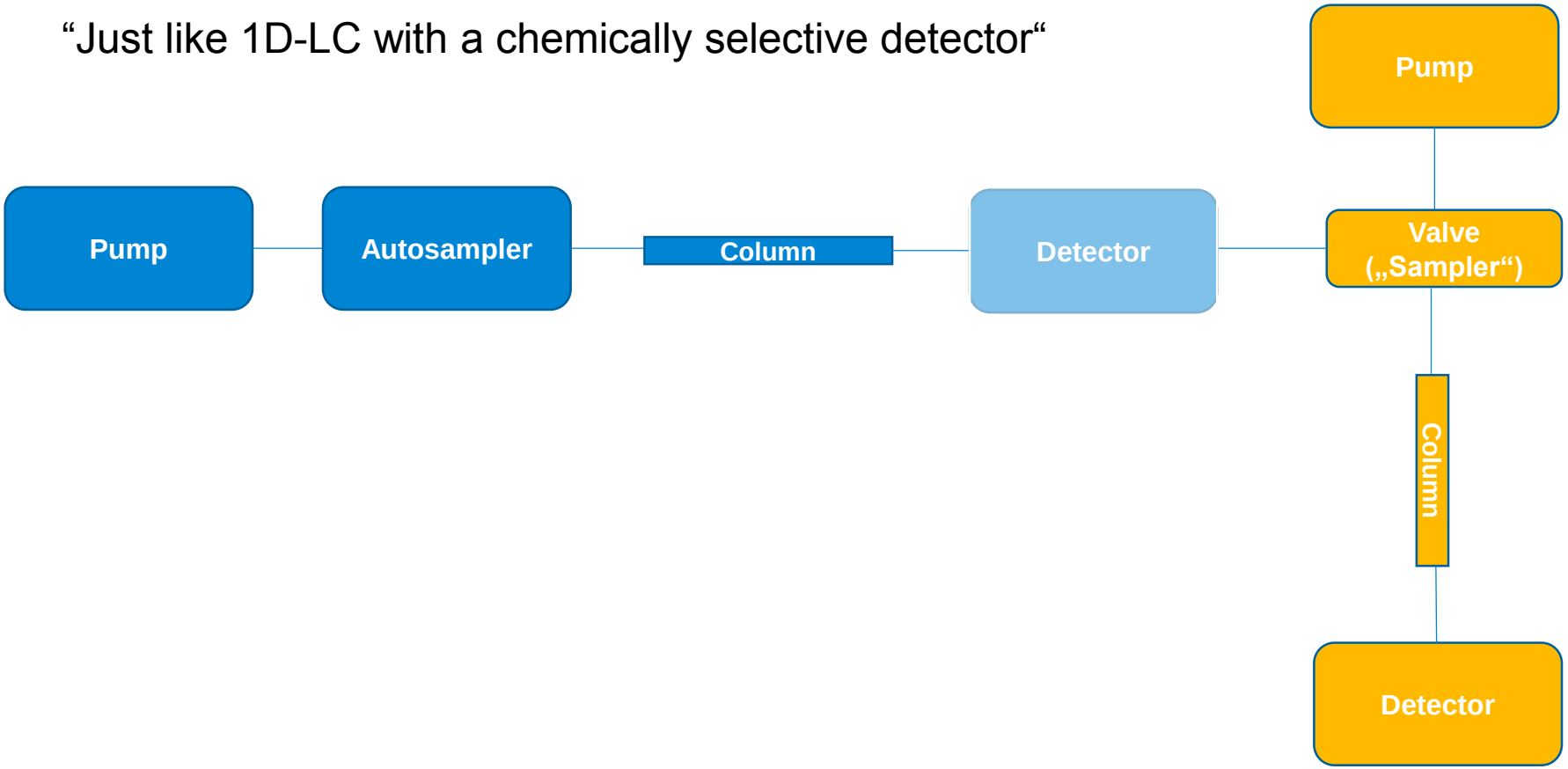
„Resolving power is what it is all about in analytical separation science.“



Pc = Peak Capacity

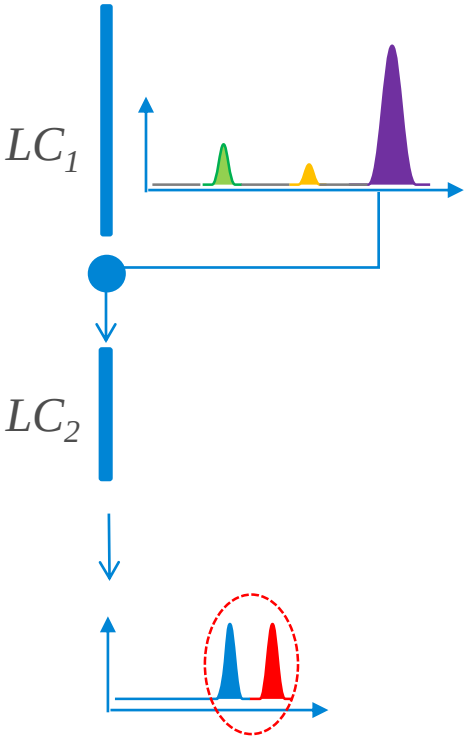
Bringing in a 2nd Dimension

"Just like 1D-LC with a chemically selective detector"



2D-LC – Heart-cutting vs Comprehensive 2D-LC

Heart-cutting 2D-LC (LC-LC):



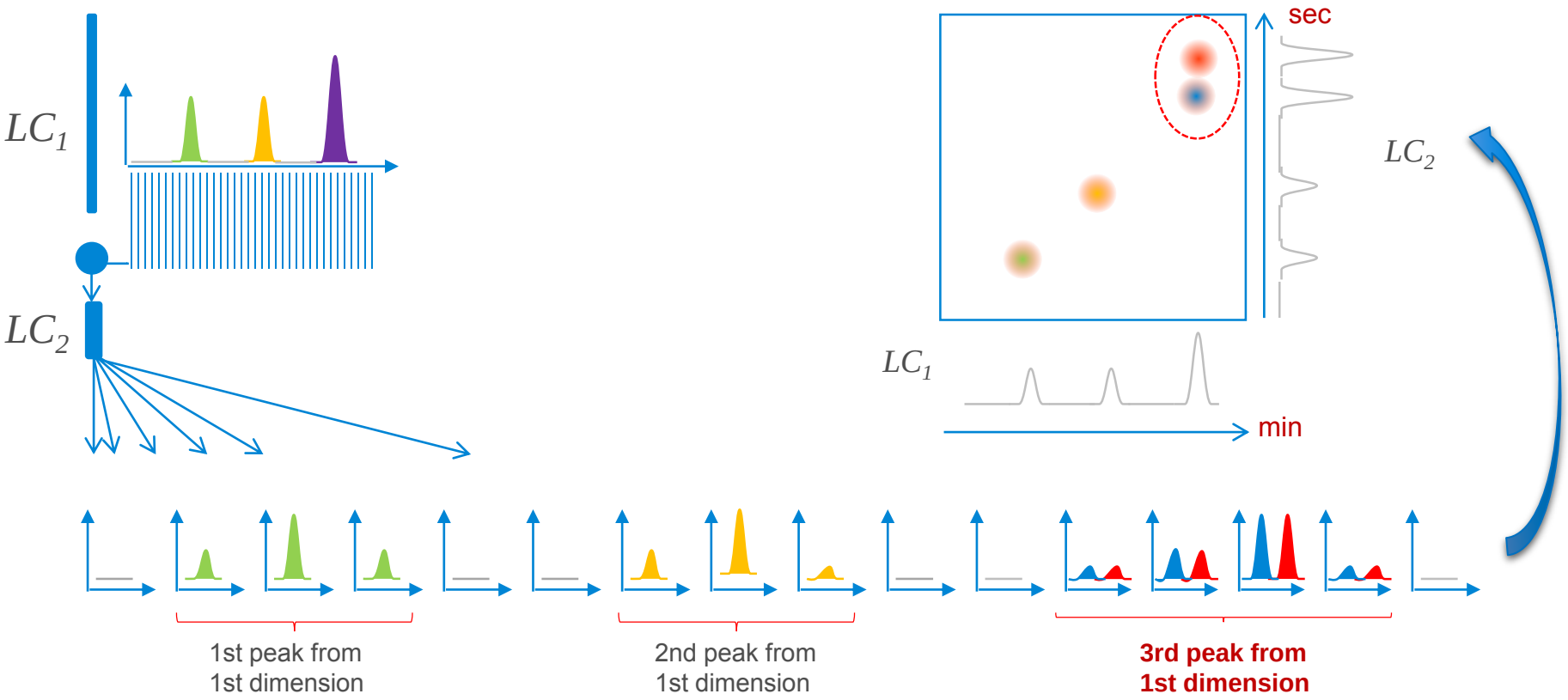
Parts of the 1D effluent are injected onto 2D system

Long 2D gradients possible → good data quality

Limited 2D information

2D-LC – Heart-cutting vs Comprehensive 2D-LC

Comprehensive 2D-LC (LCxLC):



Chiral Drug Metabolite Analysis

~50% of all marketed SM drugs are chiral

Chiral inversion: Parent or metabolite

e.g. clopidogrel, 2-arylpropionic acid derivatives

NSAIDS- ibu, flunoxoprofen, naproxen

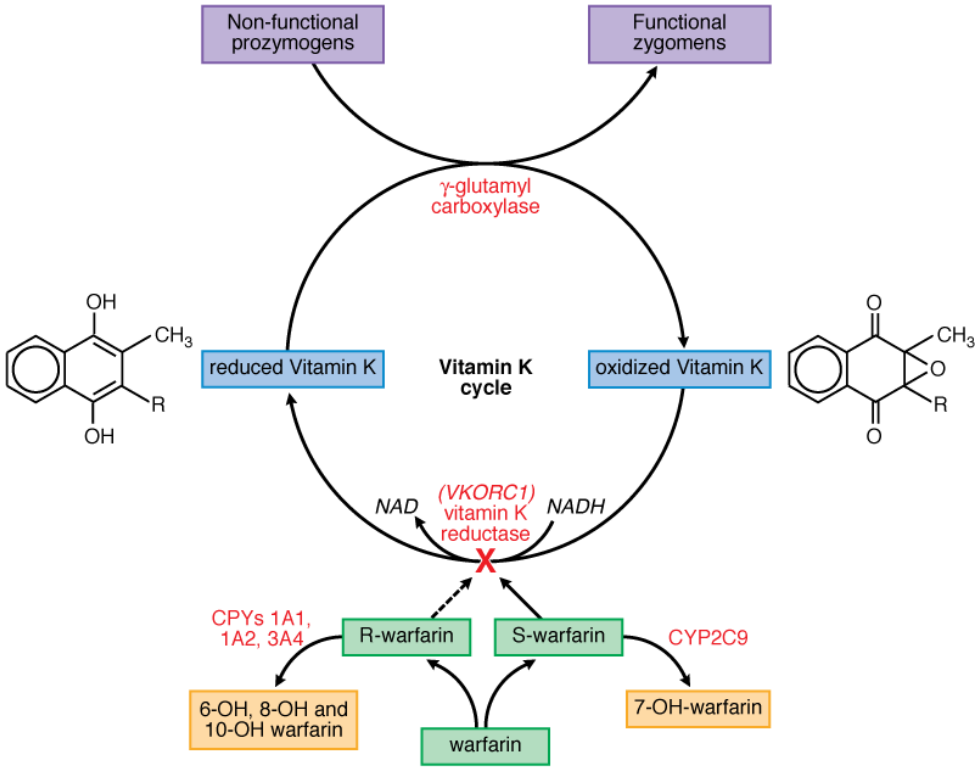
Achiral/Prochiral drug, chiral on metabolism

e.g Propanalol

Racemate drugs

Stereospecific metabolism

e.g. Warfarin



Source: Brunton LL, Chabner BA, Knollmann BC: *Goodman & Gilman's The Pharmacological Basis of Therapeutics, 12th Edition:* www.accessmedicine.com

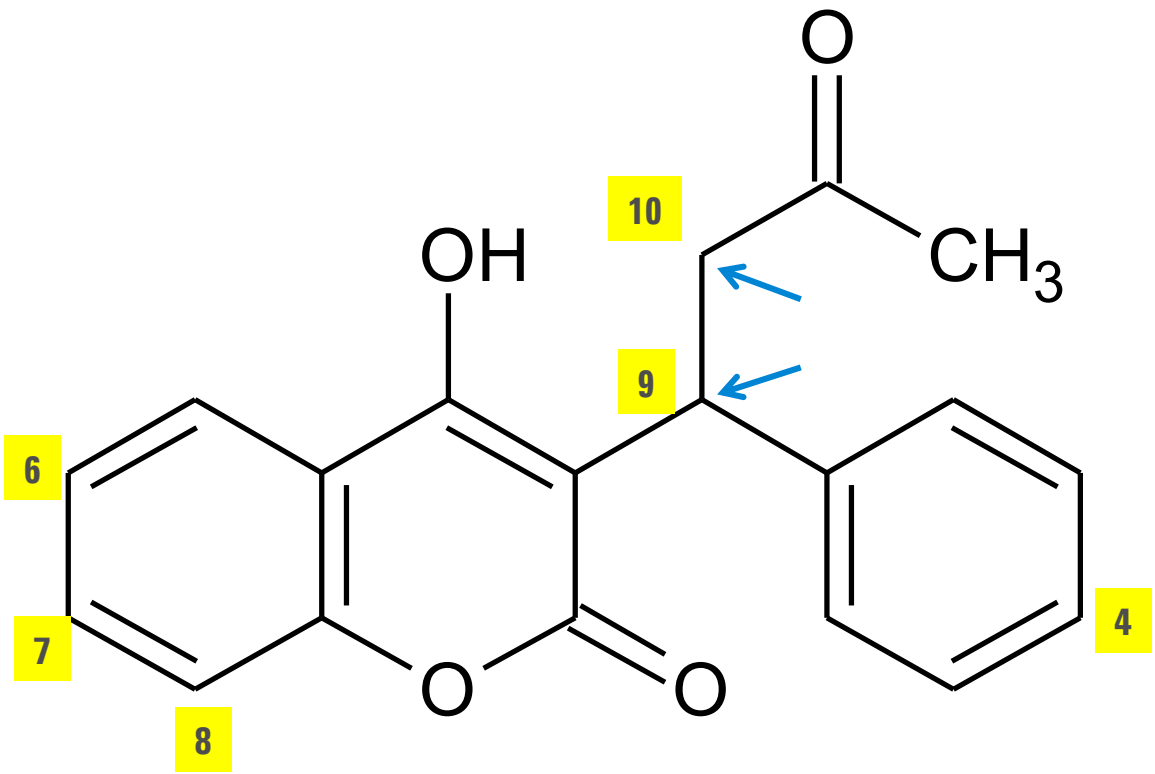
Stereospecific Analysis of Warfarin Oxidative Metabolism Using 2D LC/MS Q-TOF

Siji Joseph

Salman Lateef

LSCI Bangalore

Warfarin and Sites of Oxidative Metabolism



- Sites of warfarin hydroxylation (carbons 4, 6, 7, 8, 9 and 10 in yellow highlights)
- Stereo-centers (blue arrows) are marked

Warfarin Metabolites: Isobaric and with Similar Fragmentation

4-OH and 10-OH has unique fragmentation pattern.
6-, 7- and 8-Hydroxy warfarin affords a similar fragmentation pattern

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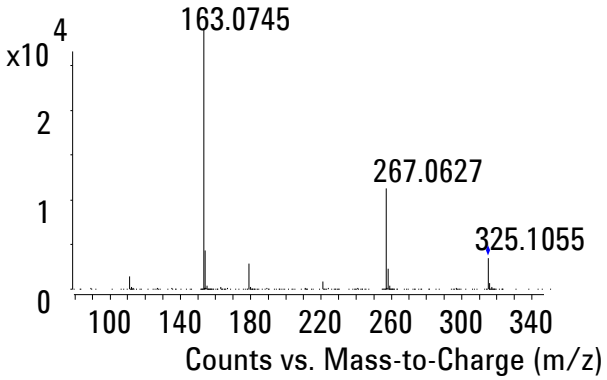
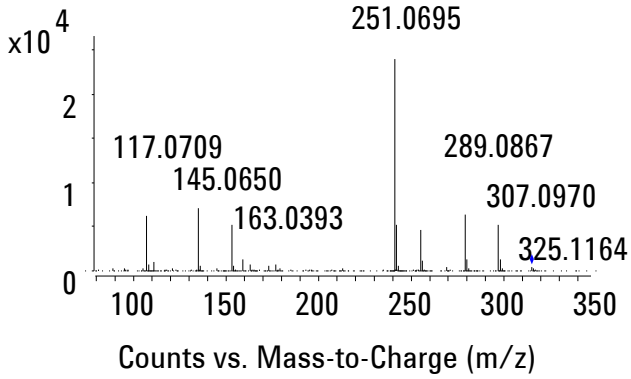


10-Hydroxy warfarin (10-OH)



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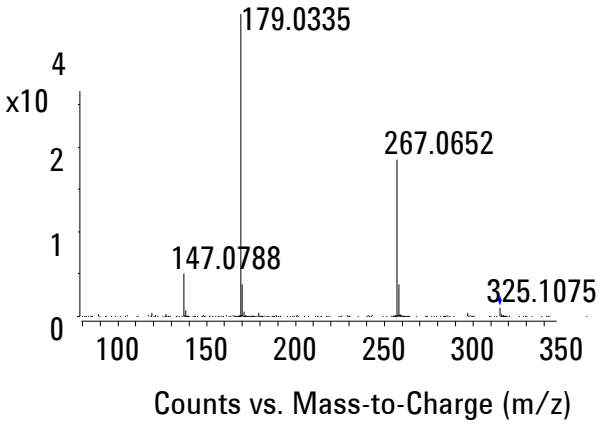

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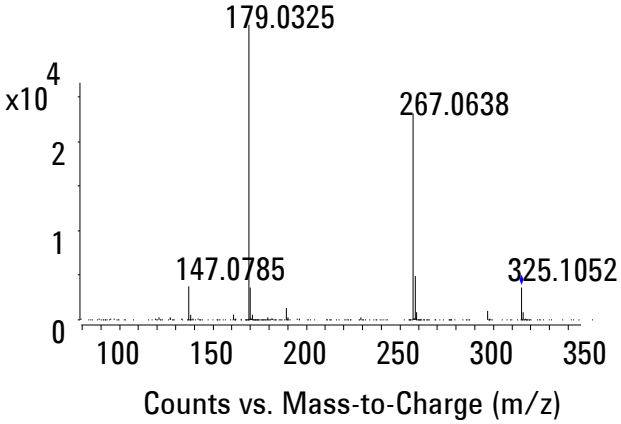

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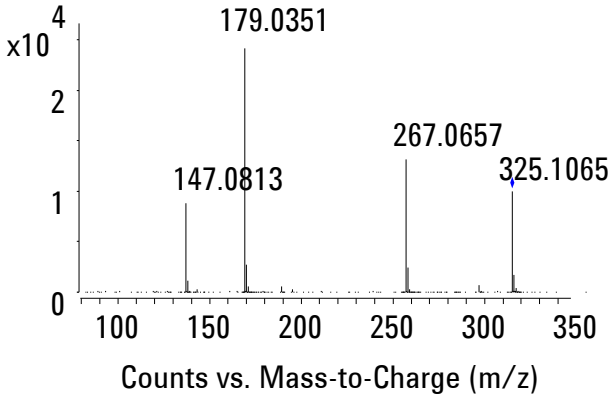
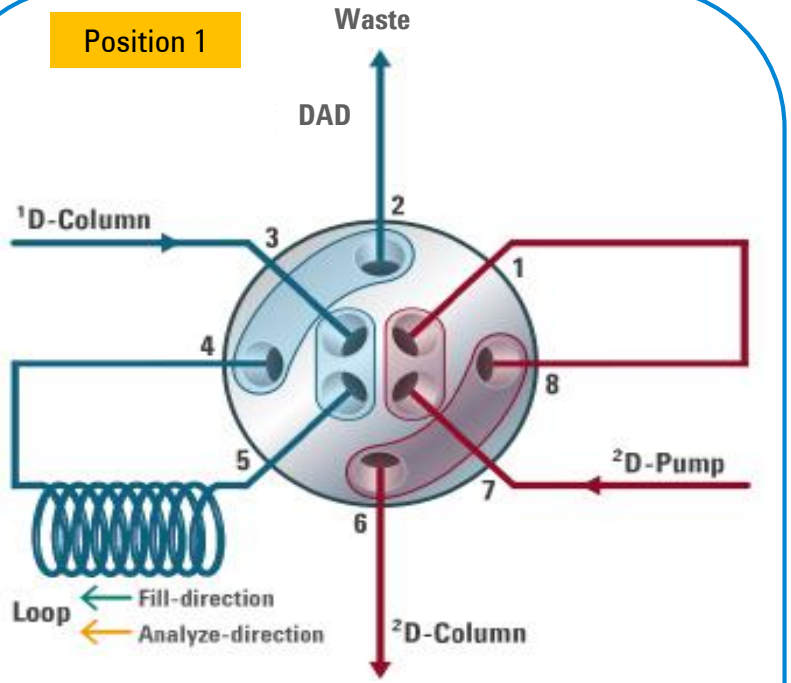


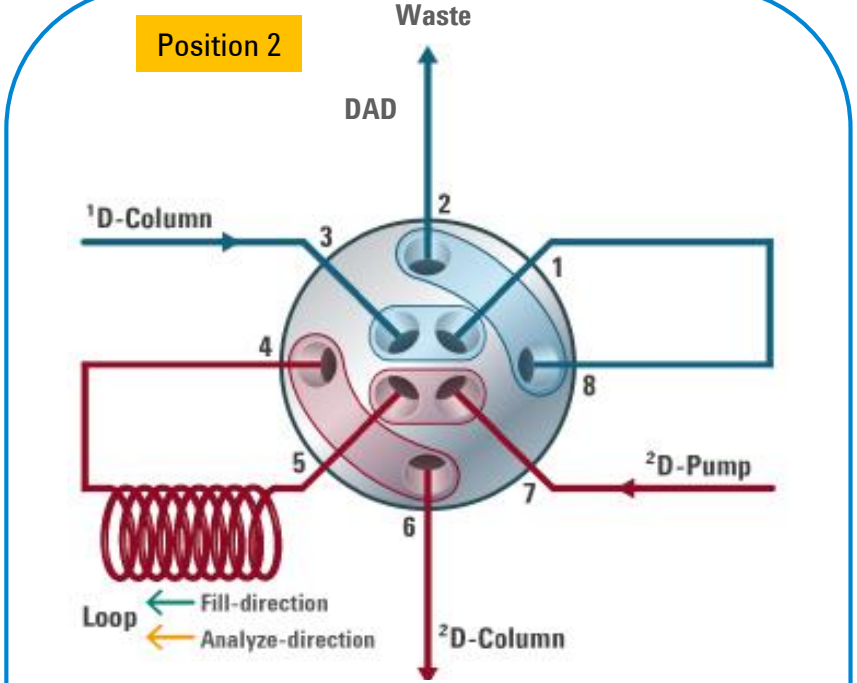
Instrumentation

Agilent 1290 Infinity 2D-LC system coupled with Agilent Accurate Mass Q-TOF LC/MS system.

2-position/4-port-duo Valve for Heart Cutting



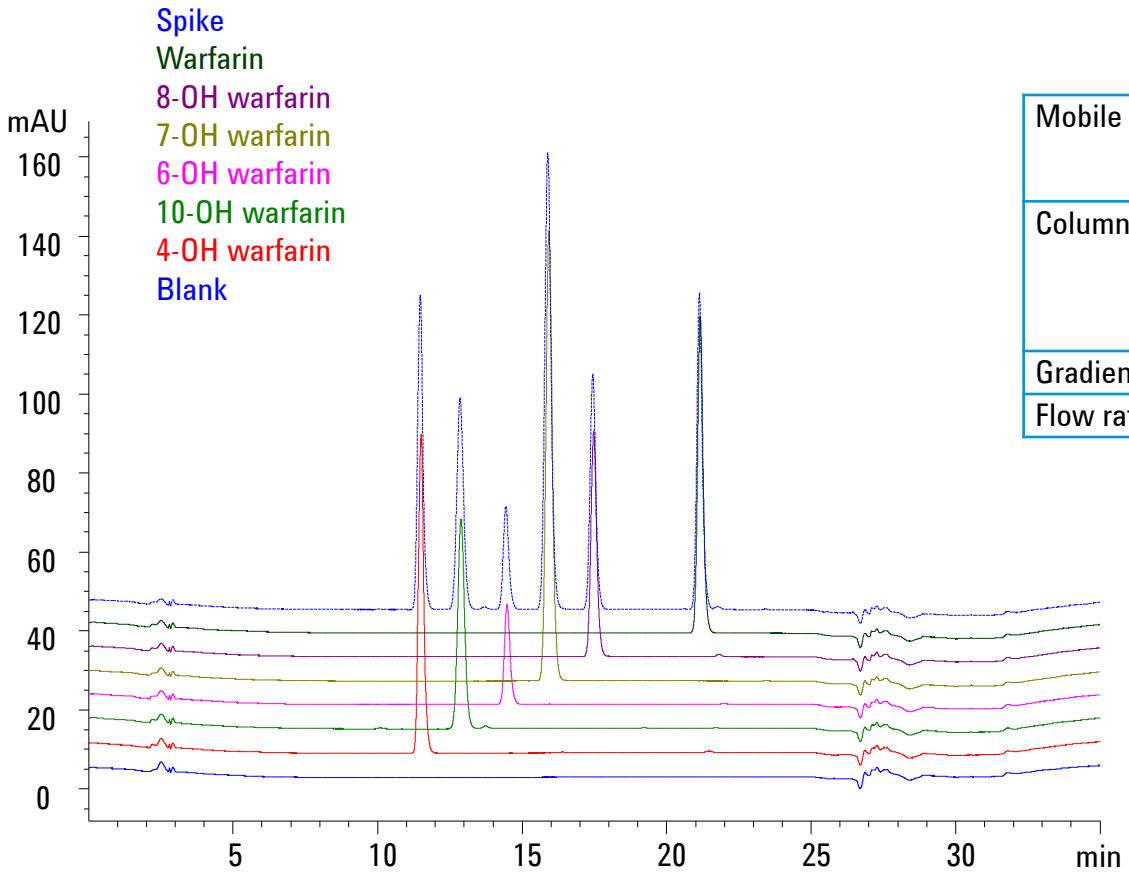
Position 2: 1D column eluent was directed onto a 80-µL loop (connected between the ports 4 and 5).



Position 1: 1D column eluent was directed to DAD and then to waste.

- After a defined time, the valve switches back to position 1 and the loop content was directed to 2D chiral column and then to the Q-TOF using pump 2.
- Second dimension column eluent was monitored by Q-TOF MS in All Ions MS/MS mode.
- Similar strategy was used to perform hear-cut for other peaks.

First Dimension LC-UV: Achiral Separation

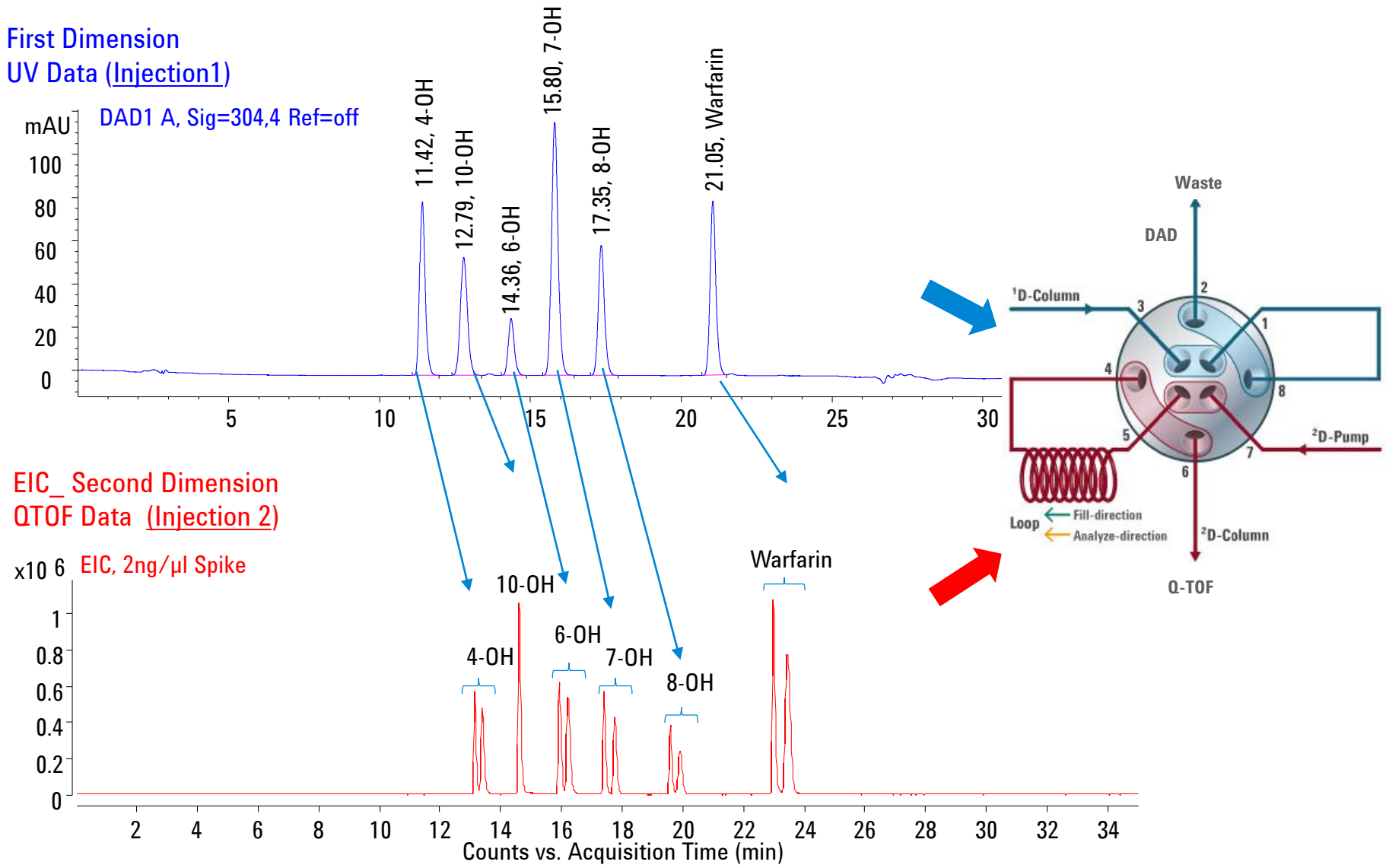


Overlay of individual and spike samples.
Y-axis off scale

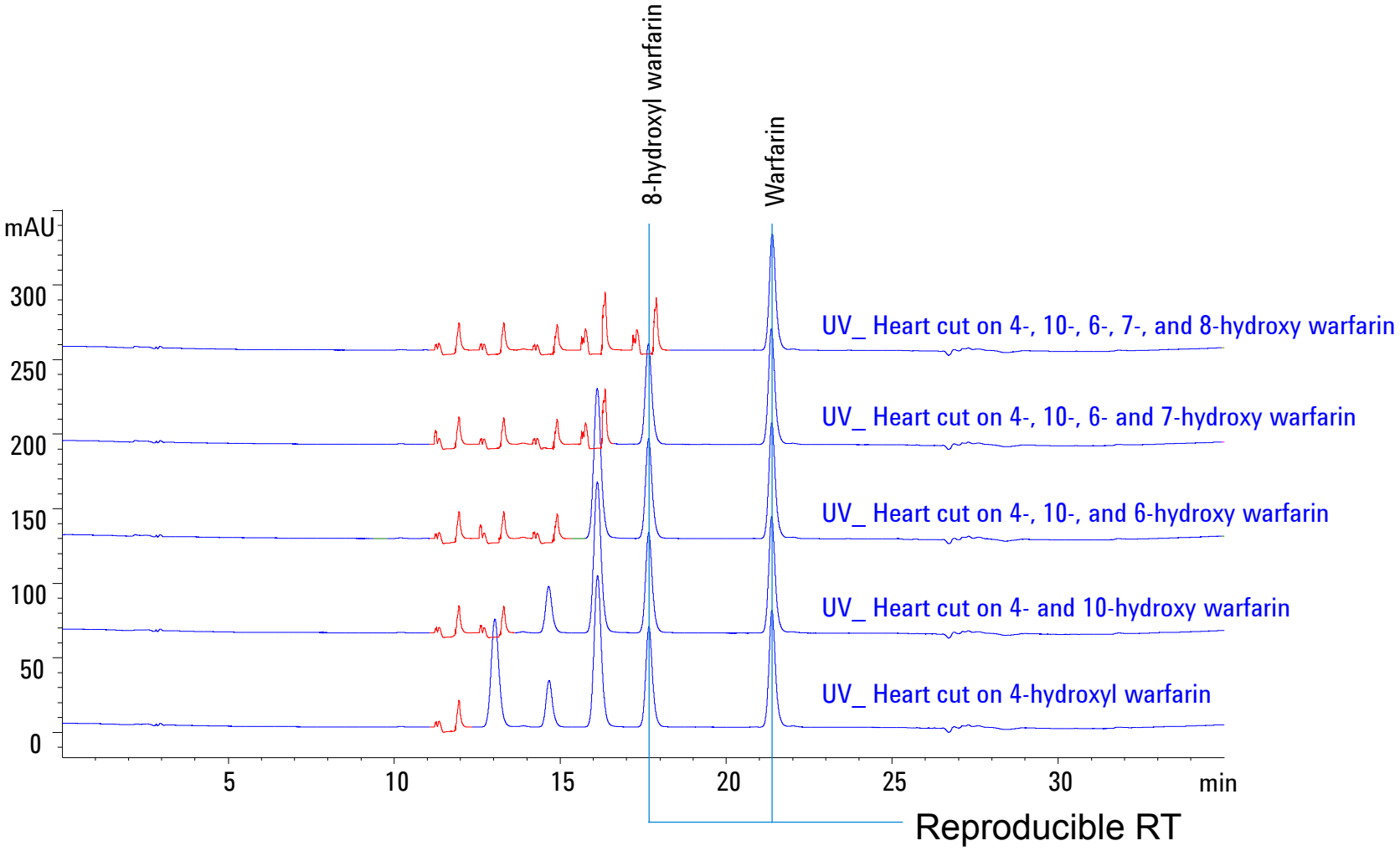
Mobile Phases	A: Formic acid in Water B: Formic acid in Methanol
Column:	Agilent ZORBAX Eclipse Plus Phenyl Hexyl, 2.1 x 150mm, 1.8 µm at 25°C
Gradient Time	25min
Flow rate	0.12 mL/min

Stereospecific Resolution in Second Dimension

Column: Chiral OD-3R (4.6 x 50mm, 3µm at 30°C)



Effect of Heart Cutting Valve Changes on RT of Later Eluting Peaks

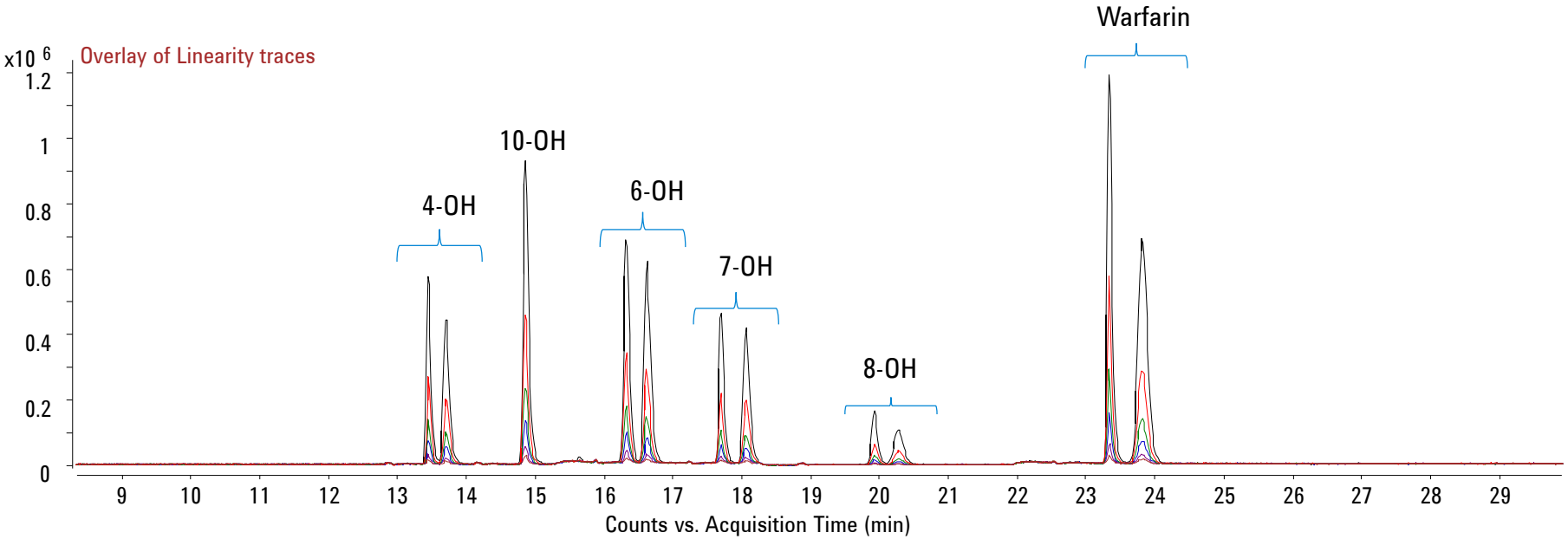


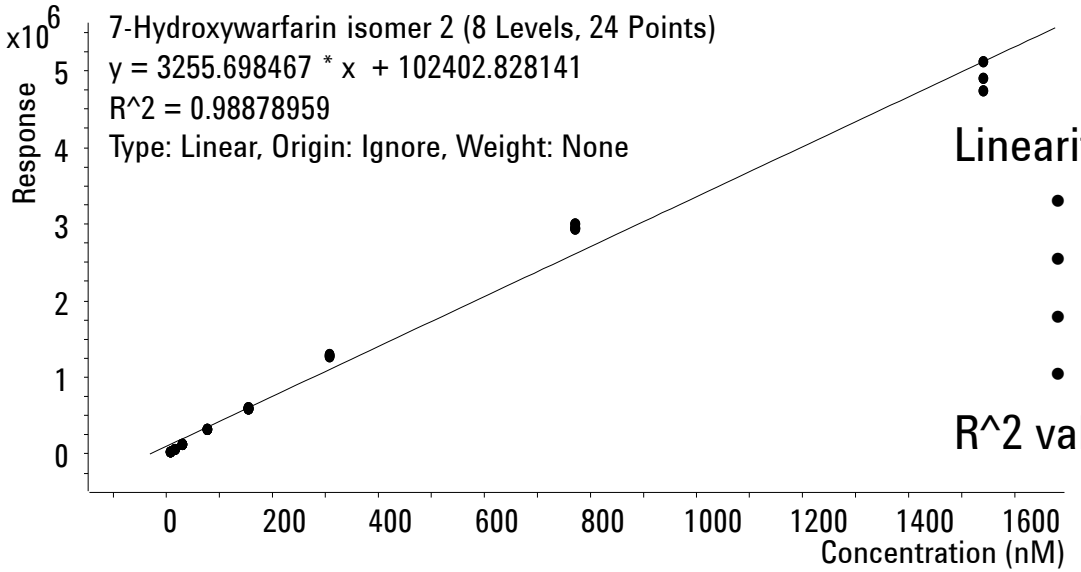
UV trace base line is shown in **red colour**

Warfarin retention time is very consistent with single and/or multi heart cuts.

This proves that, valve change is very smooth and does not affects elution of other peaks.

Linearity Results





Linearity study range

- 4-OH and 10-OH: 1.5 to 3083 nM
- 6-OH: 3.1 to 3083 nM
- 7-OH: 7.7 to 1622 nM
- 8-OH: 7.7 to 7708 nM

R^2 value for all metabolites were > 0.99

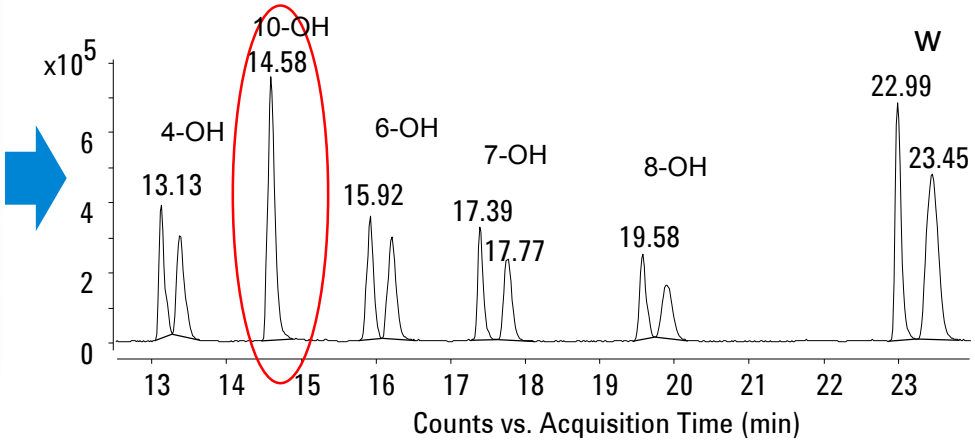
Method Summary

1st Method

1D: ZORBAX Eclipse Plus Phenyl Hexyl

2D Col: Chiral **OD-3R**

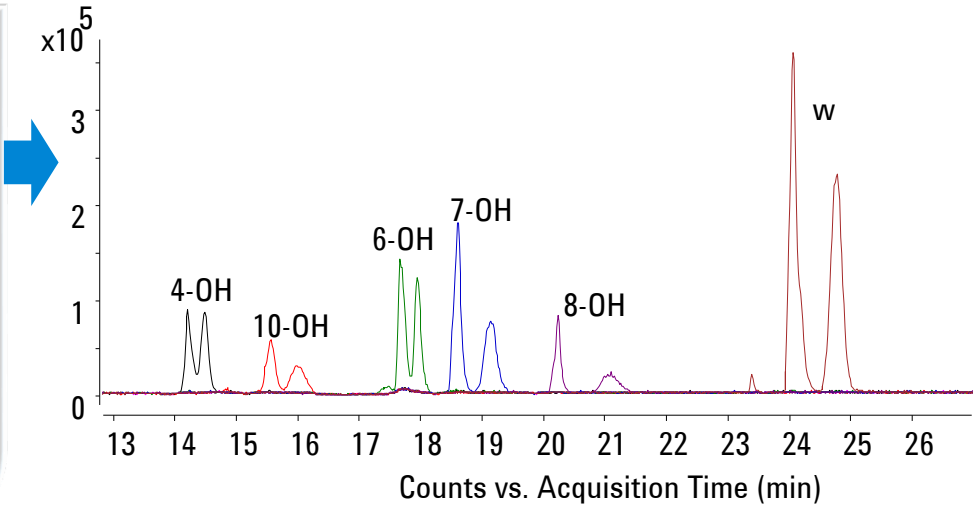
- 2D: Good separation for all isomer peaks except for 10-OH warfarin
- Best 2D sensitivity



2nd Method

2D Col: Chiral **OD-3R & AD-3R serially connected**

- 2D: Best separation for all isomer peaks

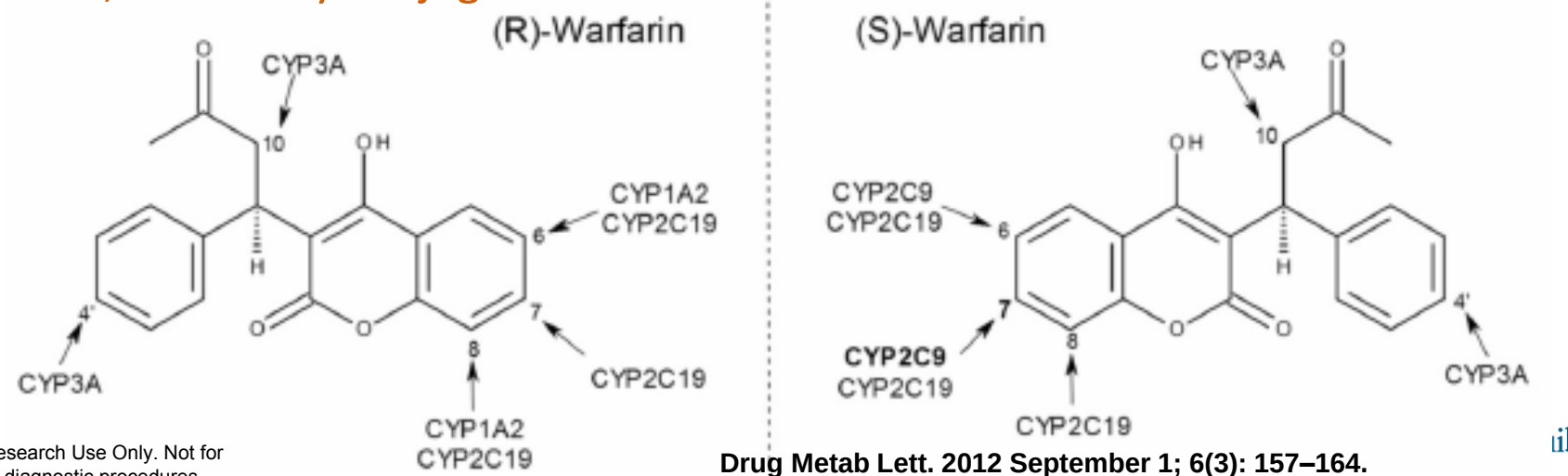


Microsomal and Hepatocyte Incubations (Performed at BBRC/Syngene)

Sample	Time points
Liver microsome incubation with 1. (*R/S*) Warfarin 2. (*R*) Warfarin 3. (*S*) Warfarin	a) 0min b) 15min c) 30min d) 60min e) 120min
hepatocytes incubations with (*R/S*) Warfarin	a) 24Hrs

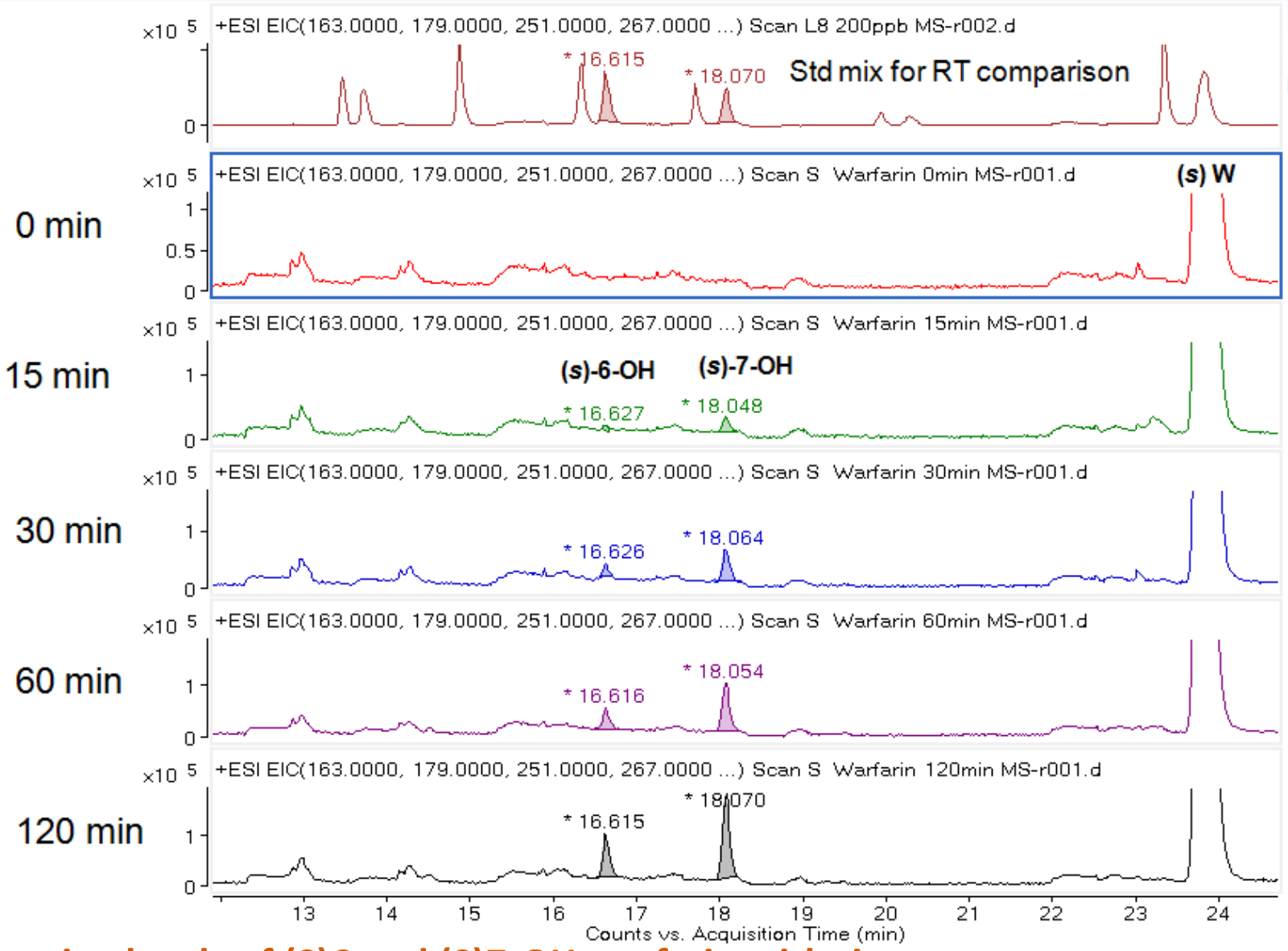
Warfarin Metabolism

- Warfarin metabolism is enantio- and regiospecific. Different metabolic profile of each enantiomer observed, and different CYP isoforms metabolize each stereoisomer. 20 unique metabolites detectable.
- S-Warfarin pathway: 7-OH>>4-OH~6-OH>10-OH~8-OH
  - 2C9 main isoform involved and 7-OH is the main pathway (80% of S-warfarin metabolism)
- R-Warfarin pathway: 10-OH>6-OH~7-OH~4-OH~8-OH
  - No predominant metabolite. 10-OH most abundant metabolized by CYP3A. CYP1A2 is also important in R-warfarin metabolism
- 10-hydroxylation and acetonyl reduction can create an additional chiral centers; secondary conjugation observed.



Drug Metab Lett. 2012 September 1; 6(3): 157–164.

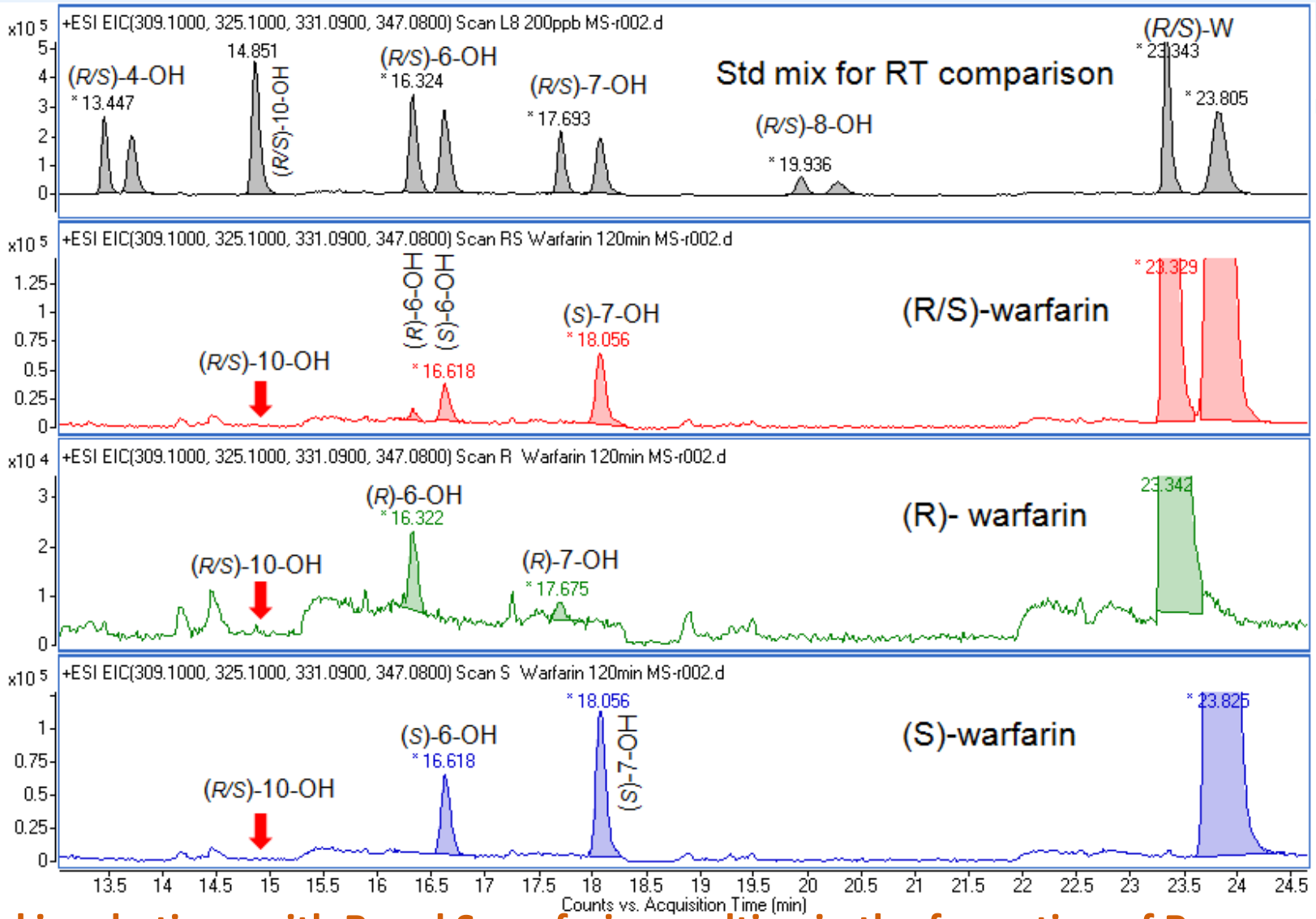
Microsomal Incubation with (S)-Warfarin



Increasing levels of (S)6 and (S)7-OH warfarin with time consistent with literature

Bristol-Myers Squibb

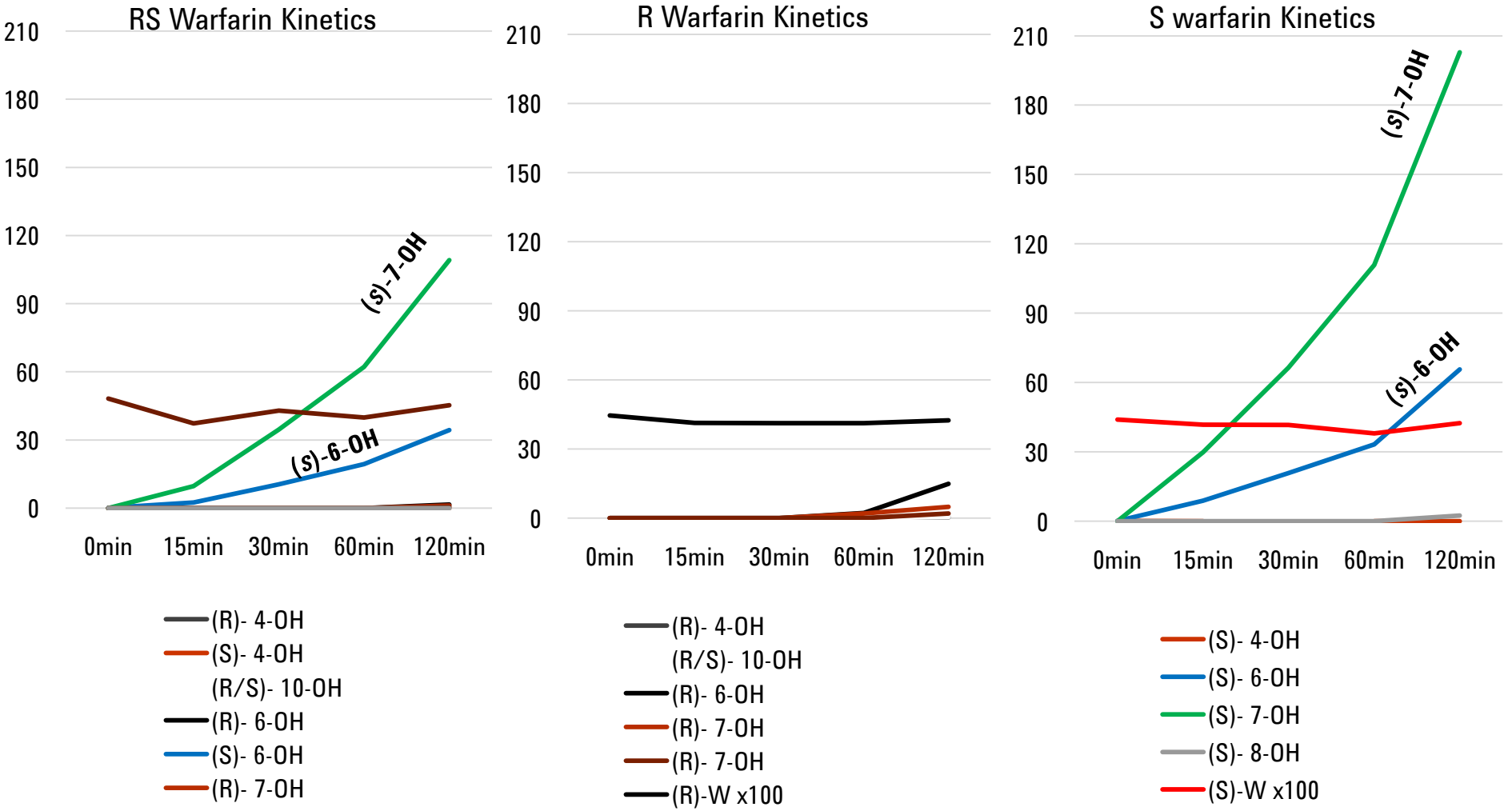
Microsomal Metabolism of R/S, R- and S-Warfarin (120 min)



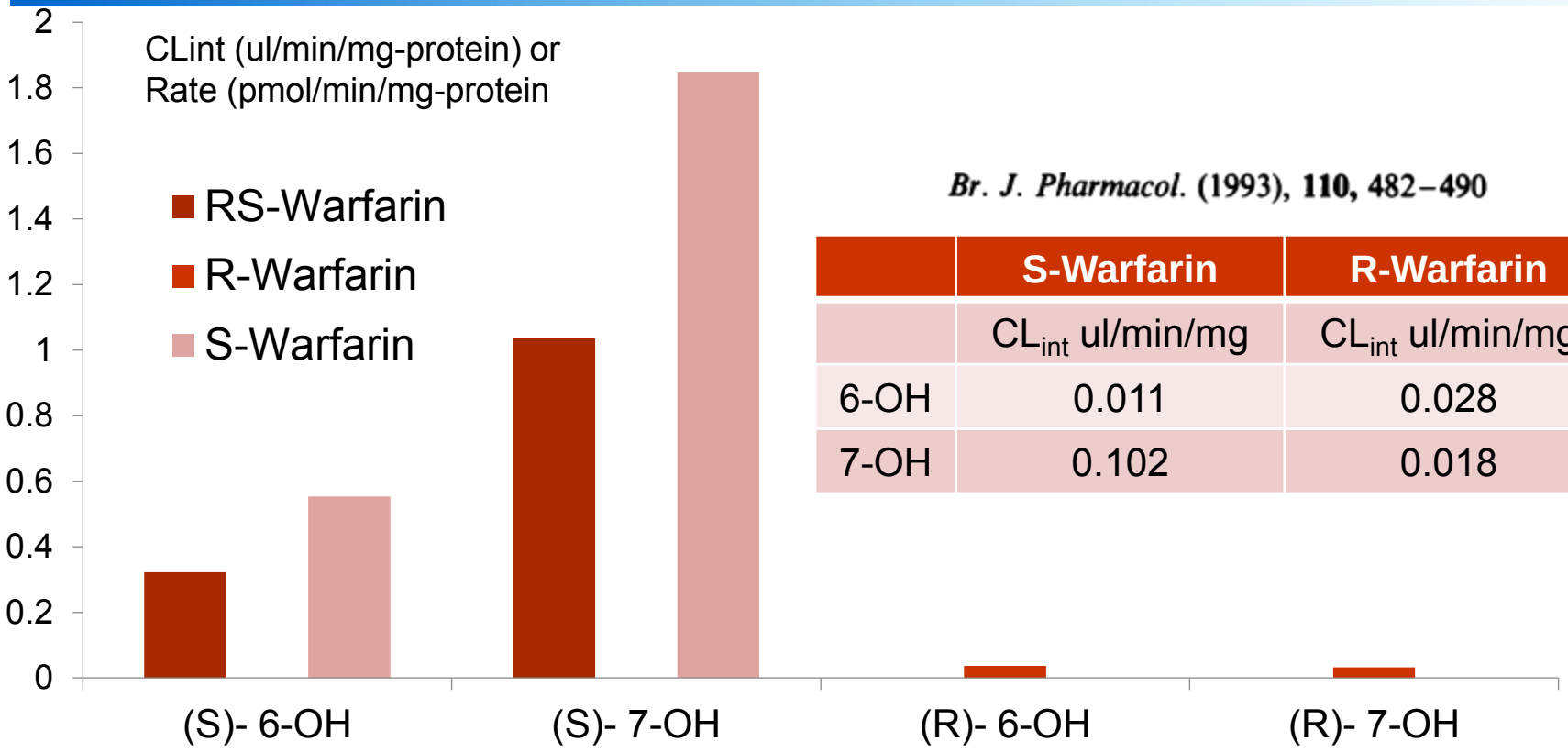
Individual incubations with R and S warfarin resulting in the formation of R and S warfarin metabolites allowed for the identification of R and S-warfarin without chiral metabolites.

Bristol-Myers Squibb

R/S, R and S Warfarin Metabolite Formation Rate



Metabolite Formation Rates

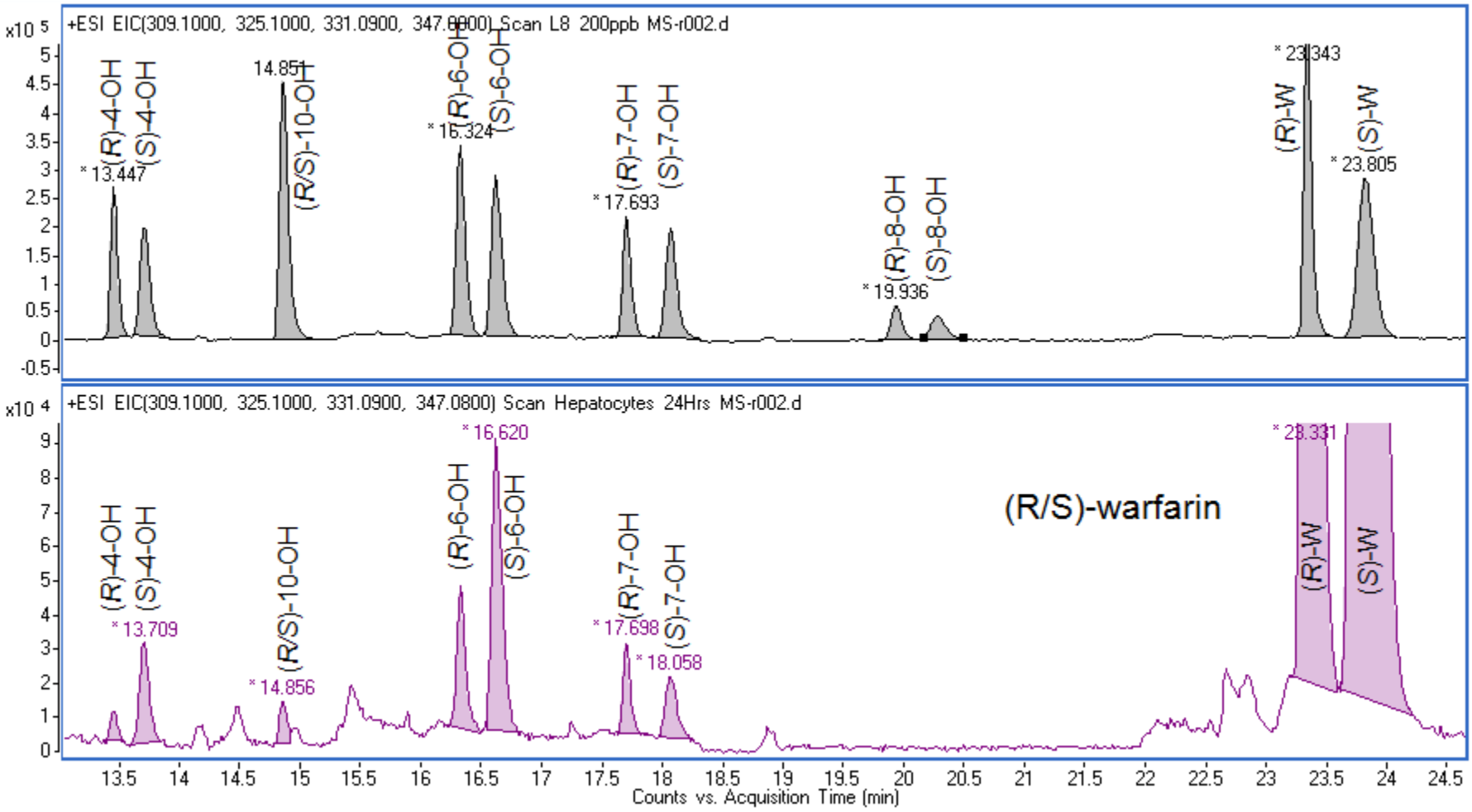


Br. J. Pharmacol. (1993), **110**, 482–490

	S-Warfarin	R-Warfarin
	CL_{int} ul/min/mg	CL_{int} ul/min/mg
6-OH	0.011	0.028
7-OH	0.102	0.018

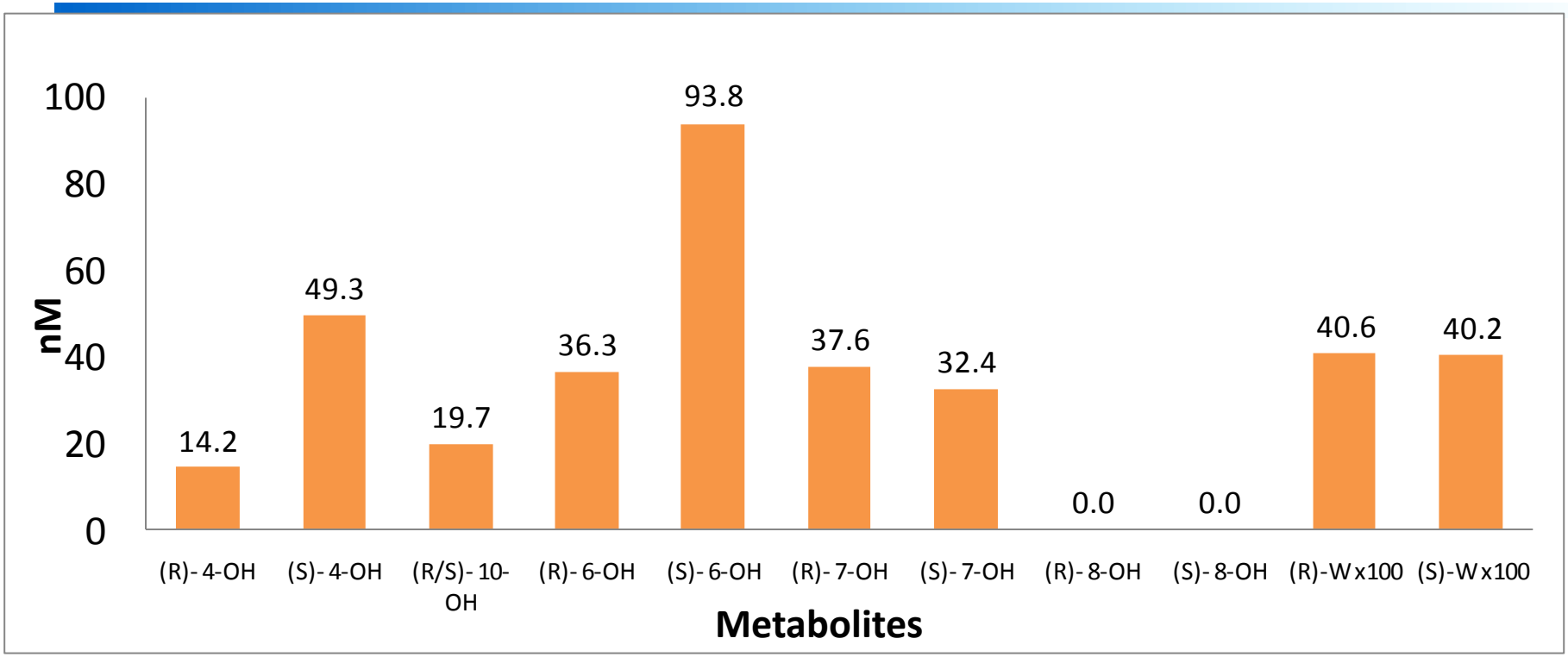
- **Higher S-warfarin and similar R-warfarin intrinsic clearances observed in our study when compared to a literature report.**

Extracted Ion Chromatogram of Hepatocytes Incubation at 24h Overlay with Standard



- All hydroxylated metabolites, except 8-OH metabolites, were formed.
- A single 2D LC-MS run capable of providing a rich data set.

Metabolites from Plated Rat Hepatocytes Incubation (24h)



- **Relative clearance rates of both the parent enantiomers can be determined. Additionally, regio and stereo-specific metabolism of both parent enantiomers can be elucidated.**
- **With MS detection, reductive and conjugative metabolism can also be assessed in a single run.**

Summary and Conclusions

- 2D LC/MS method for the stereospecific analysis of oxidative warfarin metabolism
- Better than Rs>2 for all metabolite enantiomers from single injection
- Qualitative identification of all metabolite enantiomers as well as kinetic analysis of different time points from single injection
- Results strongly support known literature characteristics of warfarin metabolism
  - (S)-warfarin primarily metabolizes to 6-OH and 7-OH
  - (*R*)-warfarin primarily metabolizes to 4'-OH, 6-OH, 10-OH and 7-OH
- 24h rat plated hepatocyte incubation showed a large number of hydroxylated metabolite formation from R- and S-warfarin.
- Assay can be applied to in vitro and in vivo samples

The new Agilent 1290 Infinity 2D-LC Solution with Multiple Heart-Cutting

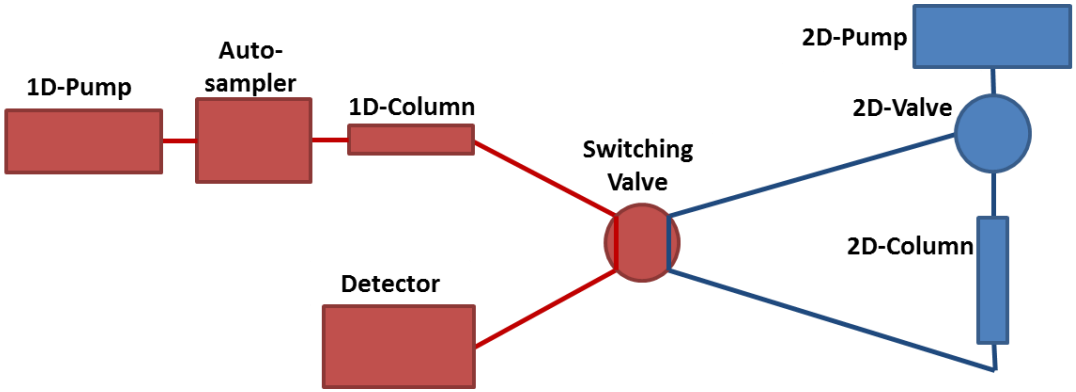
The new 1290 Infinity 2D-LC Solution

The first complete 2D-LC Solution!

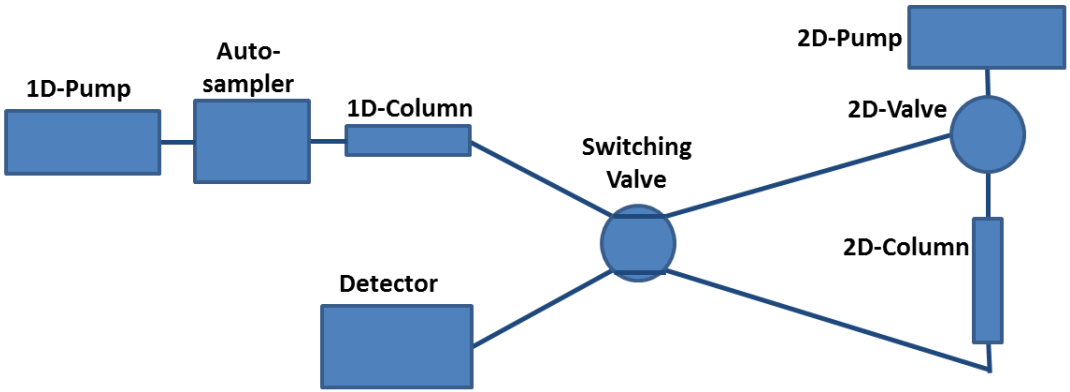
- **Not an expert tool anymore!**
- Complete pre-configured systems
- Still upgrade of existing Agilent LC systems possible
- Ready-to-go kit for easy start 2D-LC right after installation
- Easy-to-use software for fasted method setup in all available modes:
  - Heart-cutting 2D-LC
  - Multiple heart-cutting 2D-LC
  - Comprehensive 2D-LC
- Complete Ready-to-go multi heart-cutting set
- Switchable hybrid 1D/2D-LC set-up

Switchable 1D/2D Setup

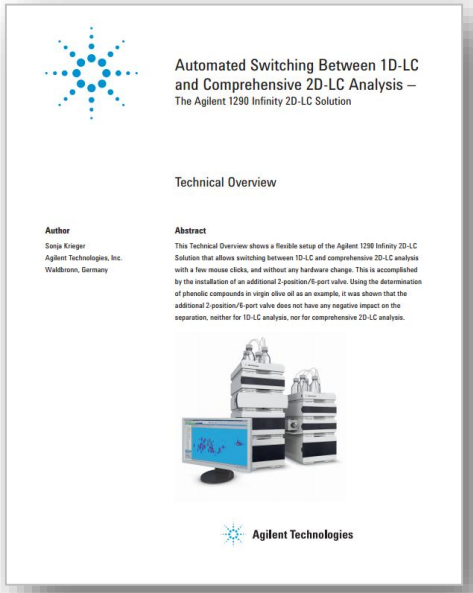
Change between UHPLC and 2D-LC without manual Interaction



1D-Setup



2D-Setup



Automated Switching Between 1D-LC and Comprehensive 2D-LC Analysis –
The Agilent 1290 Infinity 2D-LC Solution

Technical Overview

Author
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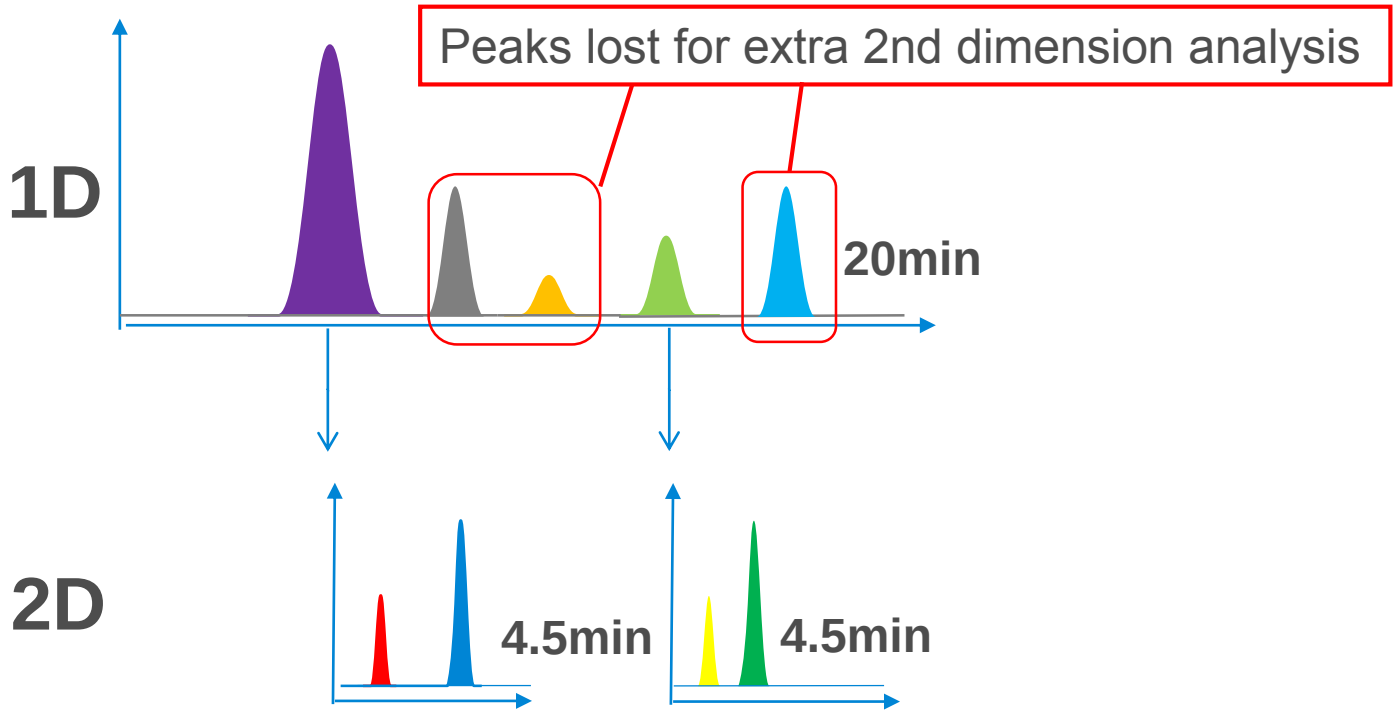
Abstract
This Technical Overview shows a flexible setup of the Agilent 1290 Infinity 2D-LC Solution that allows switching between 1D-LC and comprehensive 2D-LC analysis with a few mouse clicks, and without any hardware change. This is accomplished by the installation of an additional 2-position/6-port valve. Using the determination of phenolic compounds in virgin olive oil as an example, it was shown that the additional 2-position/6-port valve does not have any negative impact on the separation, neither for 1D-LC analysis, nor for comprehensive 2D-LC analysis.

Agilent Technologies

http://www.chem.agilent.com/Library/technicaloverviews/Public/5991-4843EN.pdf

1290 Infinity 2D-LC Solution – Scalability

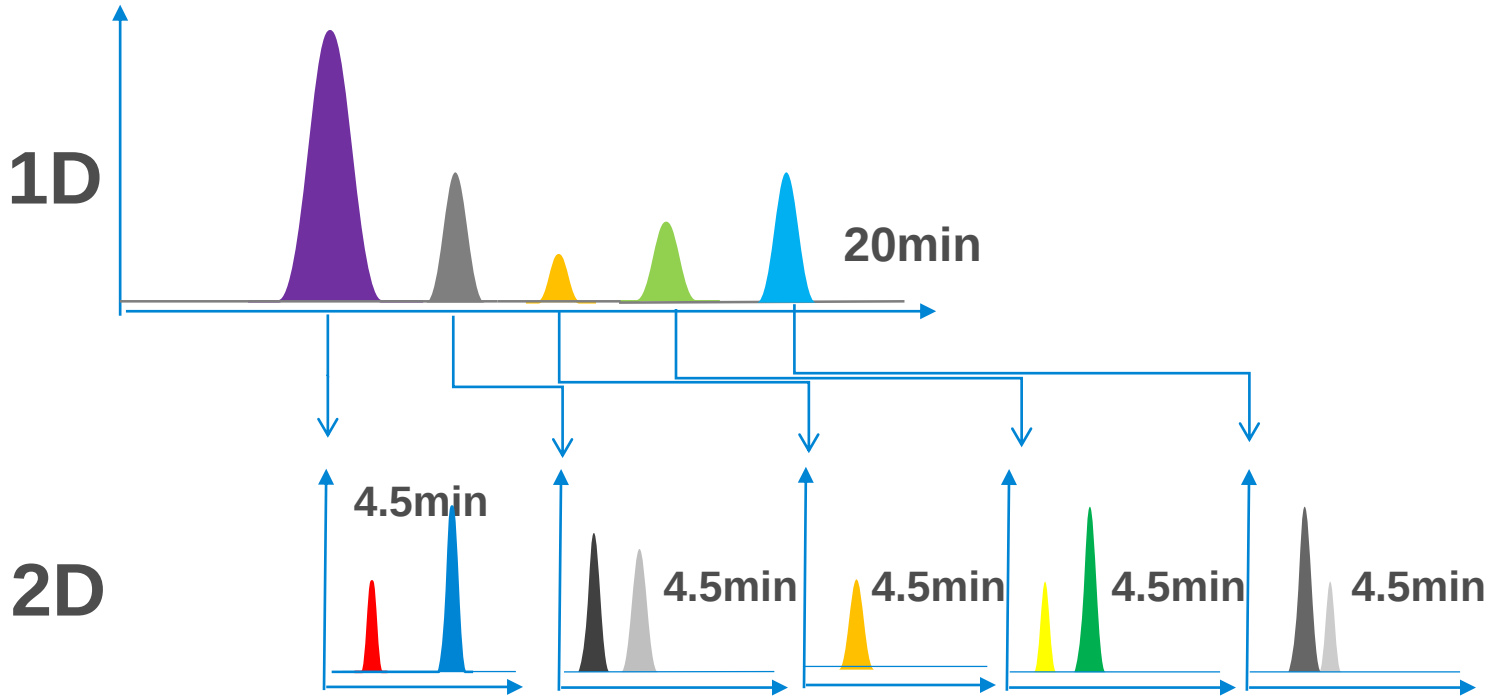
Multiple heart-cutting



Heart-cutting Data Viewer

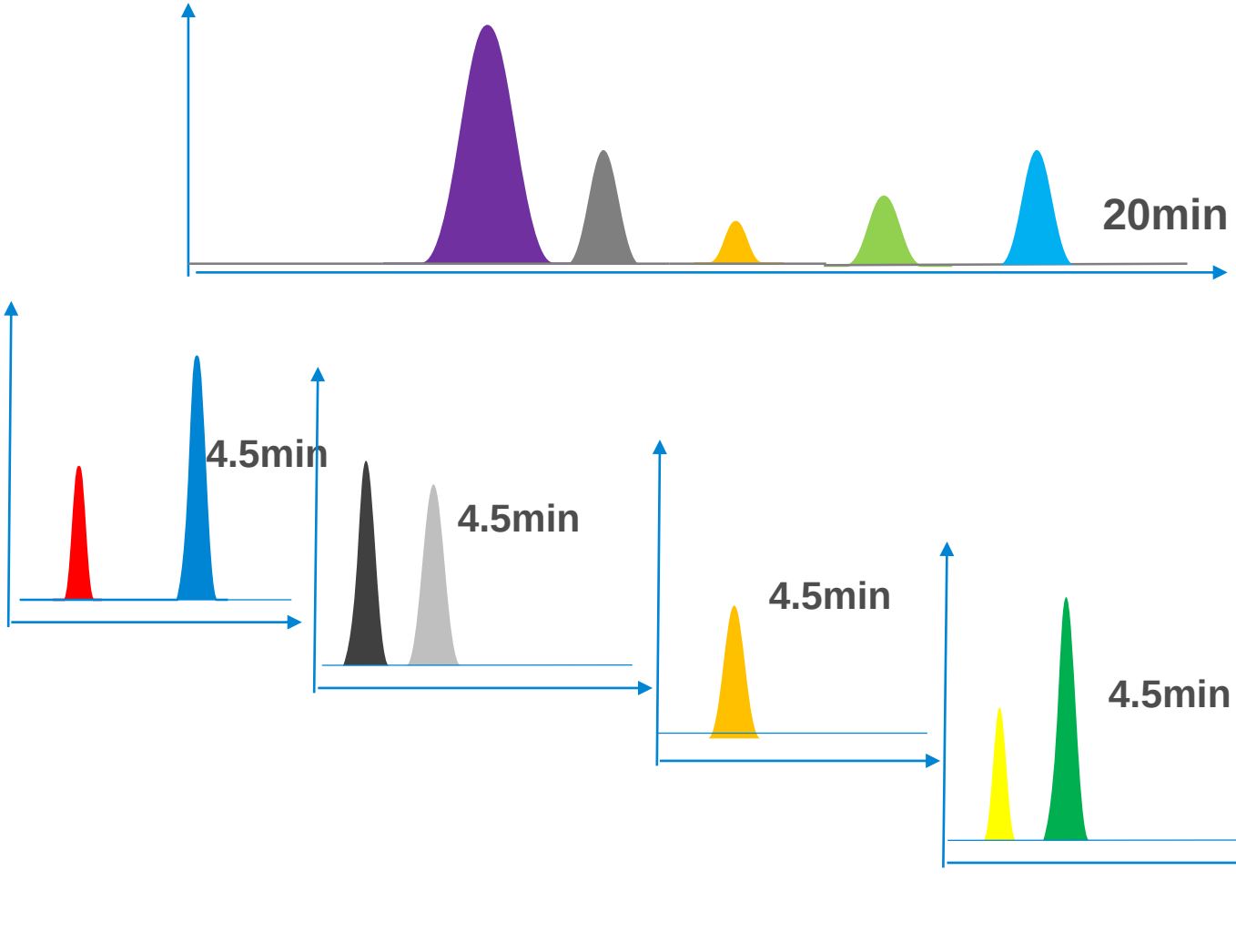
1290 Infinity 2D-LC Solution – Scalability

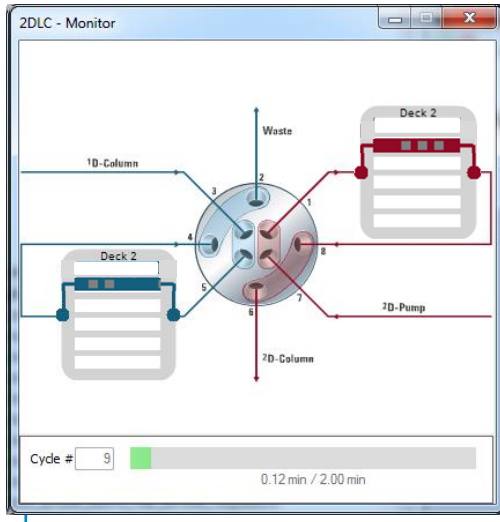
Multiple heart-cutting



1290 Infinity 2D-LC Solution – Scalability

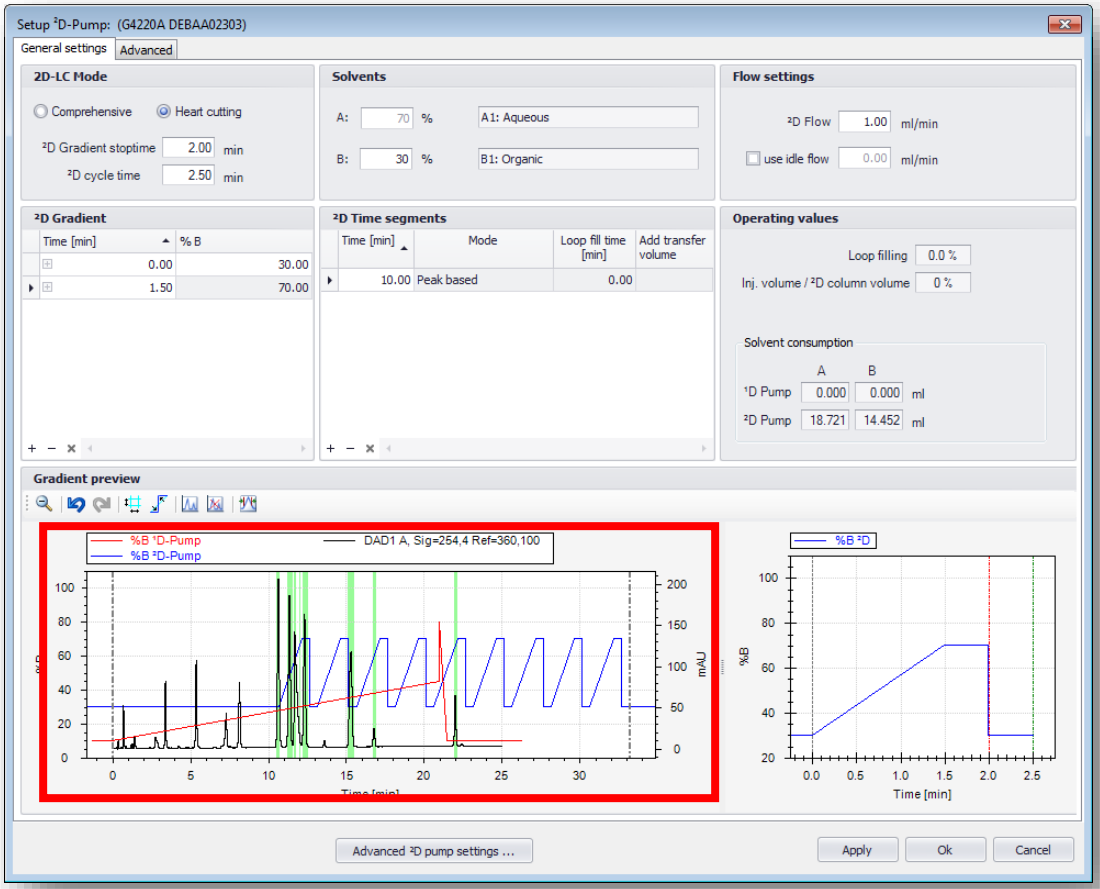
Multiple heart-cutting





2D-LC Acquisition Software

- One easy-to-use software for all operation modes

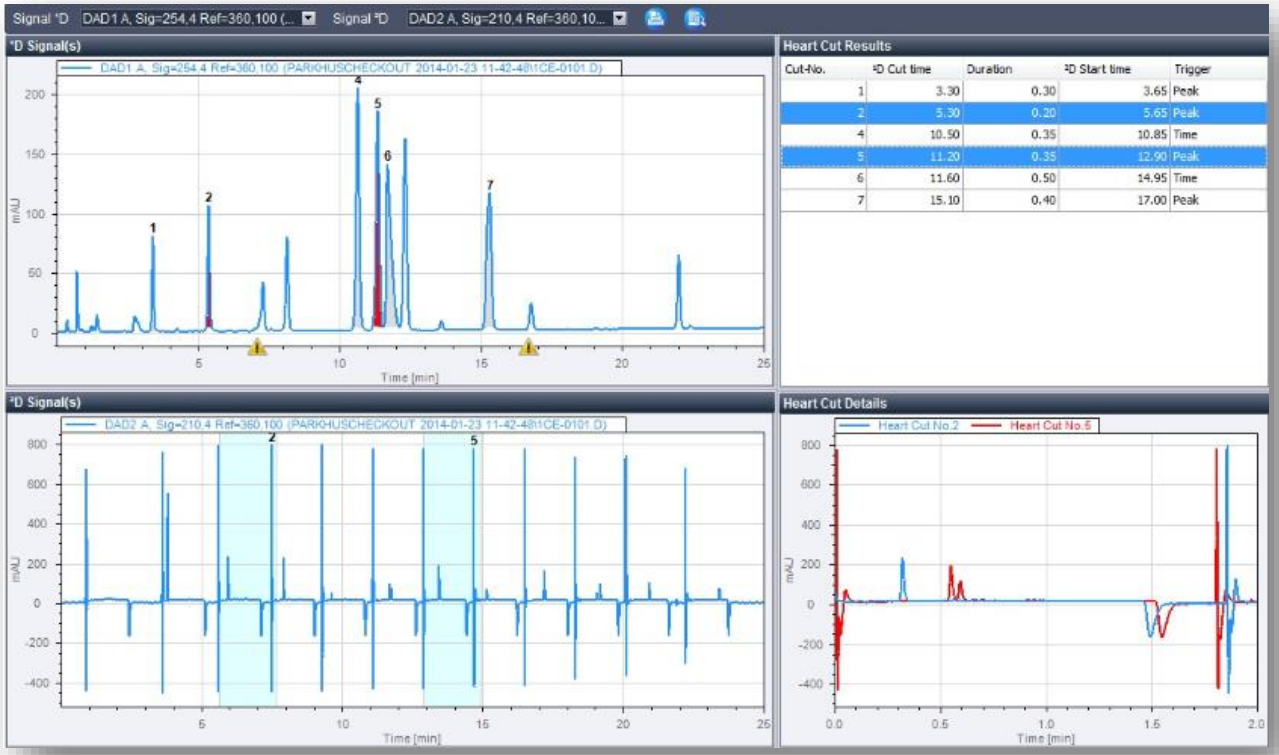


Most intuitive software to set up and edit methods within seconds:

- 1st dimension gradient
- 2nd dimension gradient
- Gradient shift
- Time-segments
- Method parameter
- Method set-up calculator
- Reference chromatogram overlay for heart-cutting
- Time-based or peak-based mode

Data Analysis Software

New Heart-Cut Data Viewer
for smartest heart-cut data evaluation and automatic reporting



THANK YOU FOR YOUR ATTENTION

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