Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

Acquity
Ultra Performance LC™

培训教程

(UPLC基础)

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内容提要

- **HPLC** 原理回顾
- **UPLC** 基本原理
- **HPLC** 与 **UPLC** 的异同
- 小结

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什么是HPLC?

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- 高效液相色谱法
  - **HPLC(High Performance Liquid Chromatography)**
  - 是一种区别于经典液相色谱,基于仪器方法的高效能分离手段:
    - 高性能色谱柱,高精度输液泵,高灵敏度检测器…
  - 广泛应用于各个领域:
    - 医药,环保,石化,生命科学,食品工业,农业…
  - 无论在技术上,理论上,还是在应用上仍有较大的发展空间

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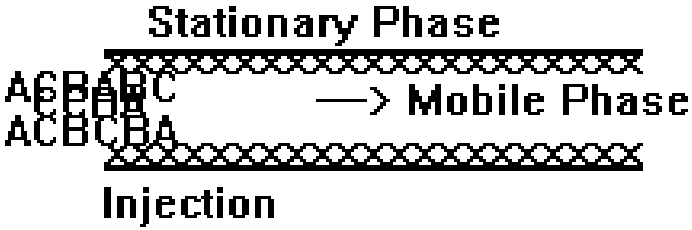
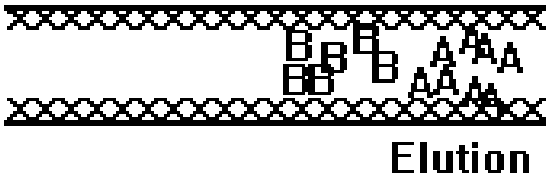
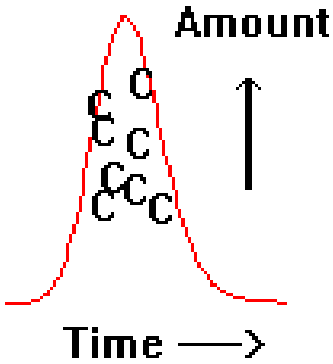
色谱分离原理



- 分离是一个物理过程。

固定相**(Stationary Phase)**
流动相**(Mobile Phase)**
进样 **(Injection)**
洗脱 **(Elution)**
相互作用**(Interaction)**





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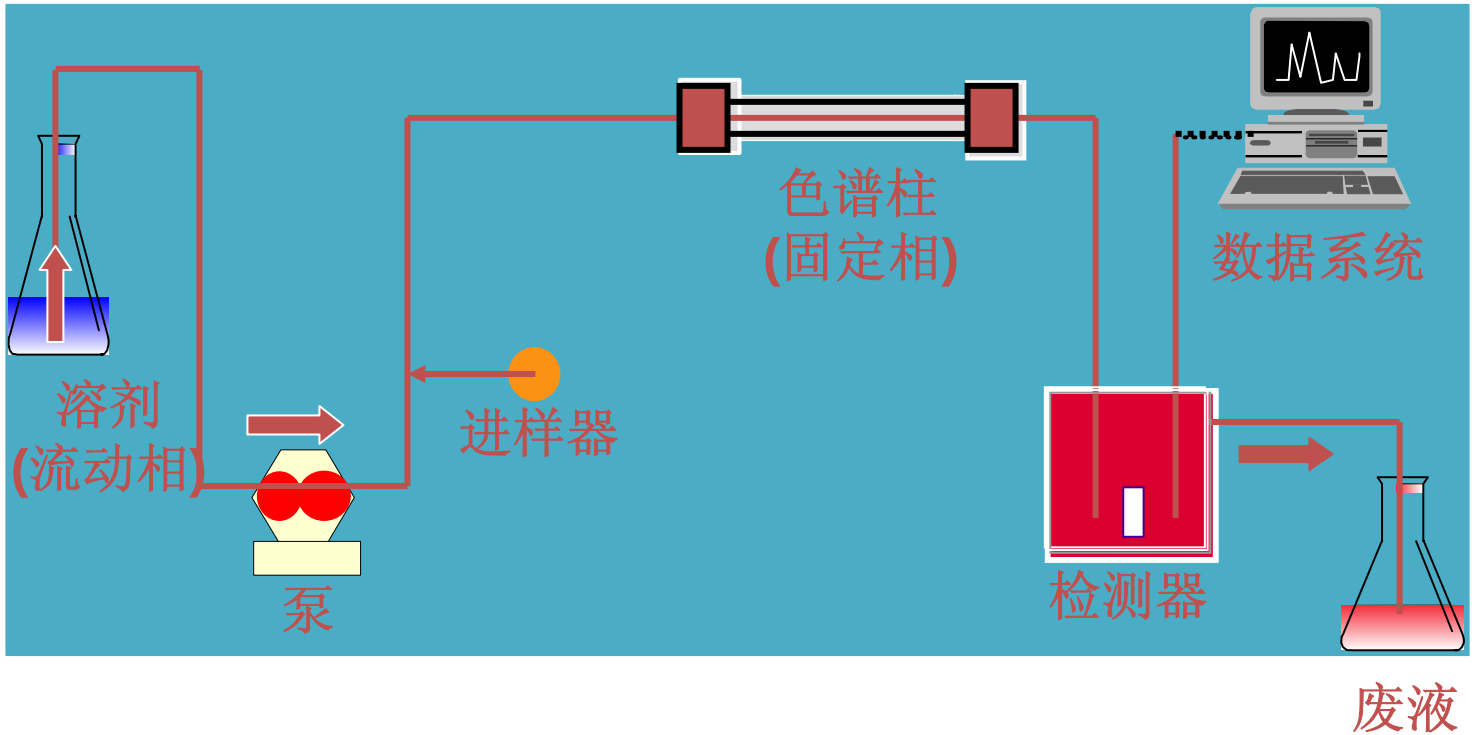
HPLC的流程



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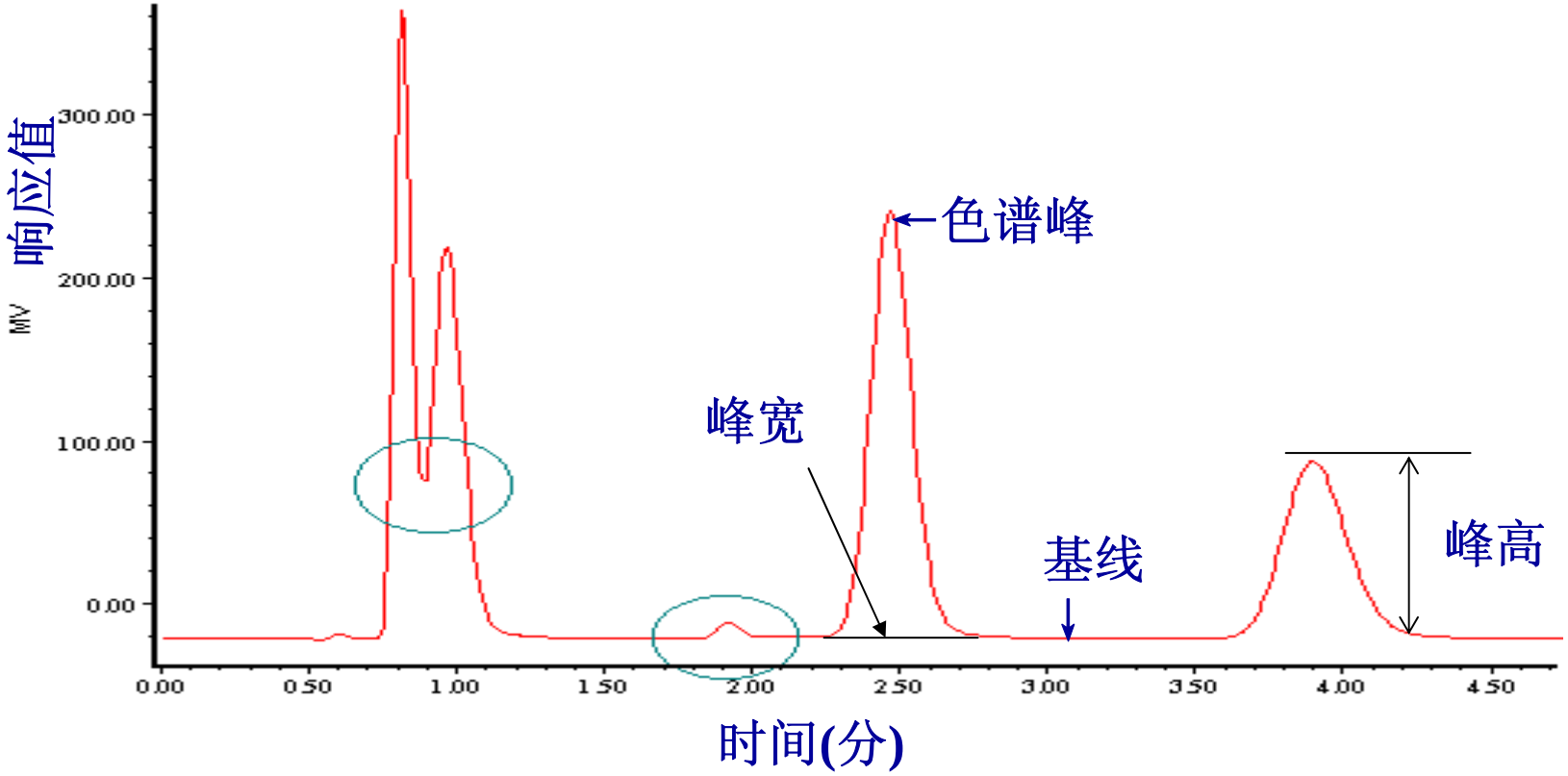
HPLC的图形结果

- 色谱图(**Chromatogram**)

  色谱柱流出物通过检测器时所产生的响应信号对时间的曲线图，其纵坐标为信号强度，横坐标为时间



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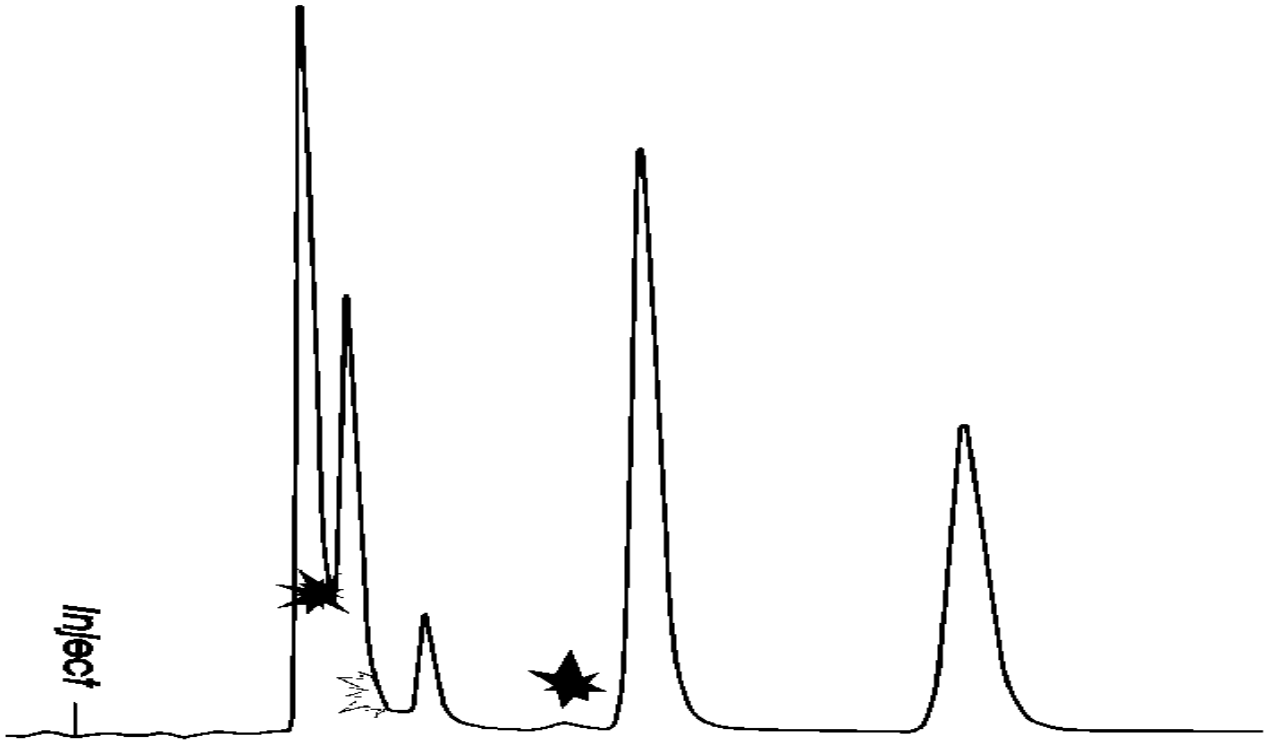
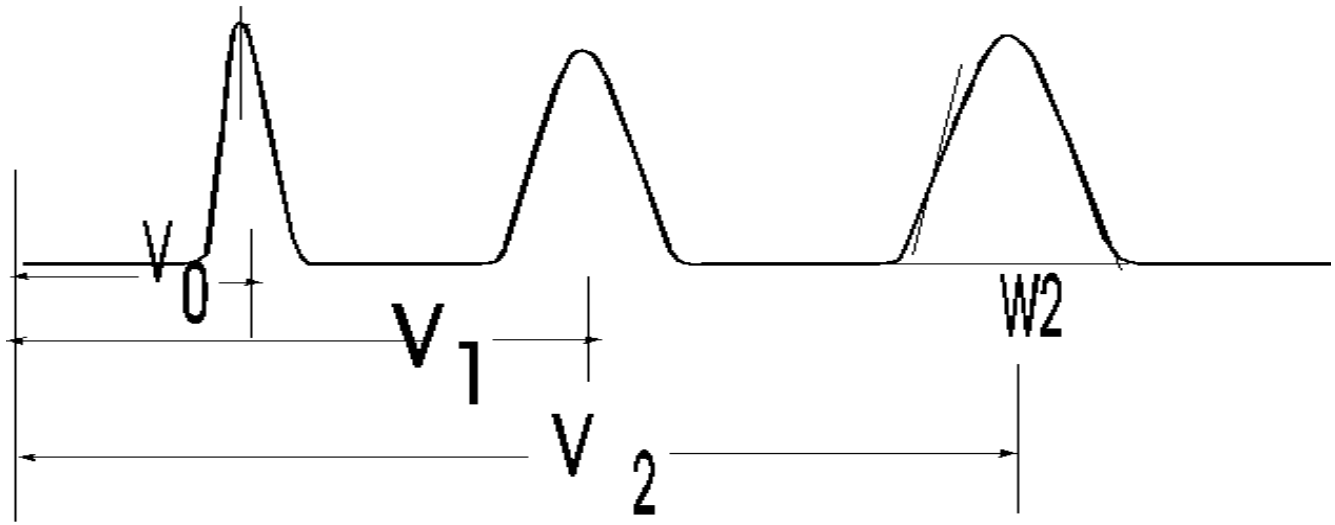
HPLC分离原理公式与术语

- 这是一张由仪器提供的表示分离状况的色谱图
- 这张色谱图含有丰富的信息



洗脱体积和峰宽

- 这些是导出理论和定义所必需的基本测量



V_0 = 无保留化合物的洗脱体积,等于一个柱体积
V_1 = 化合物1的洗脱体积
V_2 = 化合物2的洗脱体积
W_2 = 化合物2的峰宽(以体积计)

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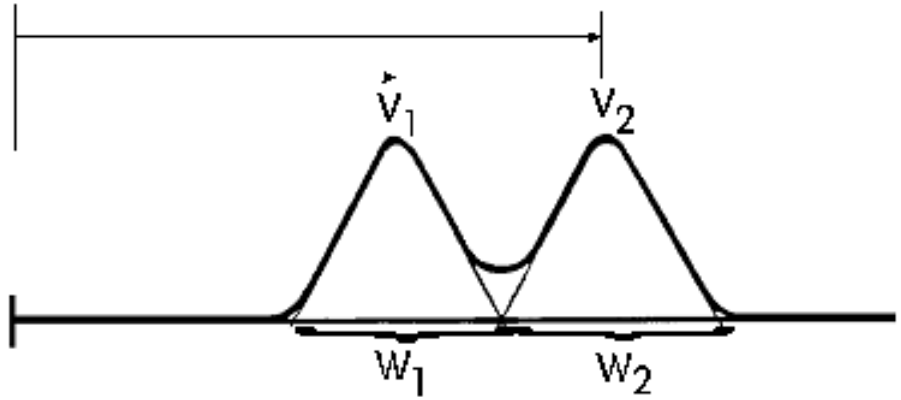
分离度(R)的定义式

- 简单说来，分离度即是两峰相对于其峰宽分开得有多远



$$R = \frac{V_2 - V_1}{\frac{1}{2}(W_1 + W_2)}$$

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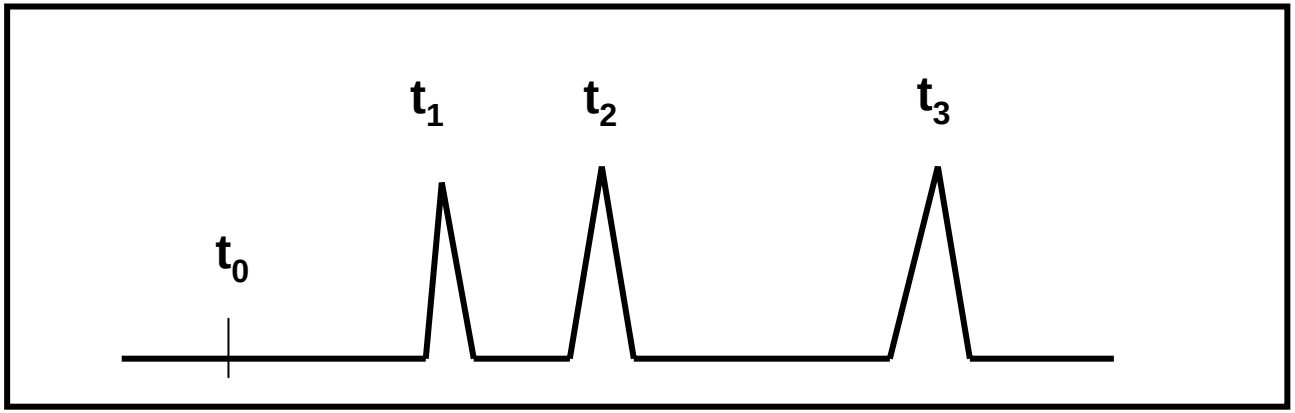
R:分离程度的量度

- 此处是几个计算分离度的实例



时间 .5 1 2 5

$$R = \frac{t_2 - t_1}{\frac{1}{2}(W_1 + W_2)}$$

$$R_{t_{2-1}} = \frac{2-1}{\frac{1}{2}(.5 + .5)} = 2$$

$$R_{t_{3-1}} = \frac{5-1}{\frac{1}{2}(.5 + .5)} = 8$$

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分离度方程式

- 分离度方程式描述了分离度与分离因子，柱效和保留因子之间的关系
- 它是评价一张色谱图以及决定如何解决、开发和优化分离方法的依据

$$R = \frac{1}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\sqrt{N}\right)\left(\frac{\kappa'}{1+\kappa'}\right)$$

分离因子　　柱效　　保留因子

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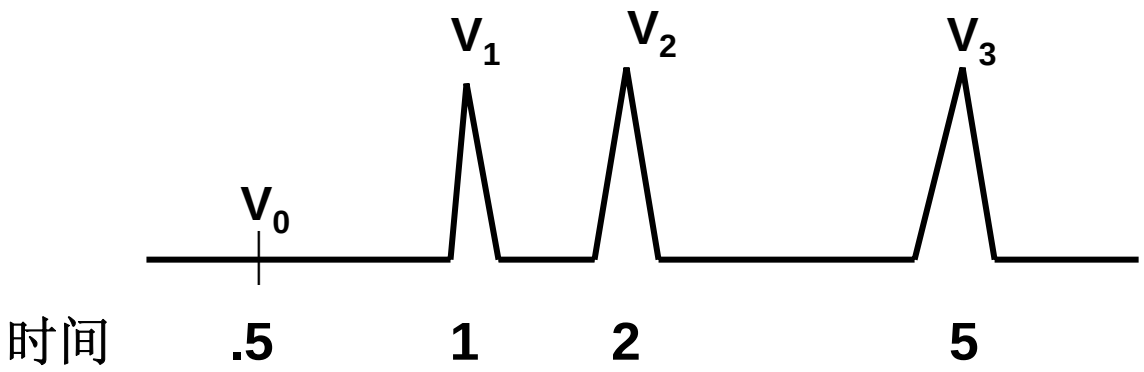
保留因子(K′):保留能力的量度

- k′ 是样品与填料作用强度的直接量度



$$k'_1 = \frac{1-.5}{.5} = 1$$

公式: $k'_1 = \frac{V_1 - V_0}{V_0}$

$$k'_2 = \frac{2-.5}{.5} = 3$$

$$k'_3 = \frac{5-.5}{.5} = 9$$

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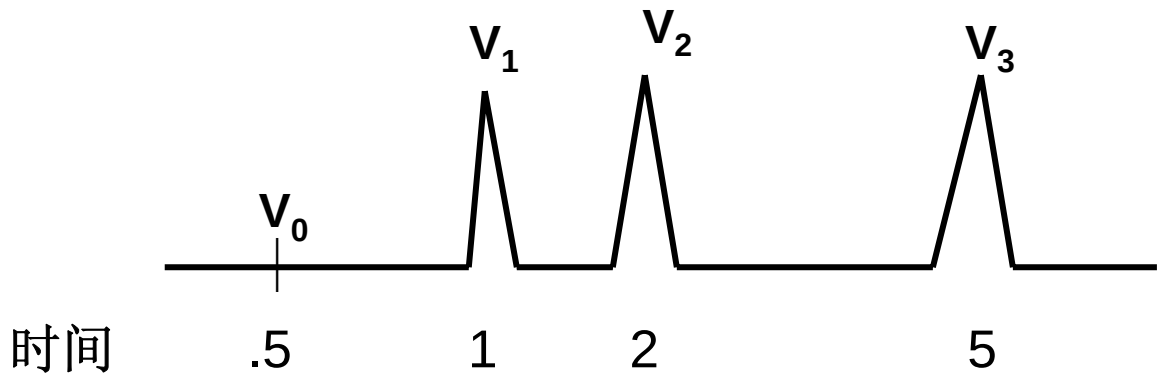
分离因子(α):峰分离程度的量度

- α 是两个化合物在同一套色谱系统上保留差异的数值表述



$$\alpha = \frac{k'_2}{k'_1} \quad 或 \quad \alpha = \frac{V_2 - V_0}{V_1 - V_0}$$

$$峰2/峰1的\ \alpha = \frac{2 - .5}{1 - .5} = 3$$

$$峰3/峰1的\ \alpha = \frac{5 - .5}{1 - .5} = 9$$

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理论塔板数(N):分离效率的量度

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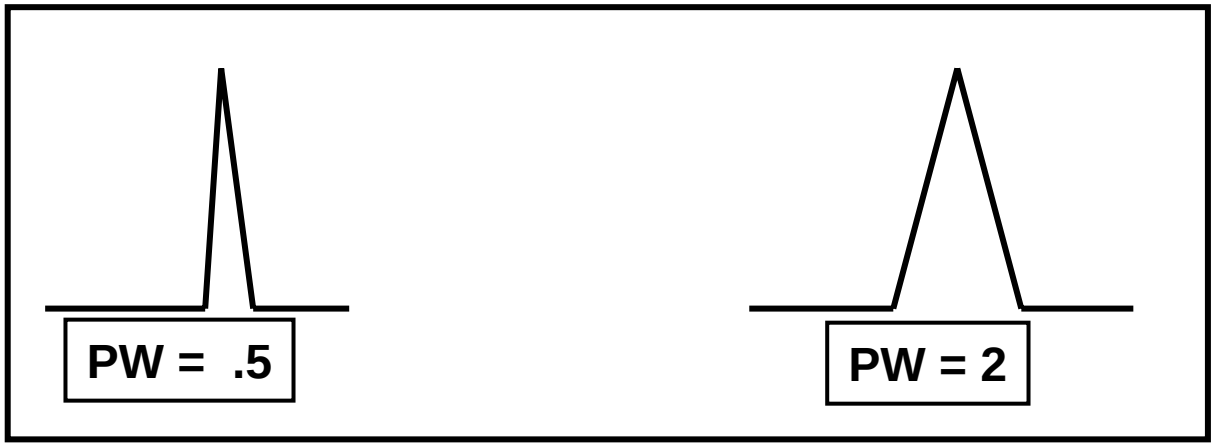
- 理论塔板数或柱效是一个数值，它表示作为保留时间函数的峰展宽的量度



$$N = 16\left(\frac{V}{W}\right)^2$$

$$N = 16\left(\frac{5}{.5}\right)^2$$

$$N = 1600$$

$$N = 16\left(\frac{V}{W}\right)^2$$

$$N = 16\left(\frac{5}{2}\right)^2$$

$$N = 100$$

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关于柱效或塔板数

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- 塔板数是综合反映色谱柱和仪器性能的关键指标
- 取决于下列两个参数:
  - —保留时间
  - —峰宽
- 窄峰 ➔ 高塔板数
- 峰形越均匀 ➔ 塔板数越高
- 分析型**HPLC**典型的塔板数值**(5σ)**约为**100,000/**米
- **UPLC**典型的塔板数值 **(5σ)** 高达 **200,000 /**米

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塔板数值

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- 塔板数值受下列因素影响:
  - 机械因素
    - 仪器
    - 联接 (端口接头, 锥箍)
    - 柱装填的好坏
  - 化学因素
    - 色谱因素 (溶剂)
    - 填料的生产制造质量

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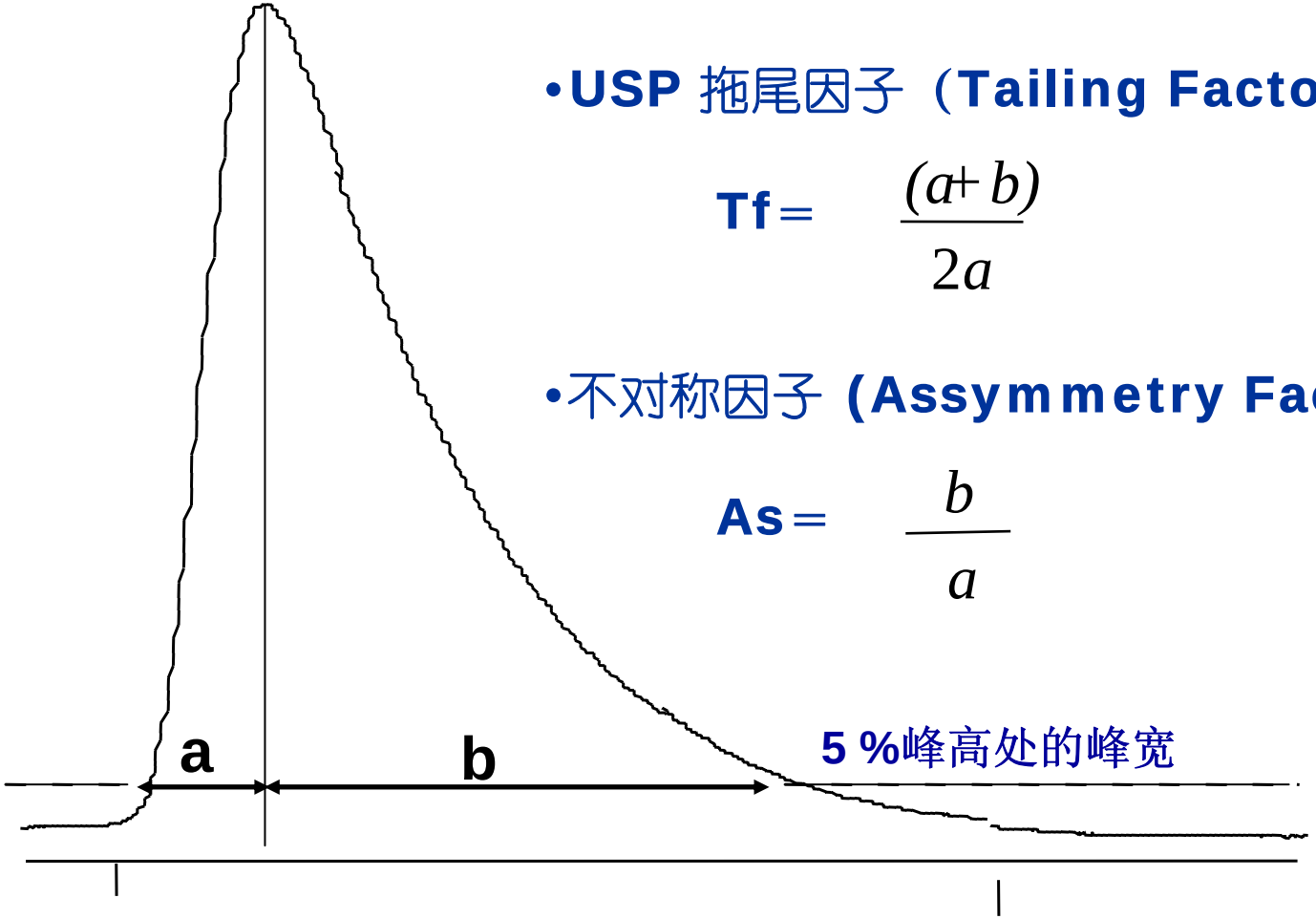
色谱峰对称性的测量方法



- **USP 拖尾因子（Tailing Factor）：**

$$Tf = \frac{(a+b)}{2a}$$

- **不对称因子 (Assymmetry Factor):**

$$As = \frac{b}{a}$$

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HPLC 实验的基本类型

- 等度
  - —ISO ➔ 相同的
  - —在整个运行过程中溶剂组成保持不变
  - —(60:40 甲醇/水)

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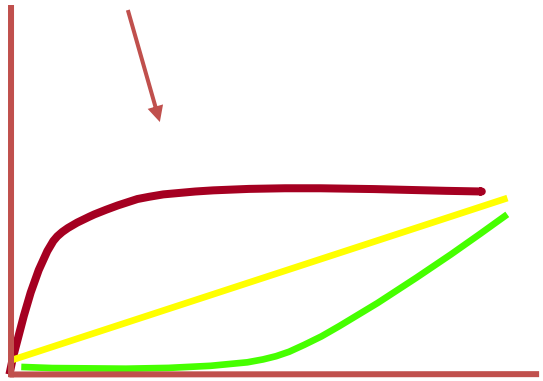
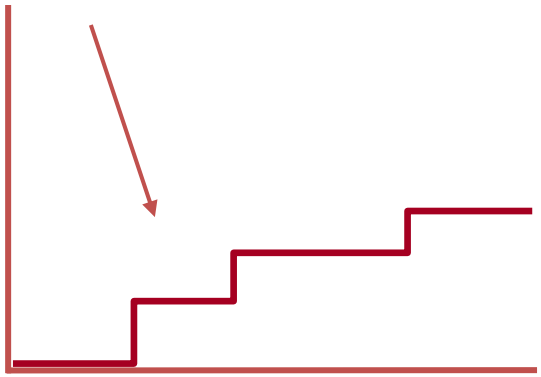
HPLC 实验的基本类型

- 梯度
  - —在运行过程中溶剂组成变化
  - —渐变或台阶式变化
    - **100% H_2O/ 0% MeOH** 到 **0% H_2O/100% MeOH**





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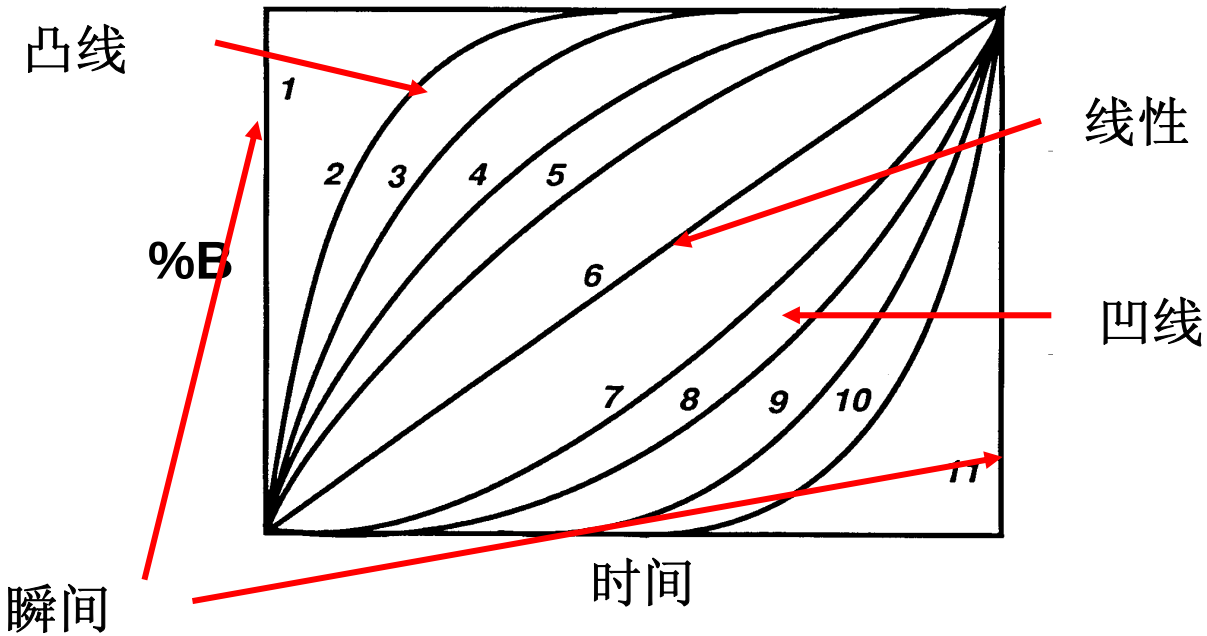
梯度曲线轮廓图

用于 Waters HPLC 系统



Curve Number	Effect
1	Immediately goes to specified conditions
2 to 5	Convex
6	Linear (Default)
7 to 10	Concave
11	Maintains start condition until next step

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关于色谱系统的反压

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- 什么是反压(**back pressure**)
  - 流动相流经管路及色谱柱时会有阻力,即所谓的反压,又称系统反压或系统压力
  - **Waters**习惯用的压力单位是**Psi**(磅/平方英吋)
  - 其它单位有:**Bar,Mpa(1Bar=0.1Mpa=14.5Psi)**
- 影响系统反压的主要因素:
  - 流动相的溶剂组成
  - 温度
  - 流速

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反压与溶剂组成的关系

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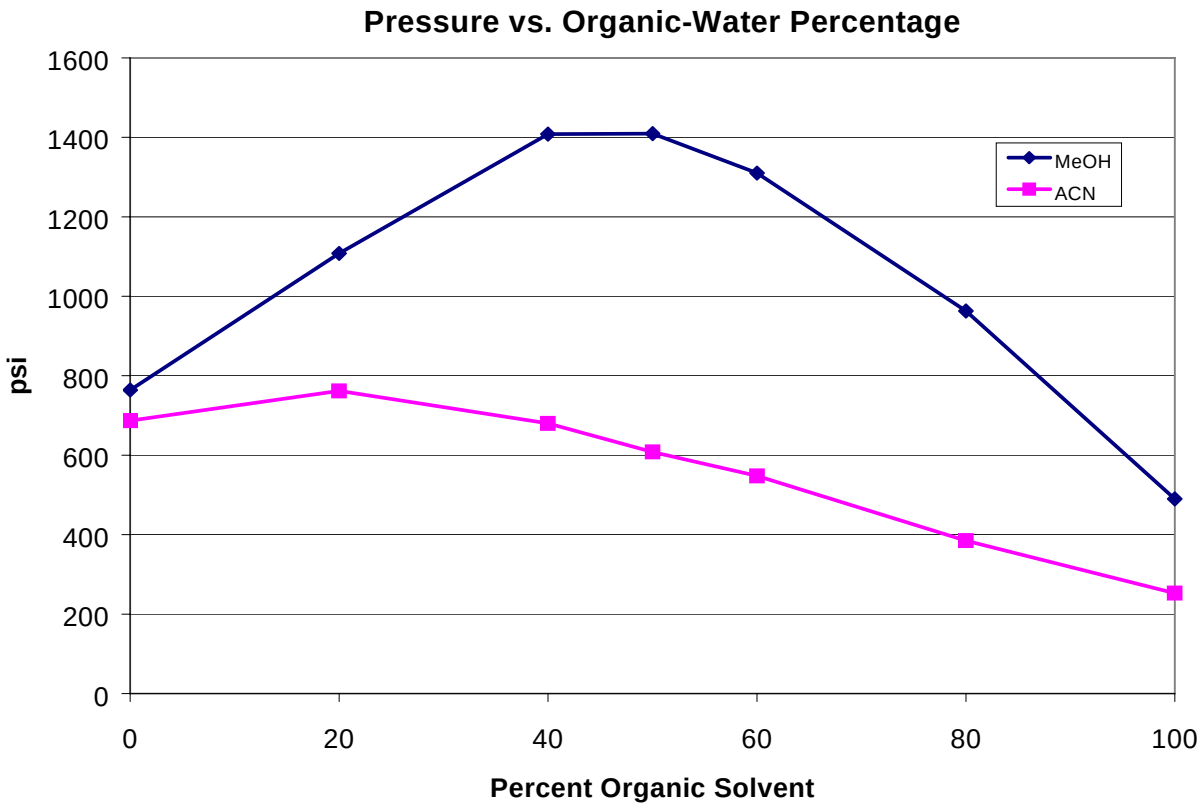
- 流动相的粘度是产生反压的主要原因
- 有机溶剂－水的粘度随组成而改变
- 其压力随组成变化如下图所示



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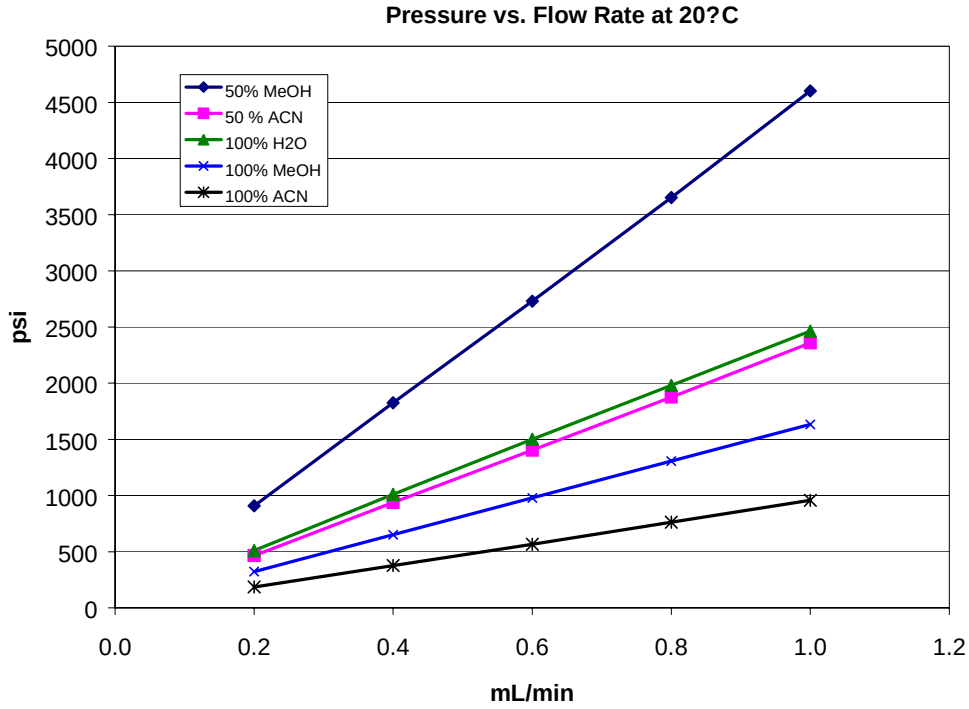
反压与流速的关系

- 流速对反压的影响是线性关系
- 流速增加，反压亦增大
- 下图的实验条件：
  - **2.1×30mm Symmetry C_{18}柱,20℃**



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反压与温度的关系

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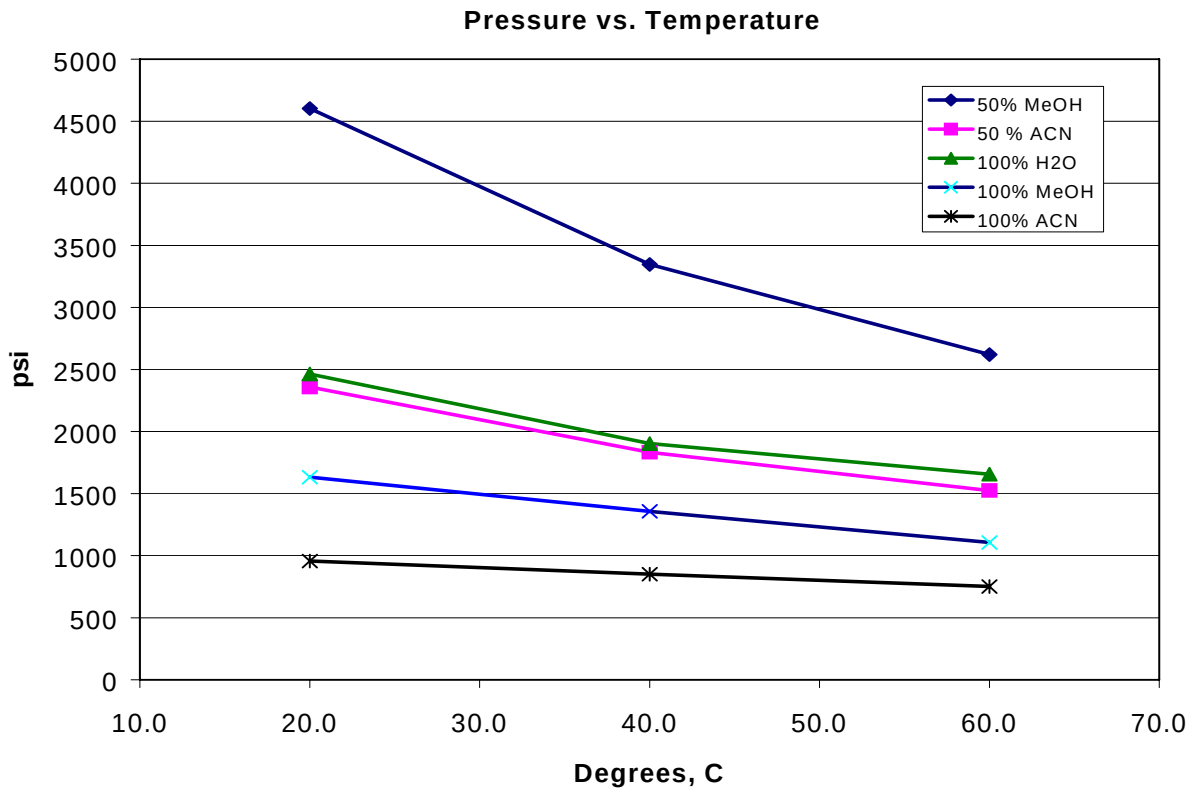
- 温度对反压的影响是反比关系
- 温度升高，反压降低，对某些流动相的影响更大一些



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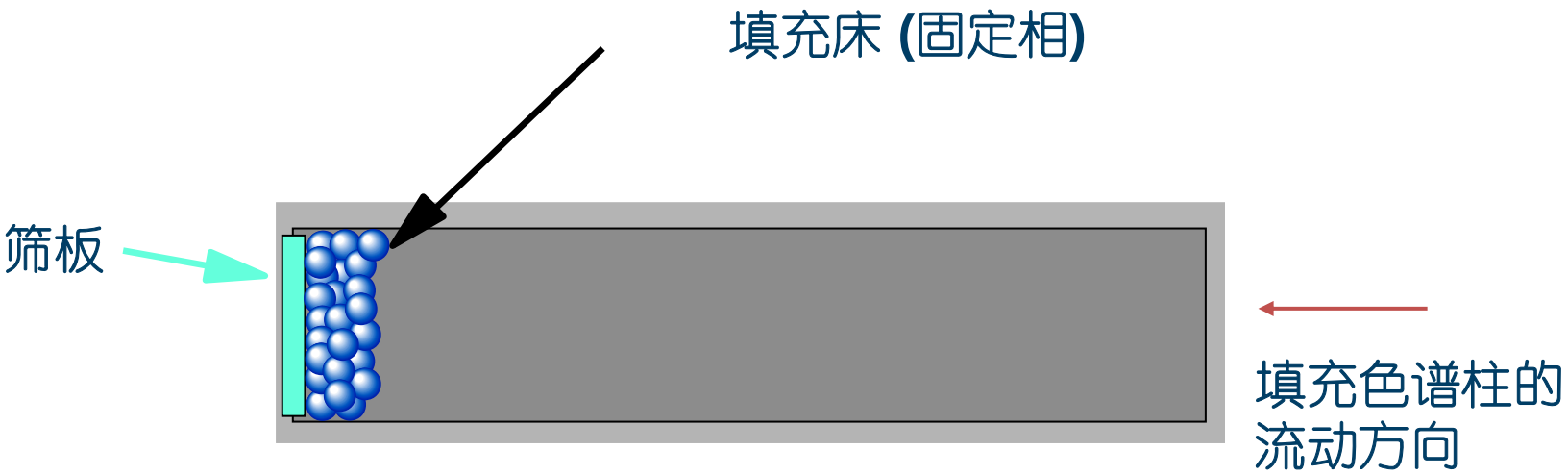
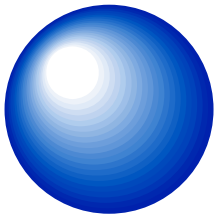
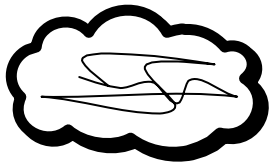
色谱柱的装填





球形颗粒



无定形颗粒

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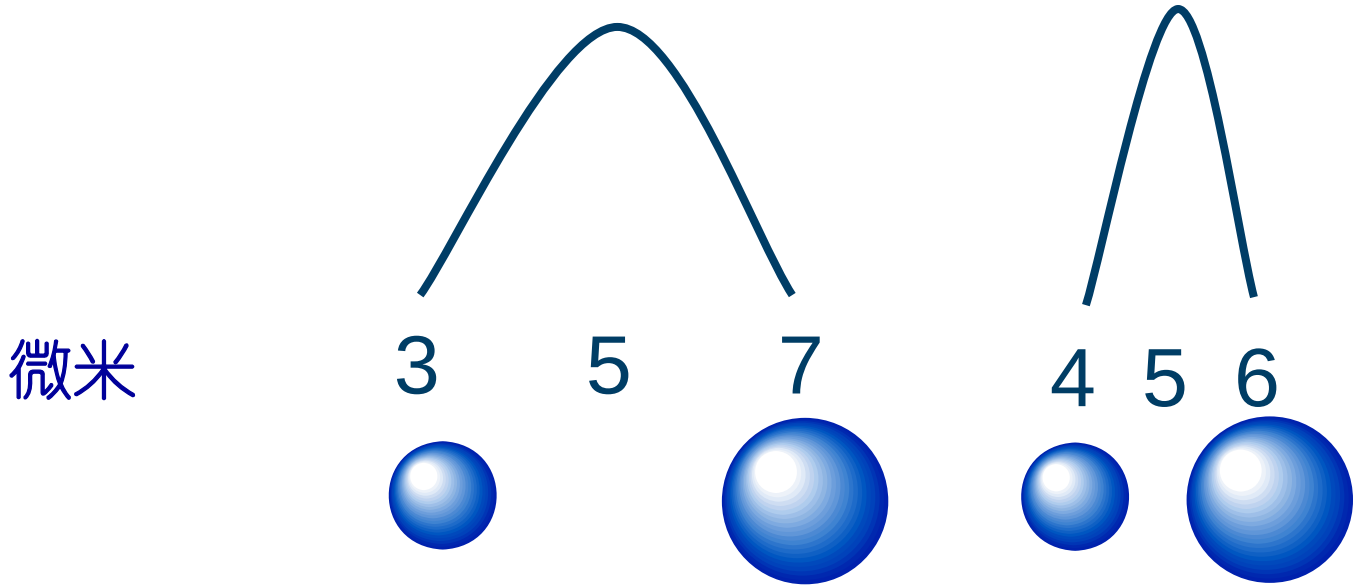
颗粒度分布

- 具名义值的填料有一个颗粒度分布的范围
  —例如: **4 μm (2 – 10 μm)**
- 窄的分布会给出较高的性能



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用于HPLC色谱柱的典型填料

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- 硅胶
  - —二氧化硅
  - —无规则或球形
- 铝
  - —氧化铝
  - —无规则或球形
- 聚合物
  - —聚苯乙烯二乙烯基苯
  - —球形
- 杂化
  - —硅胶和聚合物的结合
  - —球形

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广泛用于 HPLC 的柱填料

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- **C18**
  - —也叫作 **ODS** 或 **RP18**
    - o **OctaDecyl Silane**
  - —超过全部应用的 **70%**
  - —反相键合在硅胶基质上
  - —**“Waxed(**上蜡的**)”** 硅胶颗粒
  - —非极性的

HPLC C18 柱有各种不同品牌

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- 一个独立的生产厂商能制造出许多不同品牌的**C18** 柱
  - **XBridge, SunFire, Atlantis,…**
- 每一个品牌被设计成达到不同的色谱目的
  - 高效能
  - 没有拖尾
  - 独特的选择性
  - 高疏水性
  - 高硅醇基活性

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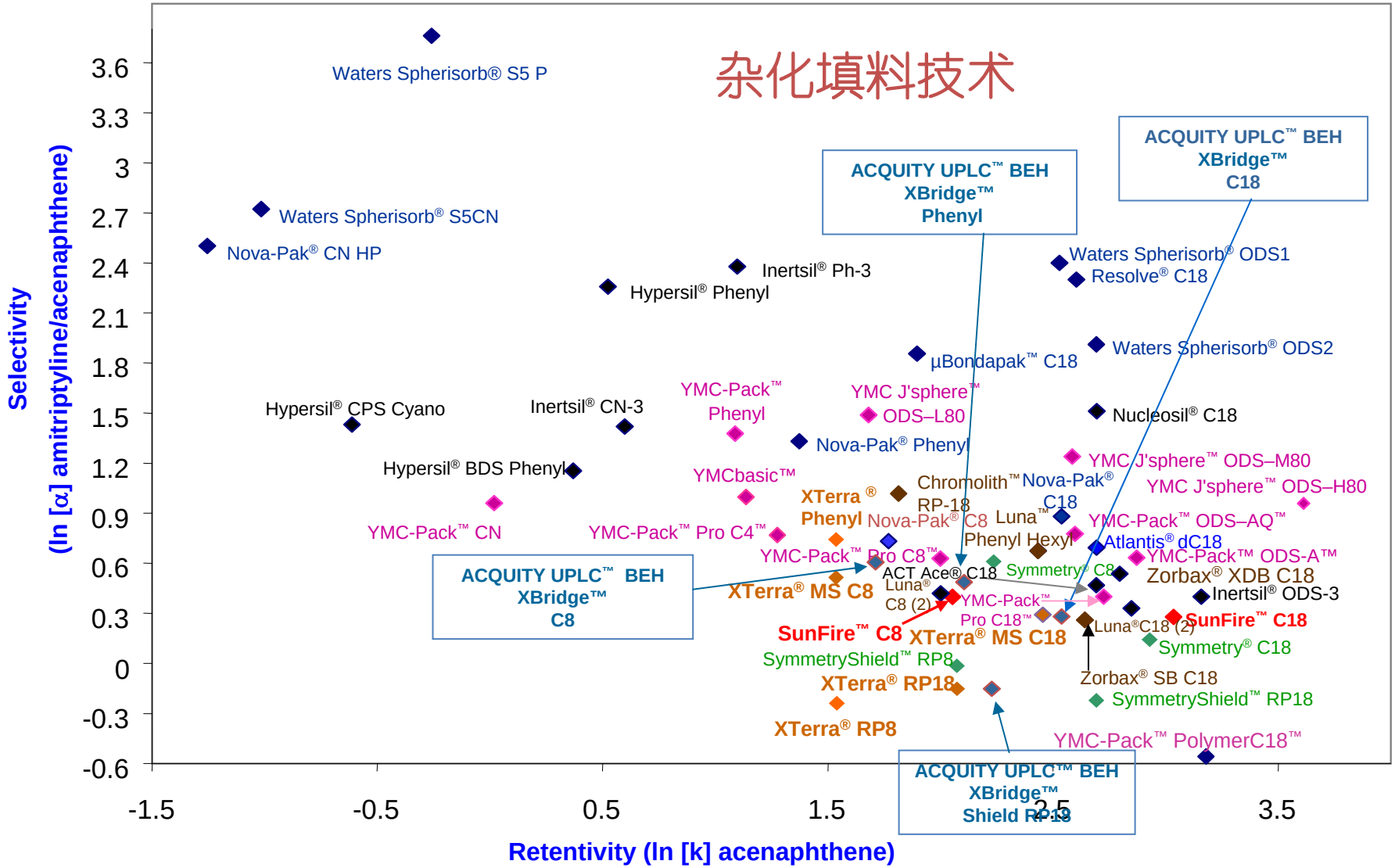
反相色谱柱选择性图表


杂化填料技术
Selectivity
(ln [α] amitriptyline/acenaphthene)
Retentivity (ln [k] acenaphthene)
3.6
3.3
3
2.7
2.4
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0
-0.3
-0.6
-1.5
-0.5
0.5
1.5
2.5
3.5
Waters Spherisorb® S5 P
Waters Spherisorb® S5CN
Nova-Pak® CN HP
Inertsil® Ph-3
Hypersil® Phenyl
Hypersil® CPS Cyano
Inertsil® CN-3
Hypersil® BDS Phenyl
YMC-Pack™ CN
YMC-Pack™ Phenyl
YMC J'sphere™ ODS–L80
Nova-Pak® Phenyl
YMCbasic™
YMC-Pack™ Pro C4™
YMC-Pack™ Pro C8™
XTerra® Phenyl
Chromolith™ RP-18
Nova-Pak® C8
Luna™ Phenyl Hexyl
ACT Ace® C18
Symmetry® C8
Luna® C8 (2)
XTerra® MS C8
SunFire™ C8
XTerra® MS C18
YMC-Pack™ Pro C18™
Luna® C18 (2)
SymmetryShield™ RP8
XTerra® RP18
XTerra® RP8
Zorbax® SB C18
SymmetryShield™ RP18
YMC-Pack™ PolymerC18™
µBondapak™ C18
Waters Spherisorb® ODS1
Resolve® C18
Waters Spherisorb® ODS2
Nucleosil® C18
YMC J'sphere™ ODS–M80
YMC J'sphere™ ODS–H80
Nova-Pak® C18
YMC-Pack™ ODS–AQ™
Atlantis® dC18
YMC-Pack™ ODS-A™
Zorbax® XDB C18
Inertsil® ODS-3
SunFire™ C18
Symmetry® C18
ACQUITY UPLC™ BEH XBridge™ Phenyl
ACQUITY UPLC™ BEH XBridge™ C18
ACQUITY UPLC™ BEH XBridge™ C8
ACQUITY UPLC™ BEH XBridge™ Shield RP18

色谱柱几何概况

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- 直径
  - —较小的直径增加灵敏度
  - —较大的直径增加柱容量
- 长度
  - —较长的柱子具有较高的分离能力和柱容量
  - —较短的柱子具有较快的分离速度和较低的柱容量

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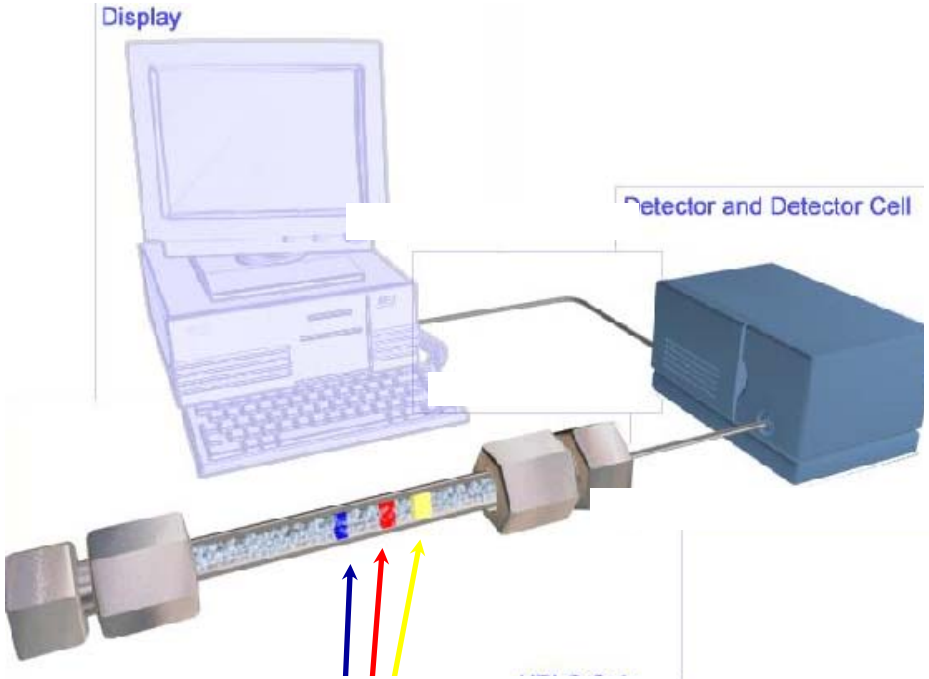
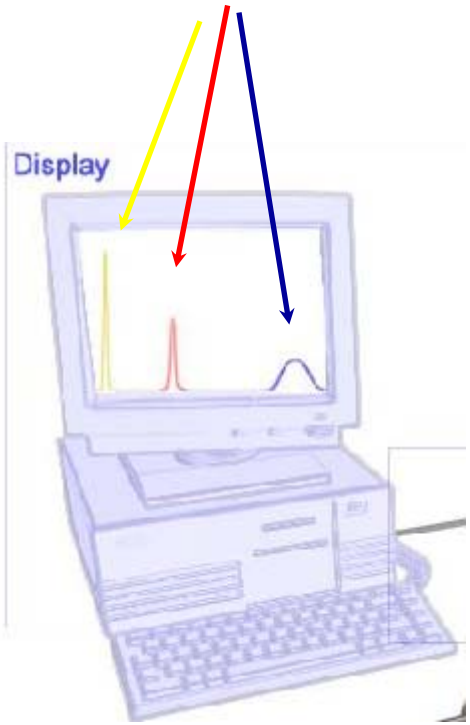
如何得到尖锐的色谱峰



柱内被分析物的谱带

色谱图上被分析物的峰



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色谱柱接头

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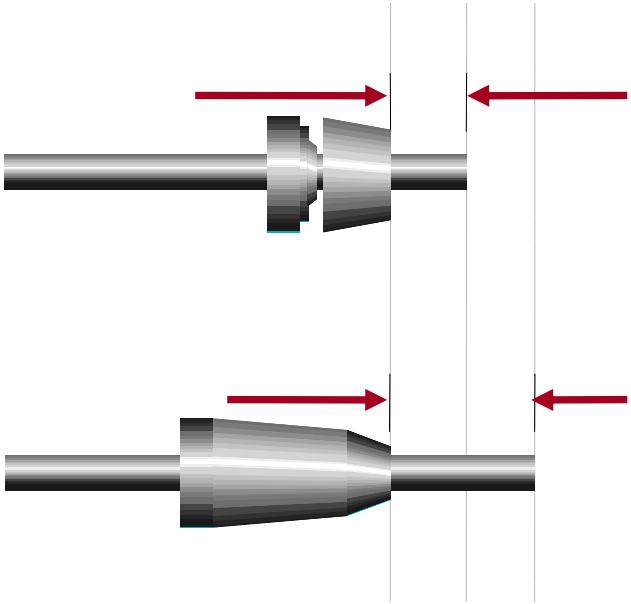
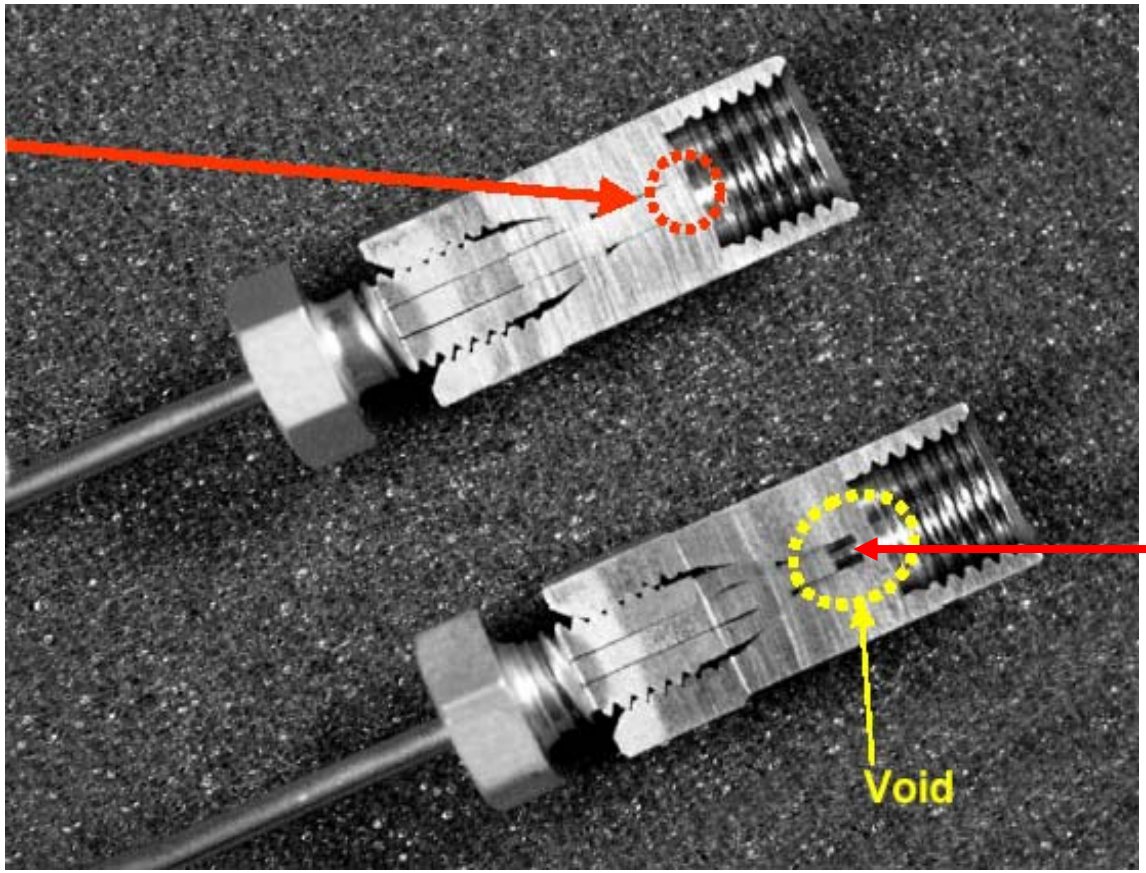


0.080 – 0.090″
Parker/Swagelok
制式

其它
Waters® 色谱柱

0.120 – 0.130″
Waters 制式

柱外谱带扩展



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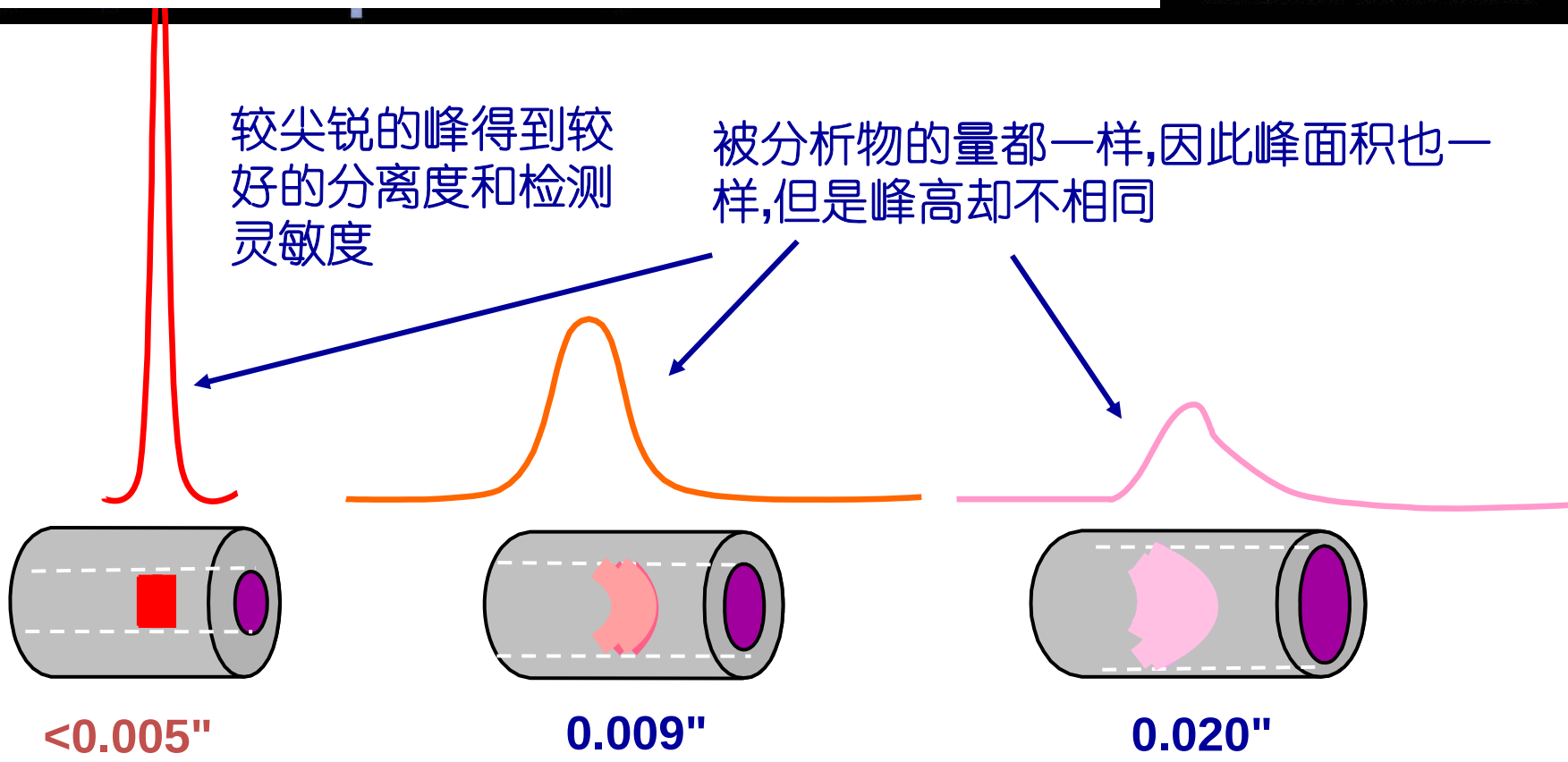
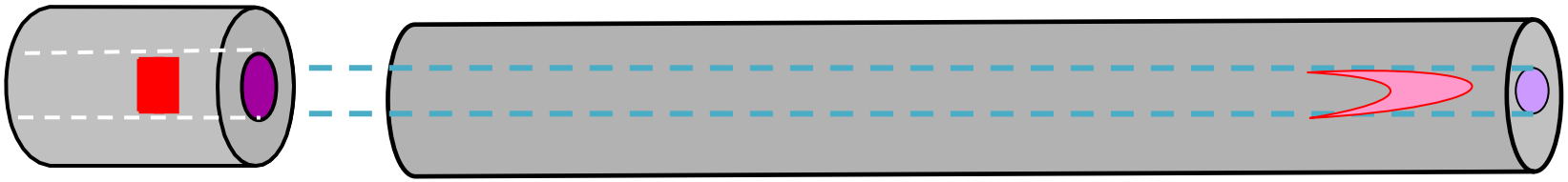
样品在管路内的谱带扩展



过长的管路也会造成谱带展宽



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UPLC 理论和原理

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

- **Ultra Performance LC**
  - —分离科学的一个新进展
- **UPLC** 使用与**HPLC**同样的理论和原理
- 基于更细的**(1.7 μm)** 颗粒化学
  - —更快的分析速度
  - —增加灵敏度
  - —增大组份分离度
  - —增高系统反压

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Ultra Performance LC™

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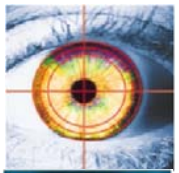
重新定义色谱科学

Acquity™
Ultra Performance LC

Acquity一词源自拉丁文“acutus”，意味着有更出色、更细致地解决问题的能力，及快速、敏锐的智能

速度

灵敏度



分离度

创新

超高效液相色谱的定义

Waters
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- 超高效液相色谱（**Ultra Performance LC™**）
  - —是分离分析科学中的一个全新类别
  - —涵盖了小颗粒填料、非常低系统体积及快速检测手段等全新技术
  - —与**HPLC**的理论及原理相同
  - —在全面提升**HPLC**的速度、灵敏度及分离度诸品质的同时，保留其原有的实用性及原理
  - —给实验室带来了新奇而强大的能力

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对当今HPLC的科学描述

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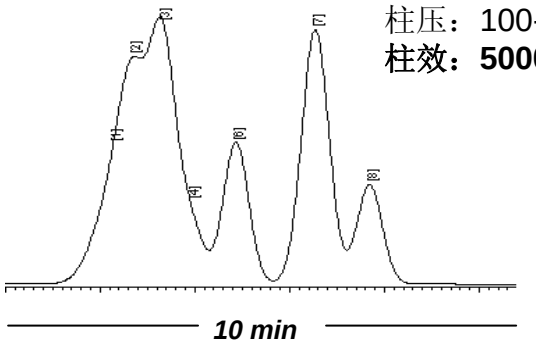
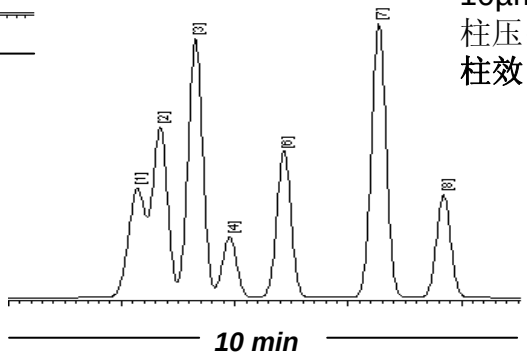
- **HPLC**的分离质量被色谱柱化学所驱动同时又受其制约
  - —优化的柱化学能够给色谱过程带来增加速度、灵敏度和分离度的诸多好处
- 颗粒度是柱效能的最主要贡献者
- 当前遇到的挑战是 **HPLC** 仪器不能实现小颗粒填料的诸多利益

颗粒尺寸的演变

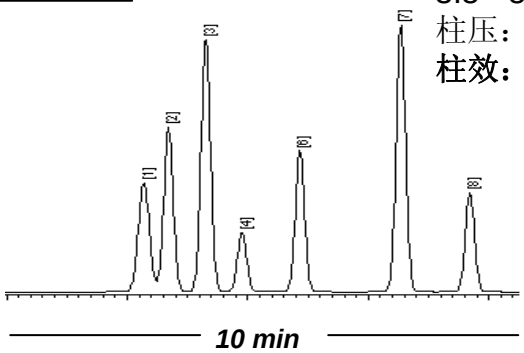
六十年代后期
40µm薄壳无孔填料
柱压：100-500 psi (7-40 bar)
柱效：5000 /米



七十年代早期
10µm无规则微孔填料
柱压：1000-2500 psi(70-180 bar)
柱效：40,000 /米



八十年代至今
3.5 - 5µm 球形微孔填料
柱压：1500-4000 psi(110-280 bar)
柱效：80,000 - 115,000/米



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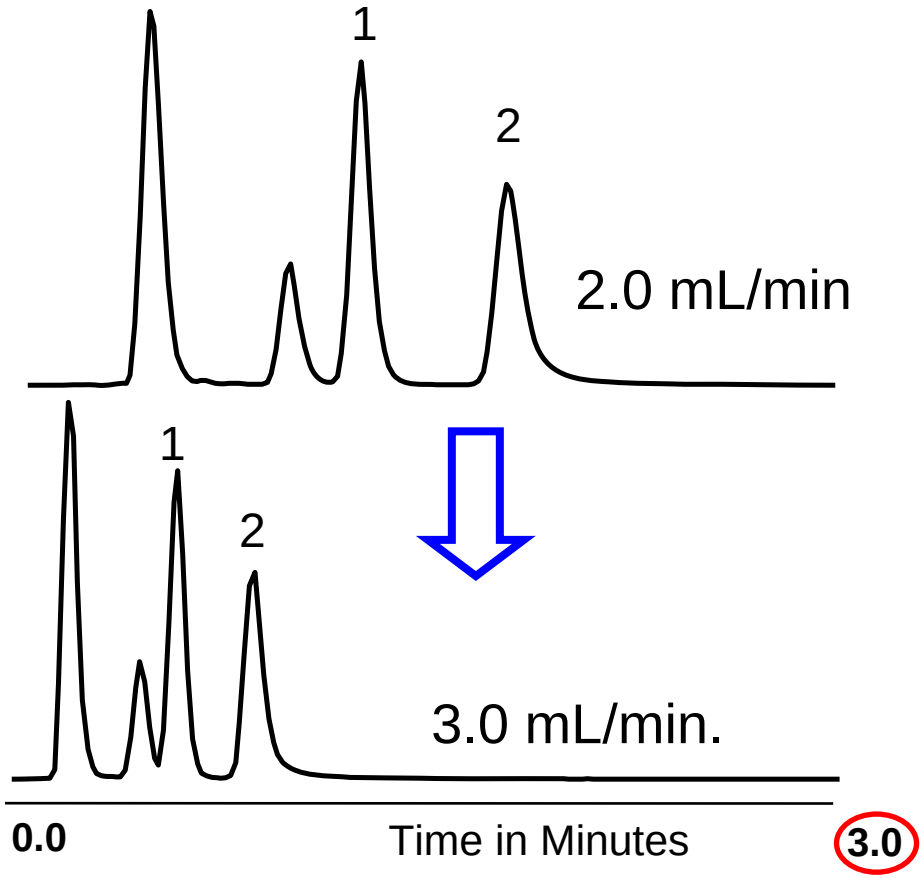
仅靠加快流速来完成的快速色谱结果是丧失分离度



5 µm 反相色谱柱

*** 50 mm 柱**

***** 较高的流速

$$R_{S_{1,2}} = 3.3$$

$$R_{S_{1,2}} = 2.3$$

未达到 **Rs = 3**的要求
(限制条件**)**

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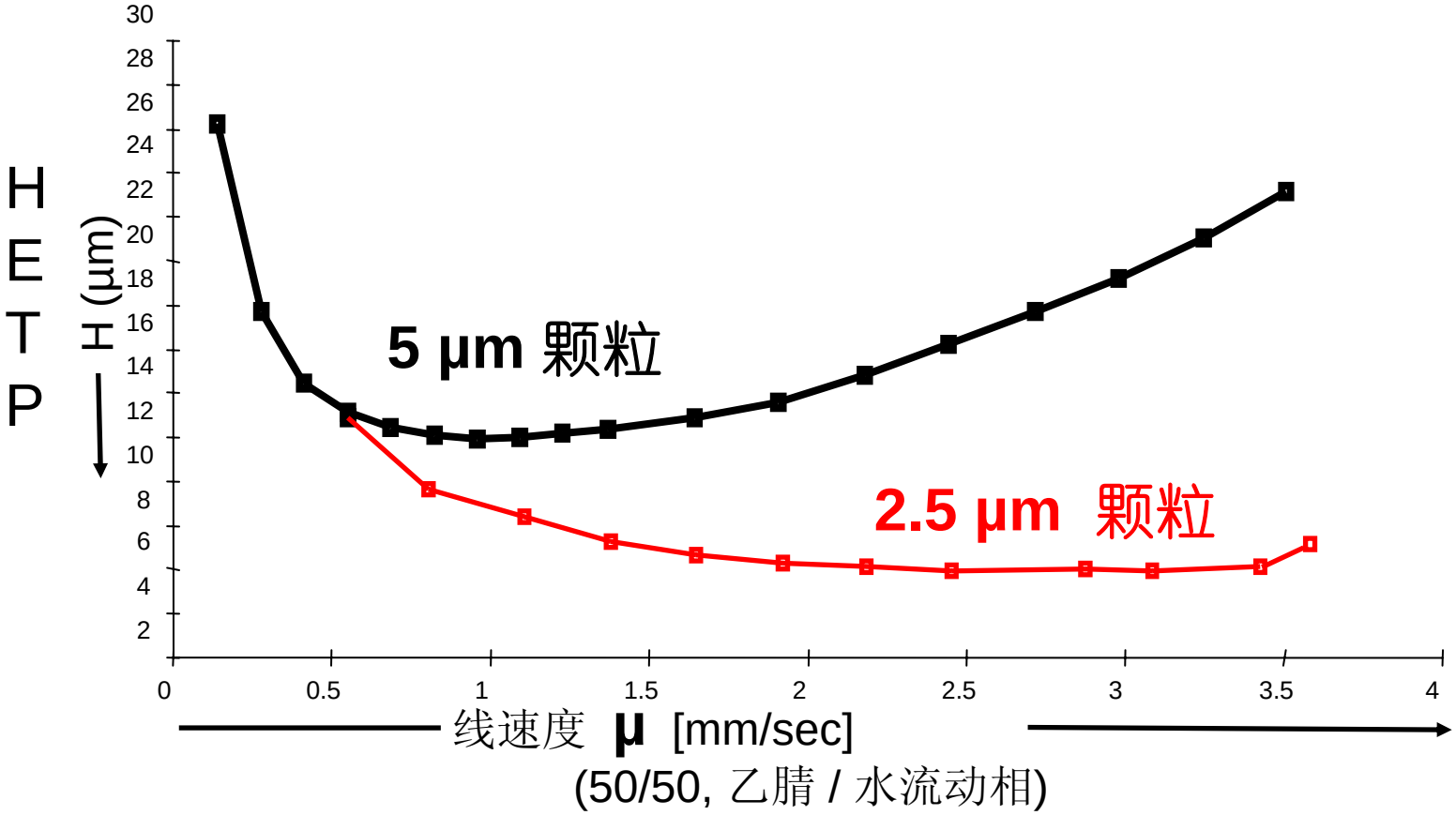
什么是 van Deemter 曲线

- 曲线是如何创建的?
- 对色谱意味着什么?



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用 HETP 表示柱效

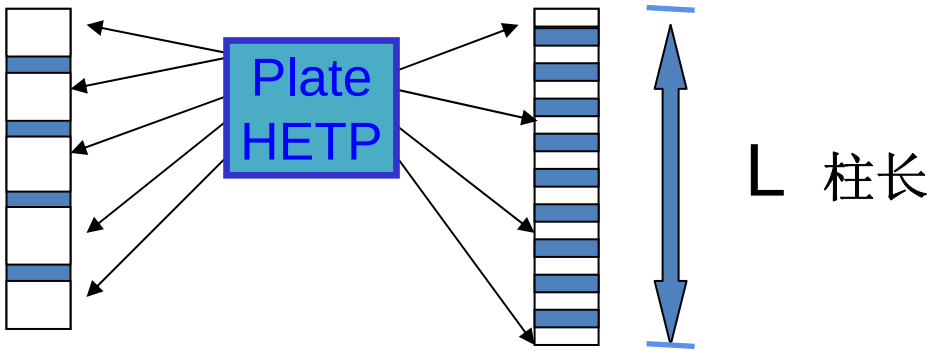
H = HETP (Height Equivalent to Theoretical Plate)

{要求 H 尽可能小,这样就可以往柱子里‘装’更多的‘板’}

$$N = \frac{L}{HETP}$$

N= # Plates

(来自蒸馏理论)



5 块板
(较大的 H)
“较厚”

10块板
(较小的 H)
“较薄”

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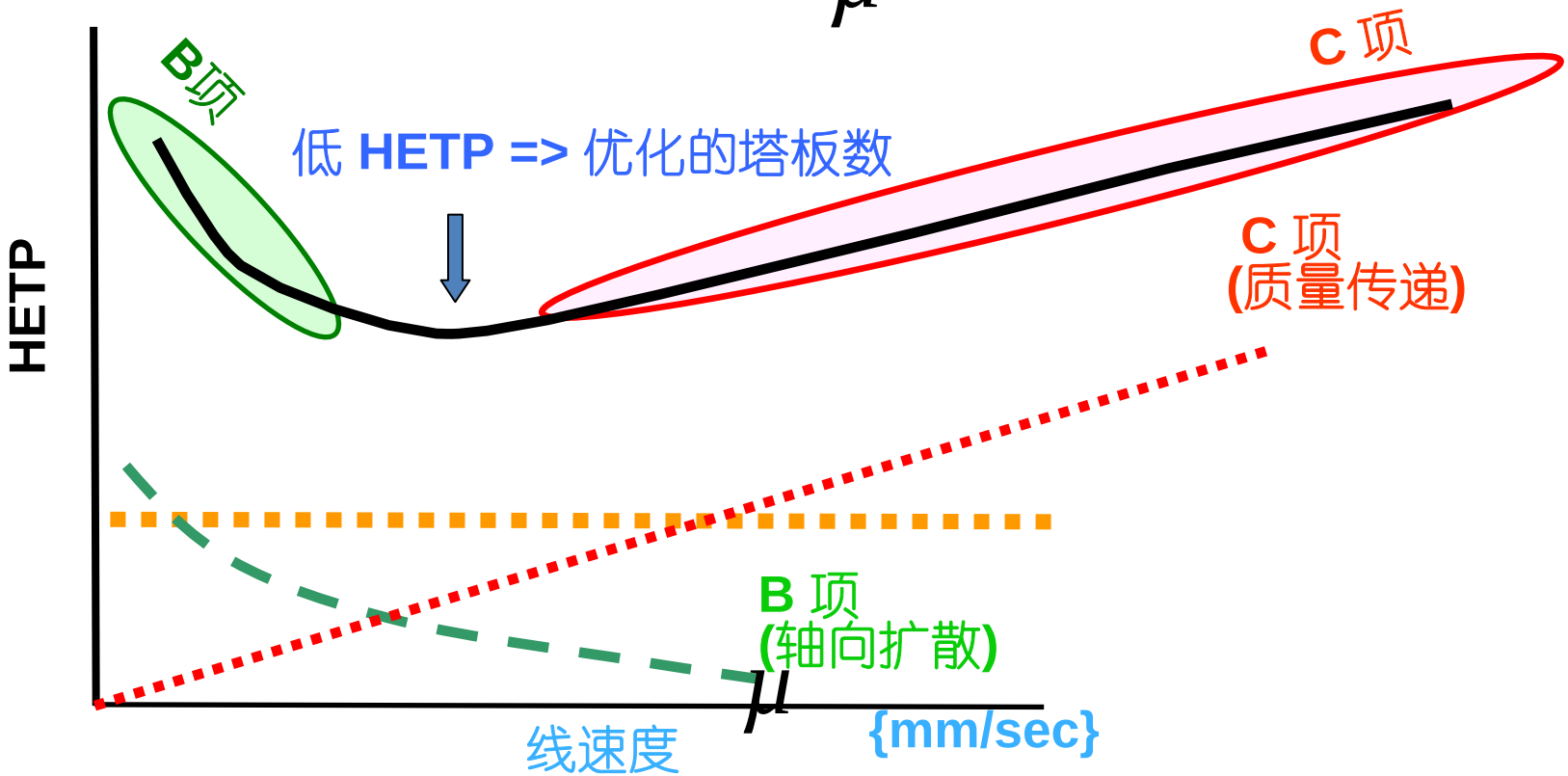
van Deemter 曲线

颗粒度的影响

$$H = a(dp) + \frac{b}{\mu} + c(dp)^2 \mu$$



三项加和得到最终的 **"van Deemter 曲线"**
它能够预报在色谱柱内将会发生的谱带展宽

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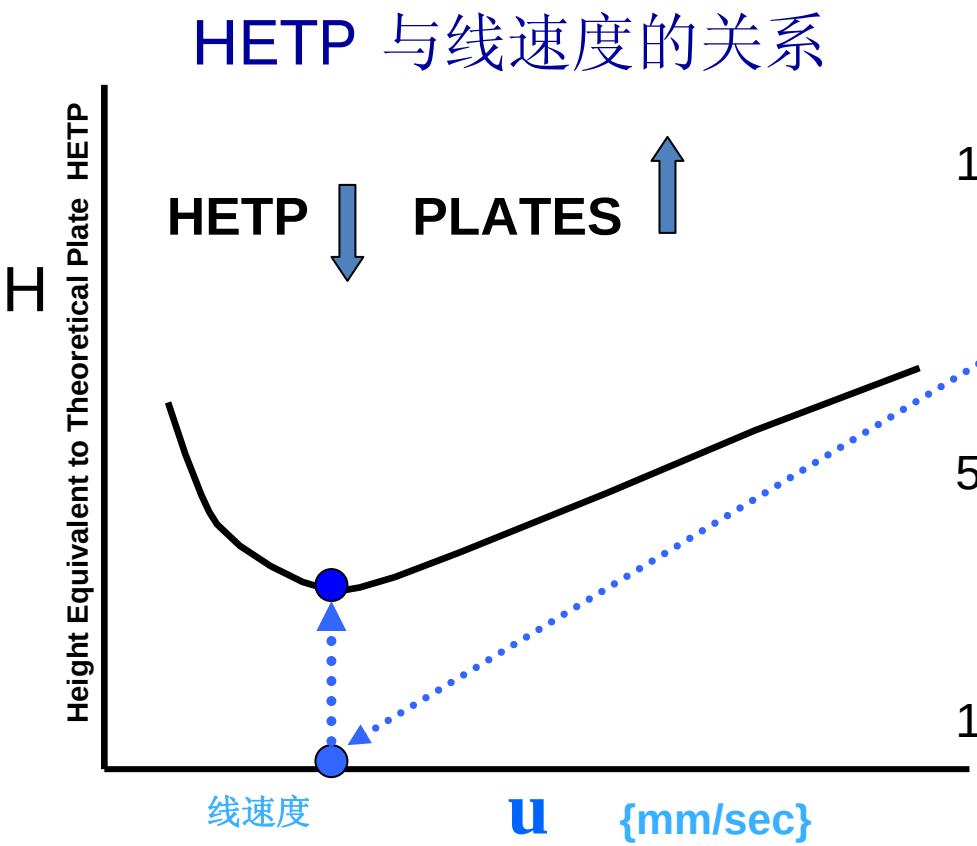
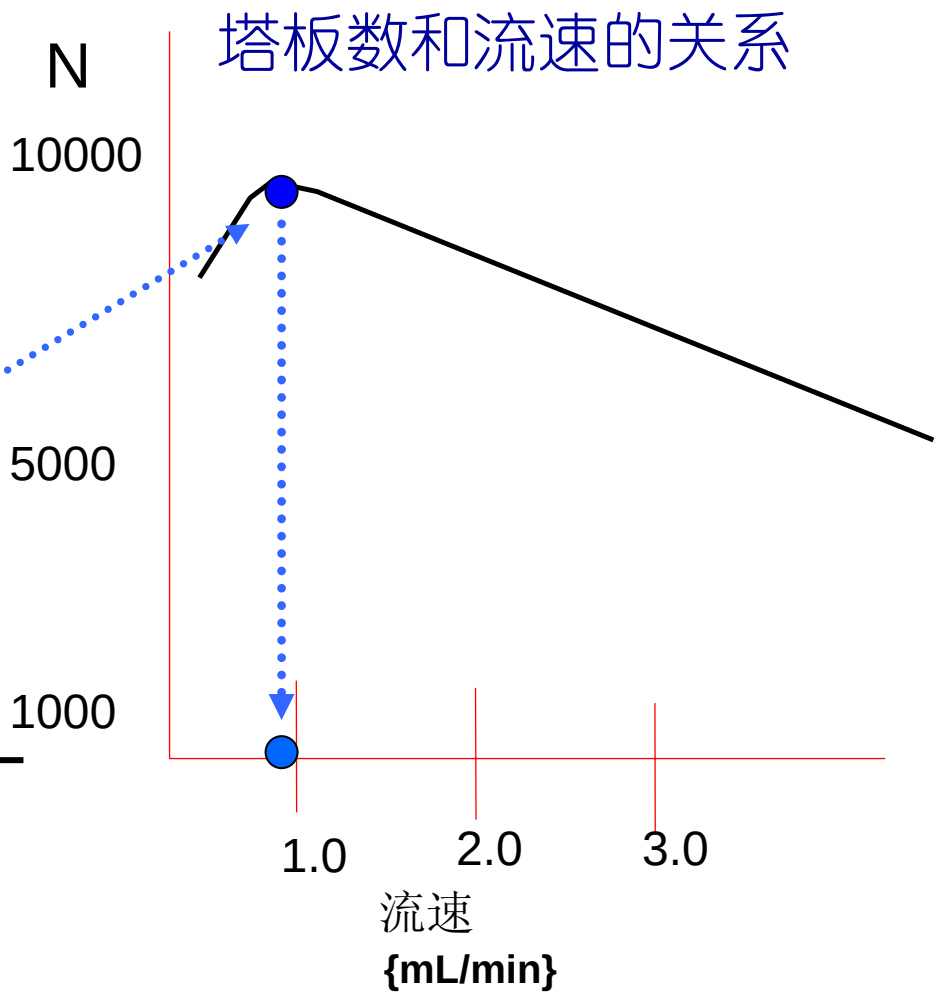
van Deemter 曲线与塔板数和流速的关系

最佳线速度与流速



$$u = L / t_0$$



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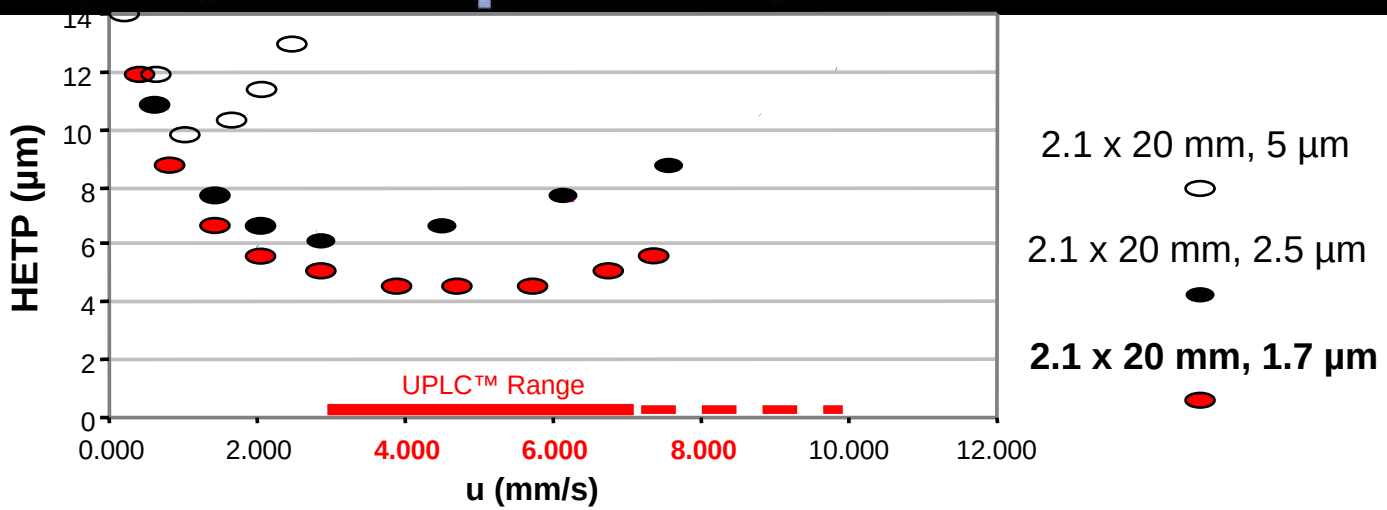
线速度与流速的换算



线速度 mm/sec	1	2	4	6	8	10
流速 1.0mm ID mL/min	0.04	0.07	0.13	0.20	0.28	0.34
流速 2.1mm ID (mL/min)	0.15	0.3	0.6	0.9	1.2	1.5
流速 4.6 mm ID	*0.7*	*1.4*	*2.8*	*4.2*	*5.5*	*7.0*

LC/MS

"Narrow-bore"

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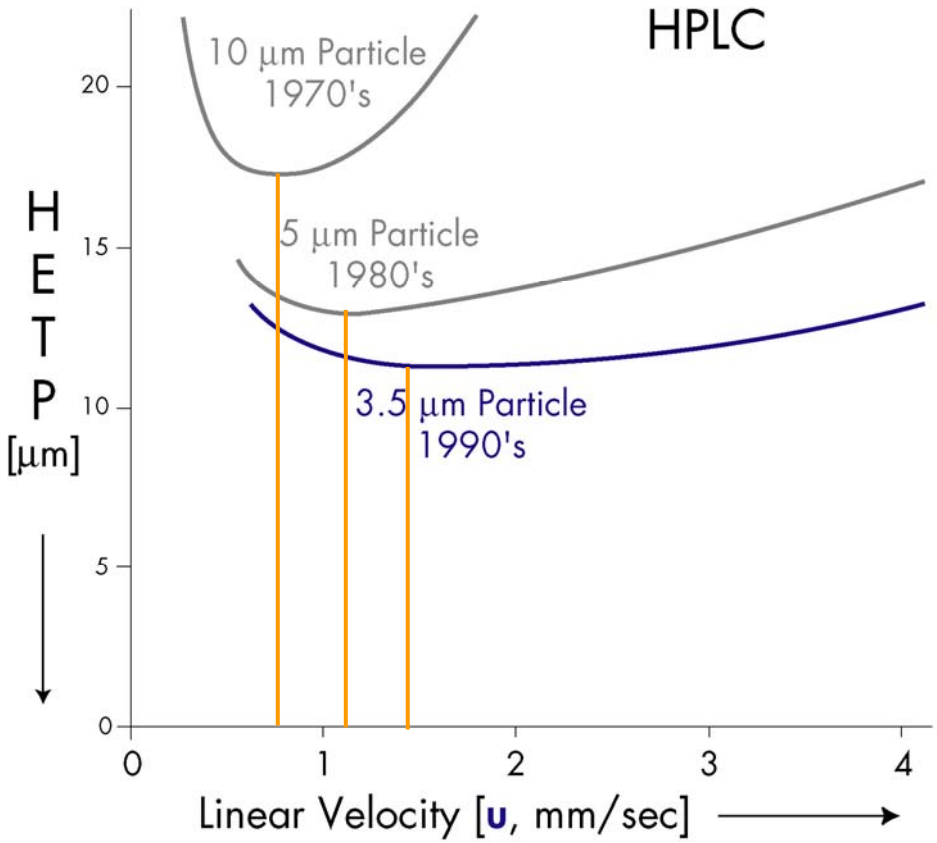
van Deemter曲线的提示

van Deemter 曲线的挑战



- 颗粒度越小柱效越高
  - 颗粒度控制着分离的质量
- 曲线上有一个最佳流速
- 更小的颗粒度：
  - 使最高柱效点向更高线速度方向移动
  - 有更宽的线速度范围
- 降低颗粒度不但可以增加柱效，同时也增加分离速度

如果填料的颗粒继续演变……

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未来的色谱:更小的颗粒度

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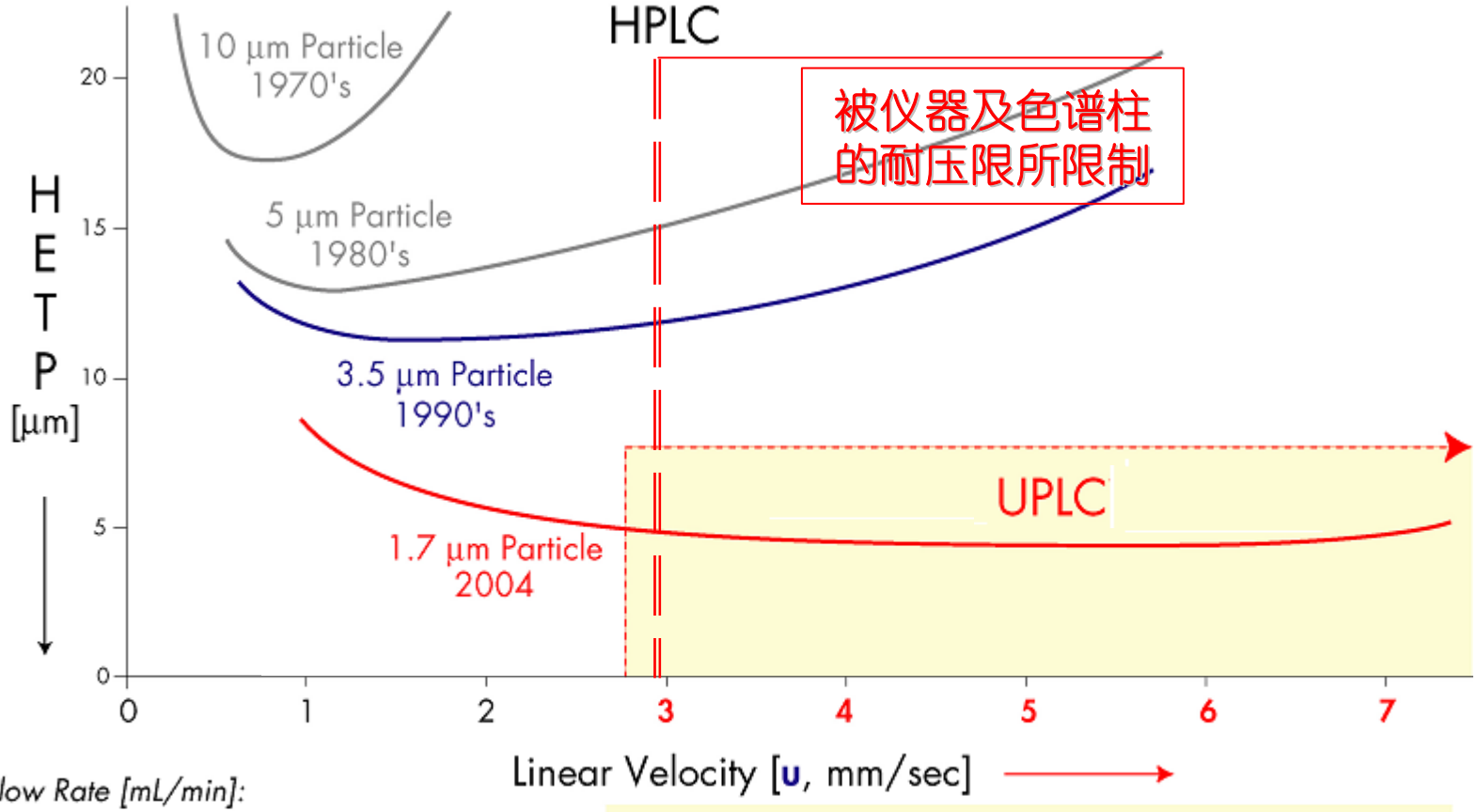

Flow Rate [mL/min]:							
ID = 1.0 mm	0.04	0.07	0.10	0.13	0.17	0.20	0.24
ID = 2.1 mm	0.15	0.3	0.45	0.6	0.75	0.9	1.05
ID = 4.6 mm	*0.7*	*1.4*	*2.1*	*2.8*	*3.5*	*4.2*	*4.9*

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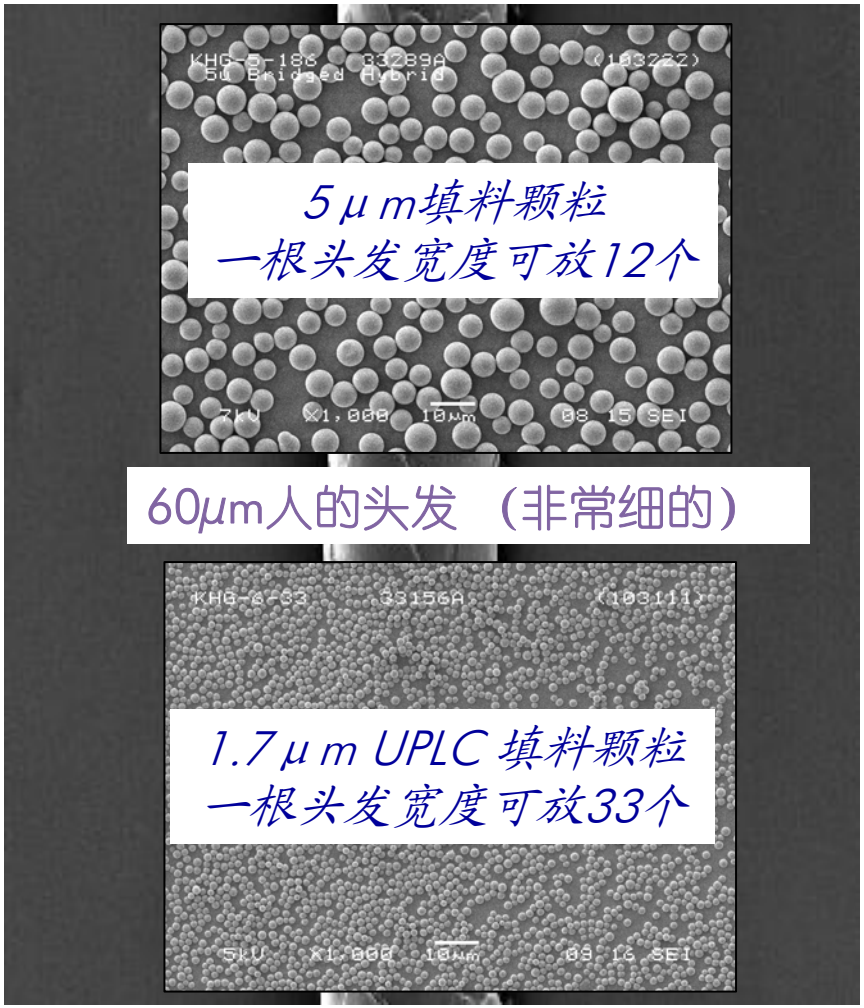
更小颗粒度所面临的挑战



- 色谱柱的性能
  - —颗粒耐压及柱结构
  - —颗粒度分布
- 系统的压力限
- 系统体积
- 检测的可行性
  - —流动池的优化
  - —检测的速度
- 系统控制及数据管理

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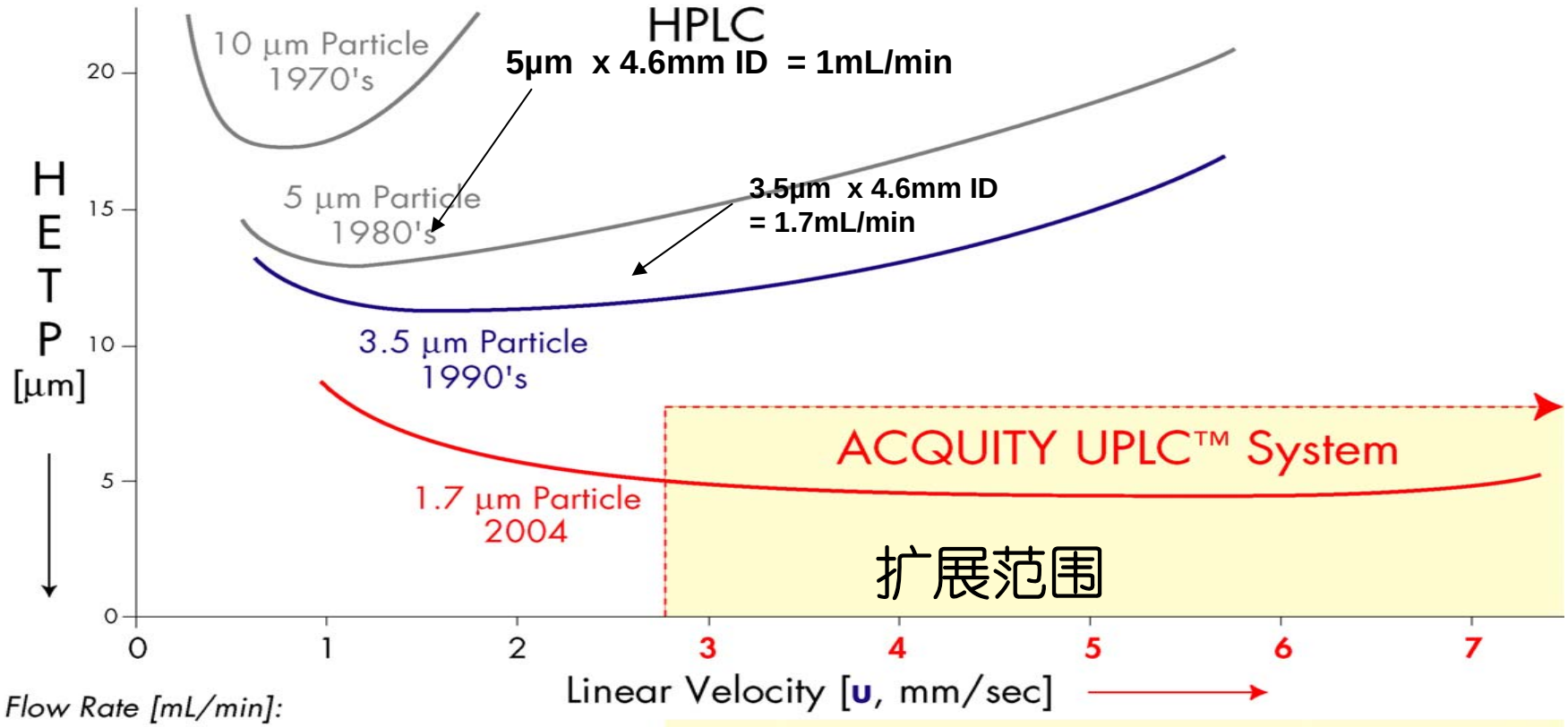
小颗粒带来生产力



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ID = 2.1 mm	0.15	0.3	0.45	0.6	0.75	0.9	1.05
ID = 4.6 mm	*0.7*	*1.4*	*2.1*	*2.8*	*3.5*	*4.2*	*4.9*

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柱长与颗粒度的比值
(最大分离能力的标记)

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$$\frac{L}{d_p} =$$

$$\frac{300\ mm}{10\ \mu m} = 30{,}000$$ 1970s ~ 30+ min.

$$\frac{150\ mm}{5\ \mu m} = 30{,}000$$ **1980s 10-15 min.**

$$\frac{100\ mm}{3\ \mu m} = 33{,}000$$ 1990s 5-10 min.

$$\frac{50\ mm}{1.7\ \mu m} = 29{,}500$$ 2004 ~1-2 min.

$$\frac{100\ mm}{1.7\ \mu m} = 58{,}820$$ ← ***2x 最大分离能力***

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保持 / 改善分离度的同时提高速度

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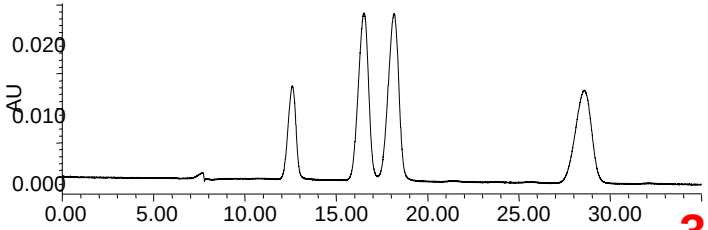
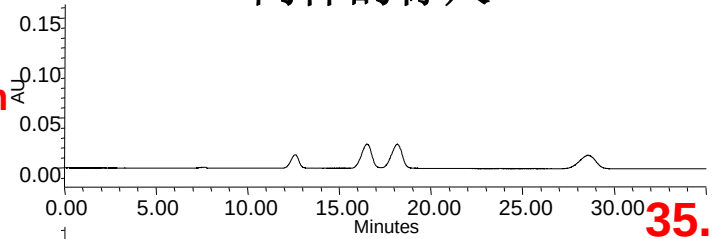
L/dp不变

独立的 Y- 轴

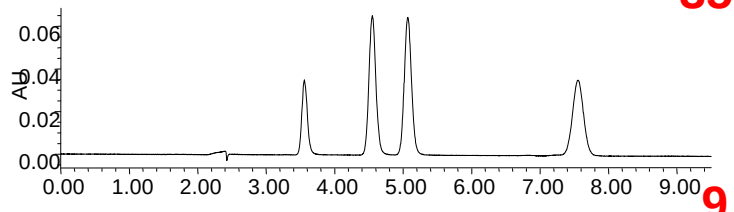
同样的标尺

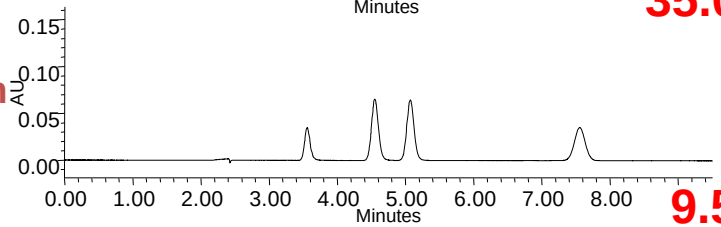




10 µm – 250 mm
Rs $_{(2,3)}$ = 1.54

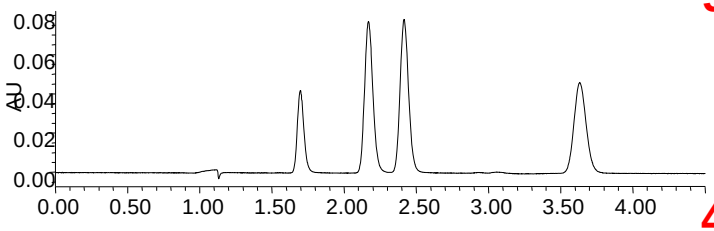
35.00

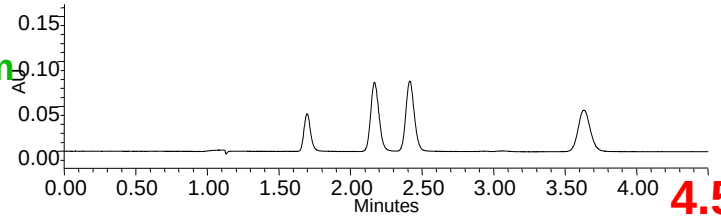




5 µm – 150 mm
Rs $_{(2,3)}$ = 2.69

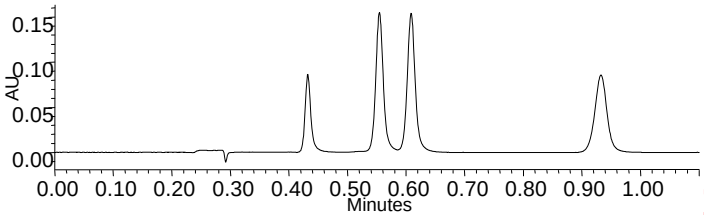
9.50

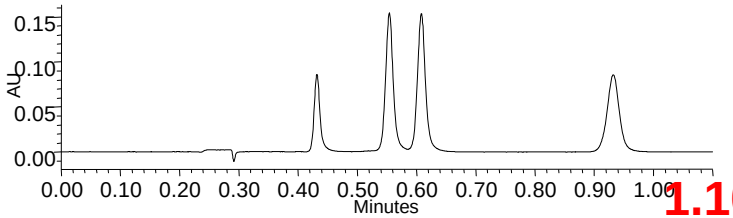




3.0 µm – 100 mm
Rs $_{(2,3)}$ = 2.29

4.50





1.7 µm – 50 mm
Rs $_{(2,3)}$ = 2.25

1.10

灵敏度 ⬆ 颗粒度 ⬇

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固定 L/dp 的峰表

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分离度				
	颗粒度 (µm)			
峰	10	5	3	1.7
1				
2	4.22	5.86	4.93	5.92
3	1.54	2.69	2.29	2.25
4	7.46	10.04	9.11	10.3

峰高				
	颗粒度 (µm)			
峰	10	5	3	1.7
1	13465	34883	41936	86087
2	24316	65209	76885	153625
3	24184	64468	77871	152450
4	13487	35144	46233	85613

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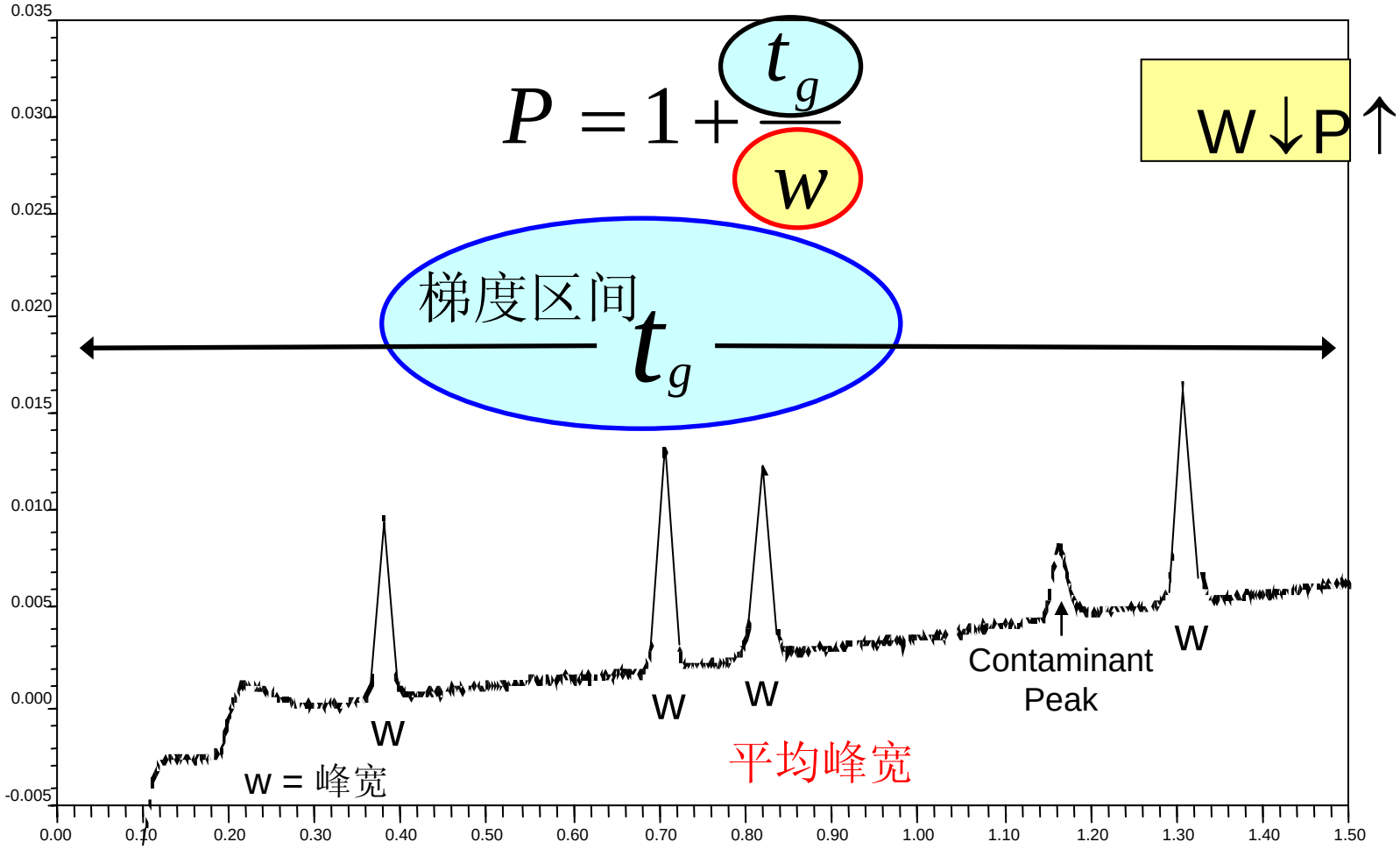
峰容量 (P)
梯度分离能力的量度



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ACQUITY UPLC 分离能力小结

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- van Deemter 方程和曲线预言了UPLC 的分离能力
- ACQUITY UPLC
  - 在保持分离度的同时扩展了线速度范围 ,同时减少了分析时间

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Ultra Performance LC 速度

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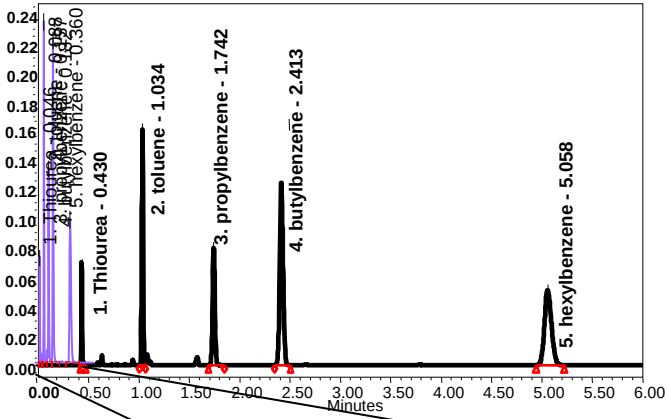
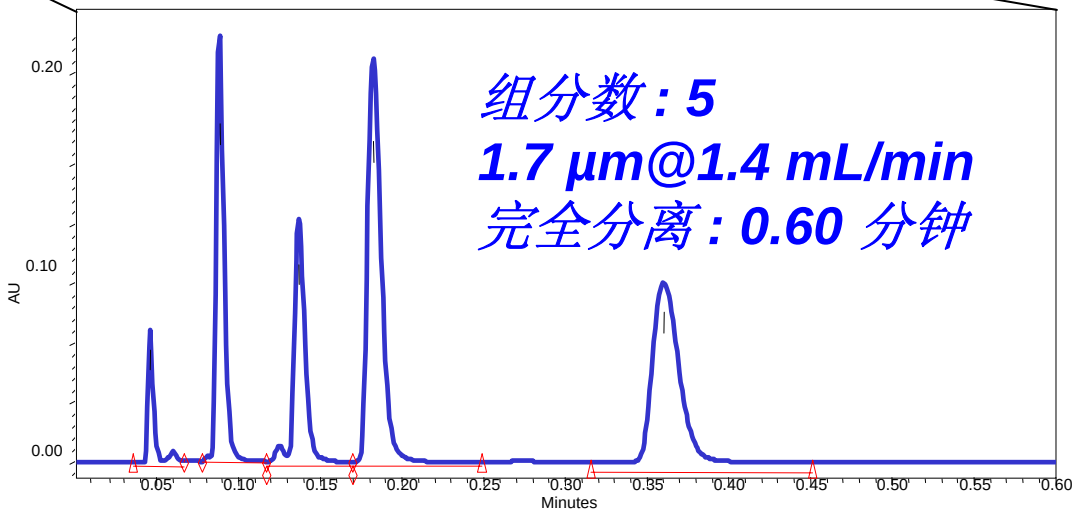
HPLC



组分数 : **5**
2.2 µm@0.7 mL/min
完全分离 : **6.00** 分钟

UPLC™

以 **2**倍流速,**10x**速度
0.6 分钟对 **6.0** 分钟
(0.85 mL 对 **4.2 mL)**



组分数 : 5
1.7 µm@1.4 mL/min
完全分离 : 0.60 分钟

Ultra Performance LC
速度，灵敏度和分离度

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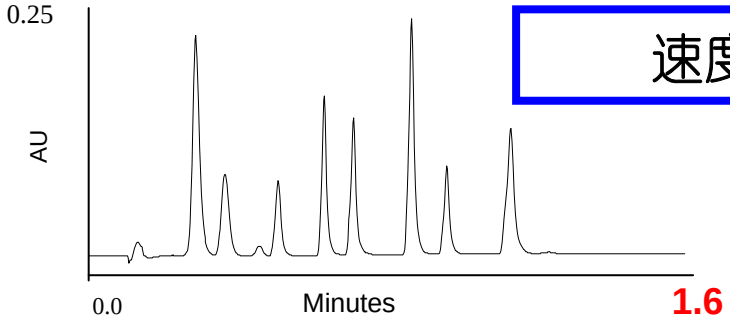
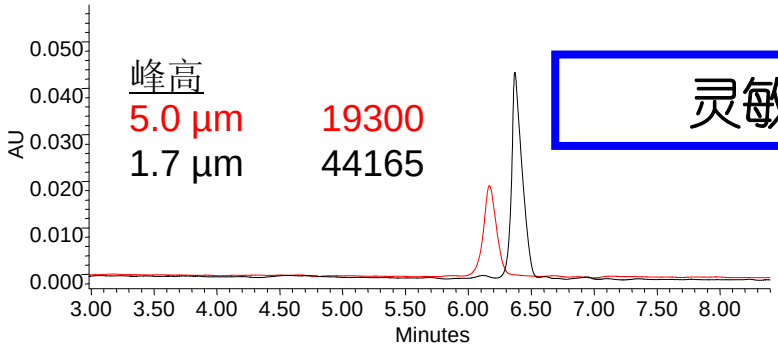
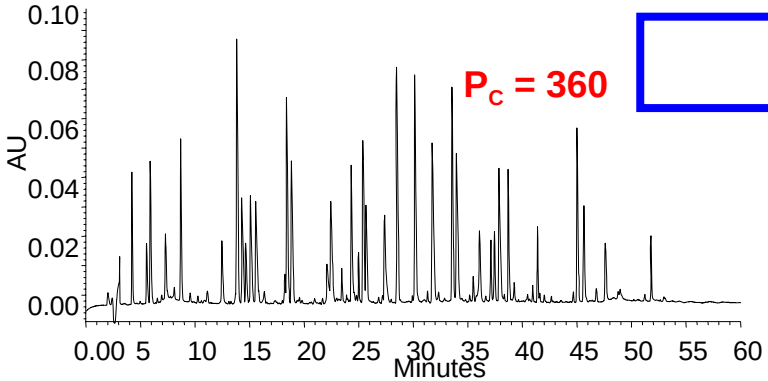


速度: **9X (900%)** 更快的分离



灵敏度: **3X (300%)** 增长



分离度: **1.7 (70%)** 增加

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UPLC 与 HPLC的异同

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- 相同:
  - —相同的色谱原理
  - —同样的色谱理论
- 相异:
  - —填料颗粒度
  - —系统工作压力
  - —色谱分离能力
- 相互关联:
  - —**UPLC**以**HPLC**的极限为起点,全面提升液相色谱的性能
  - —**UPLC**是分离科学的新领域

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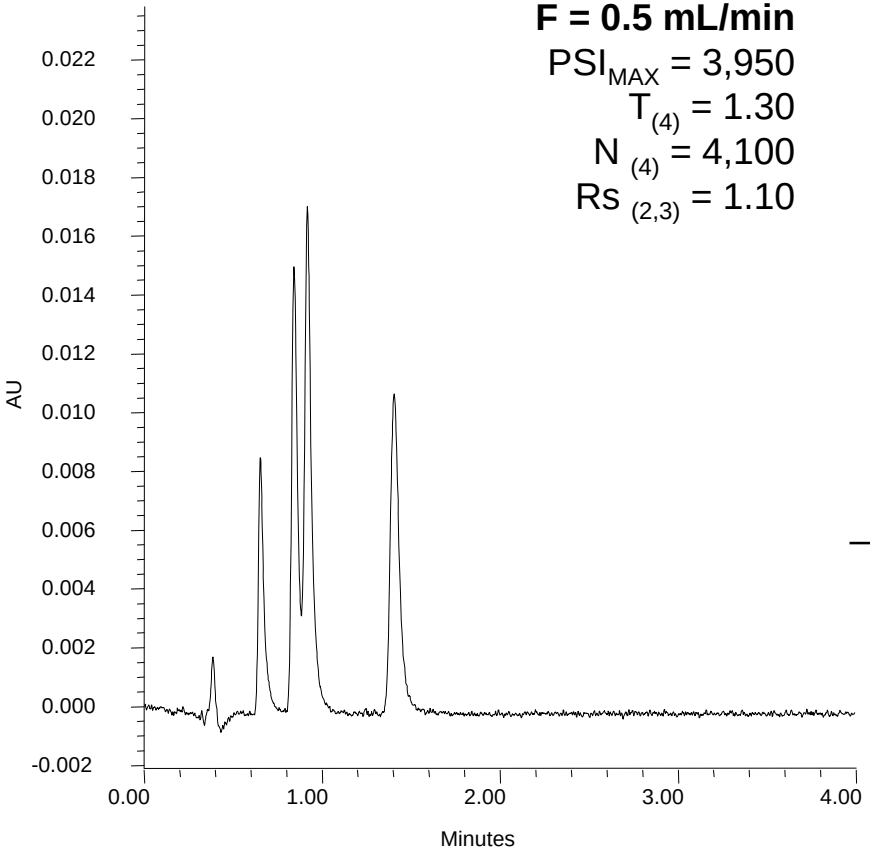
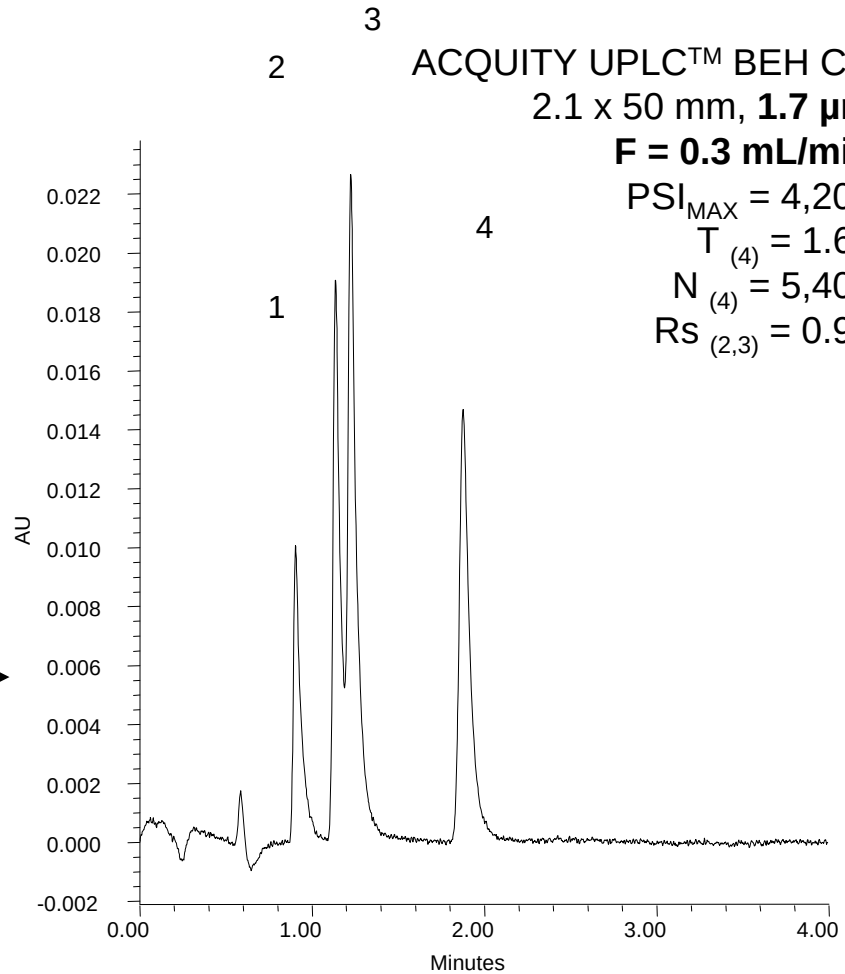
HPLC和UPLC比较(在HPLC系统上)

咖啡因代谢物

XTerra® MS C_{18}
2.1 x 50 mm, **2.5 µm**
F = 0.5 mL/min
PSI_{MAX} = 3,950
$T_{(4)}$ = 1.30
$N_{(4)}$ = 4,100
$Rs_{(2,3)}$ = 1.10



ACQUITY UPLC™ BEH C_{18}
2.1 x 50 mm, **1.7 µm**
F = 0.3 mL/min
PSI_{MAX} = 4,200
$T_{(4)}$ = 1.63
$N_{(4)}$ = 5,400
$Rs_{(2,3)}$ = 0.97



非优化的线速度，差的峰形和柱效

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在ACQUITY UPLC上优化线速度

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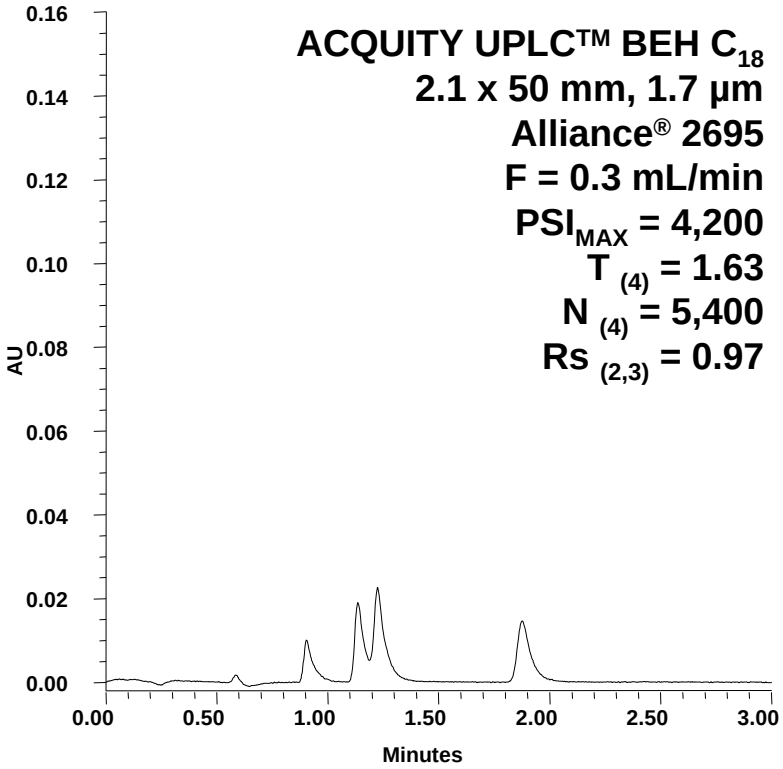
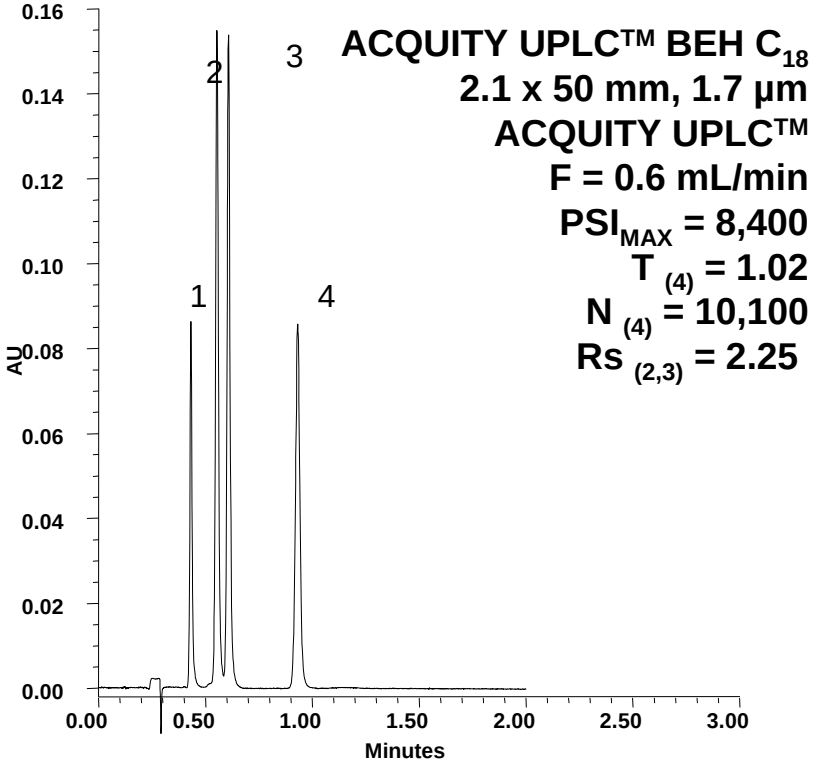
咖啡因代谢物

HPLC
非优化的线速度

UPLC
优化的线速度





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从HPLC 到 UPLC

用ACQUITY 做 HPLC

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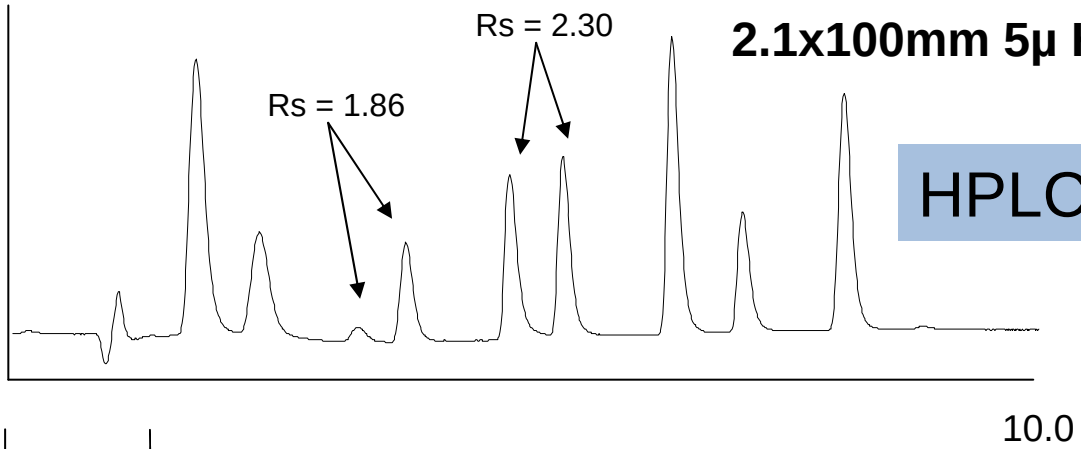
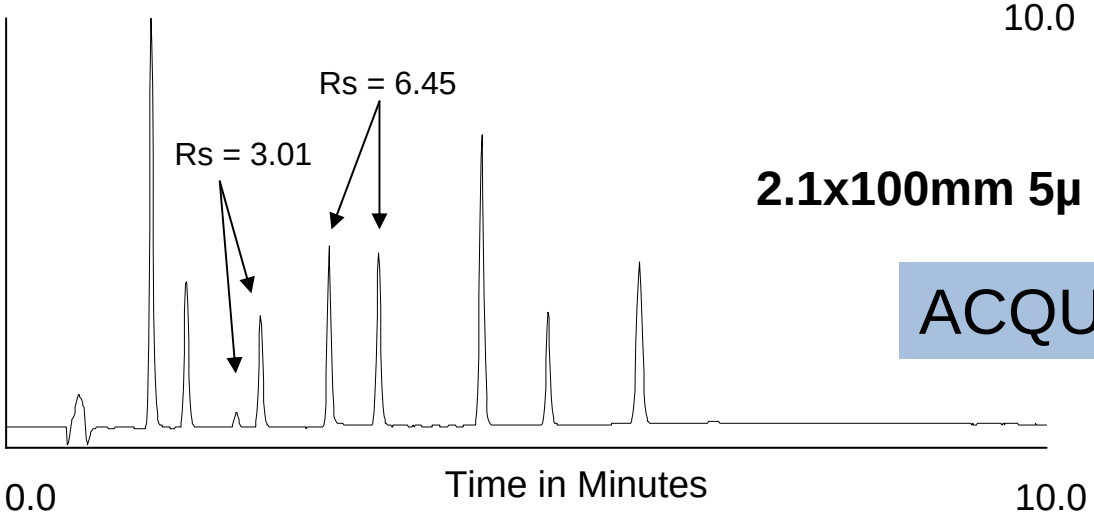
1



2.1x100mm 5µ Hybrid C18

HPLC

2



2.1x100mm 5µ Hybrid C18

ACQUITY (HPLC)

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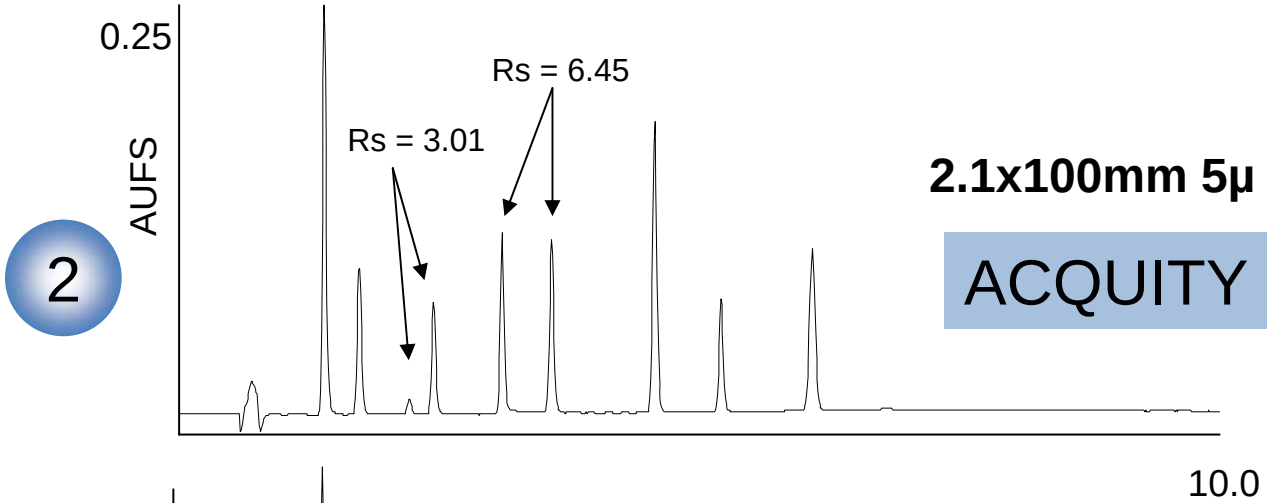
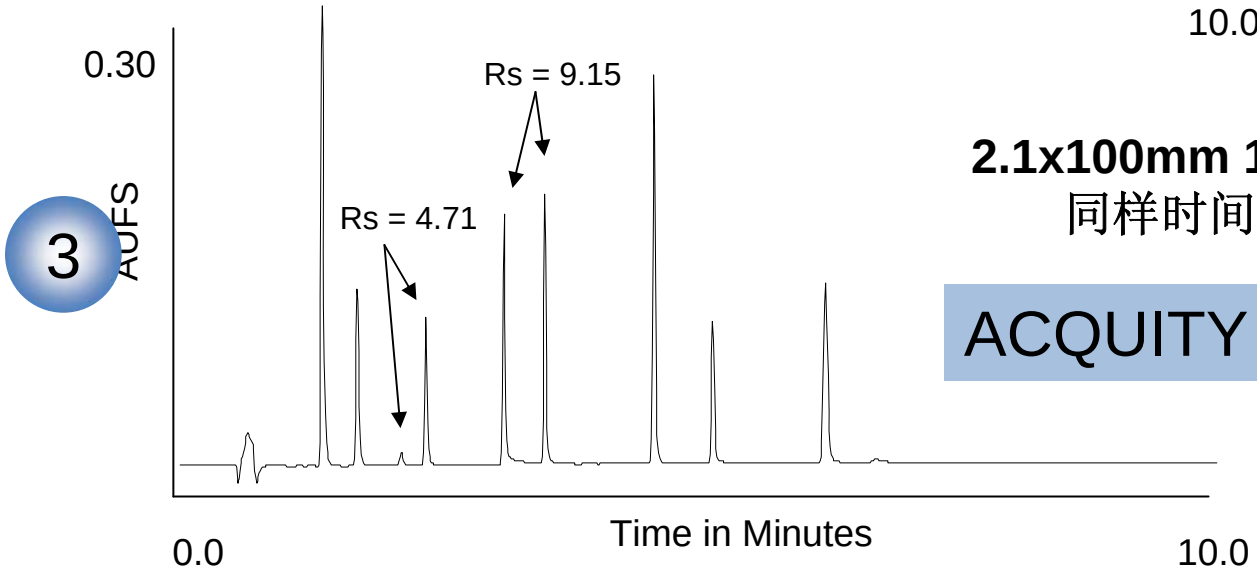
用ACQUITY做HPLC 和 UPLC™



2.1x100mm 5µ Hybrid C18

ACQUITY (HPLC)



2.1x100mm 1.7µ Hybrid C18
同样时间,分离度更高

ACQUITY UPLC™

ACQUITY UPLC™

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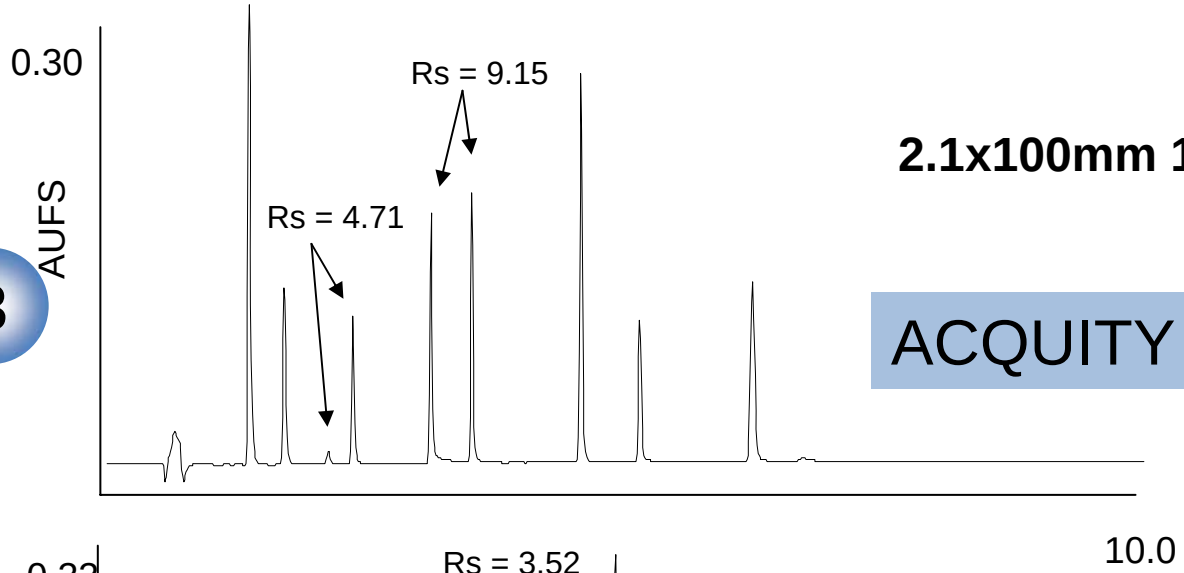
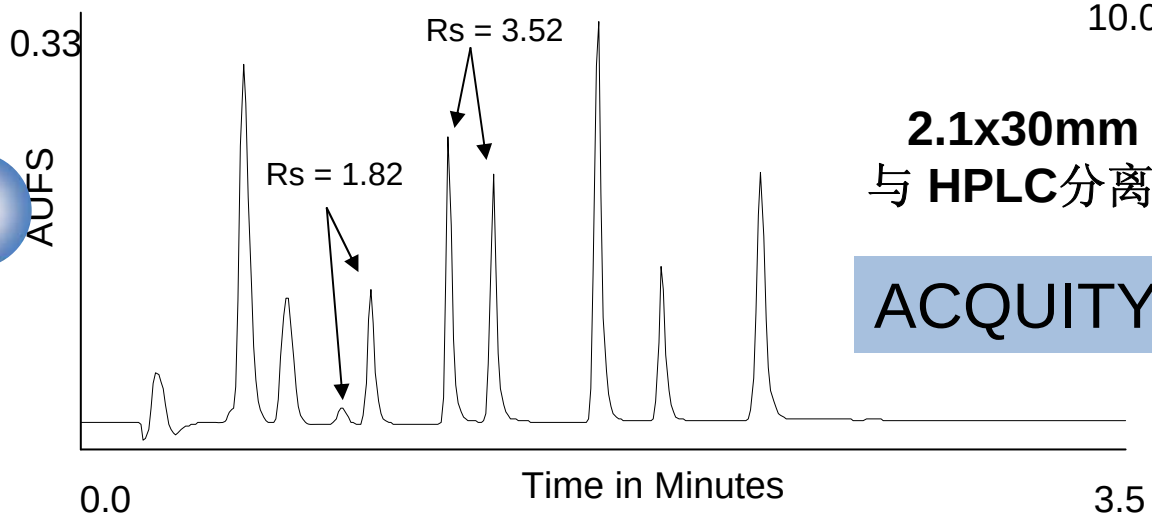
3



2.1x100mm 1.7µ Hybrid C18

ACQUITY UPLC™

4



2.1x30mm 1.7µ Hybrid C18
与 **HPLC**分离度相同**,** 但时间缩短

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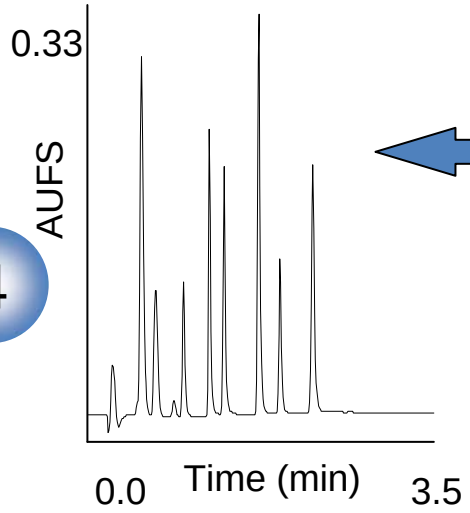
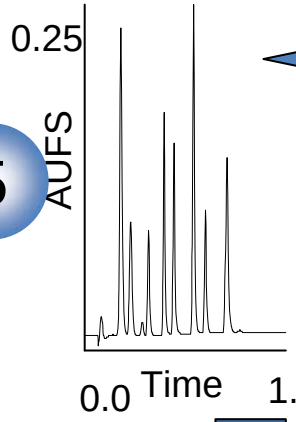
4



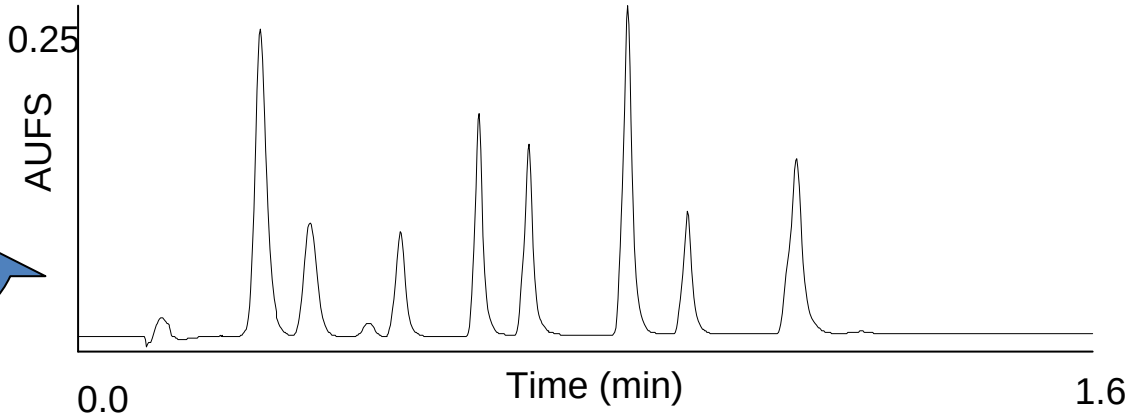
2.1x30mm 1.7µ Hybrid C18
与 **HPLC**分离度相同**,** 但时间缩短

ACQUITY UPLC™

5



2.1x30mm 1.7µ Hybrid C18
流速加倍，相似分离度，更短分离时间



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HPLC 和 UPLC

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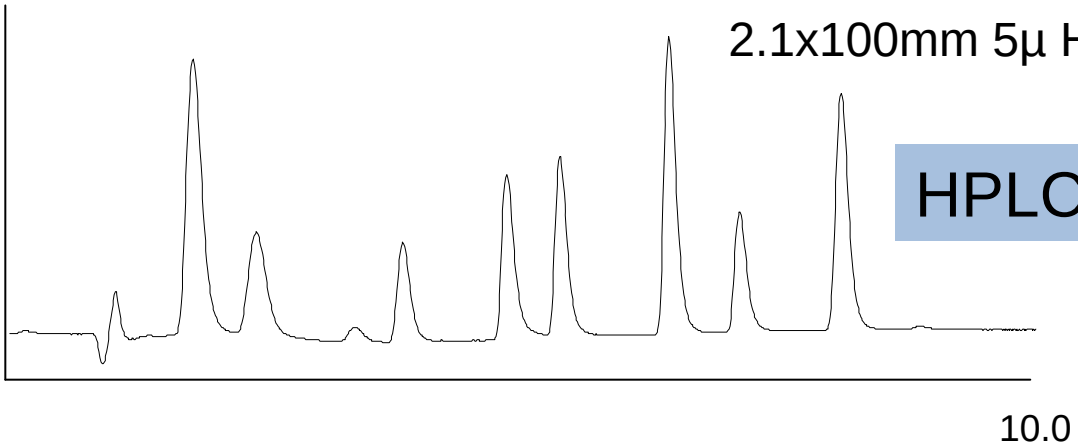
1

2.1x100mm 5µ Hybrid C18

HPLC

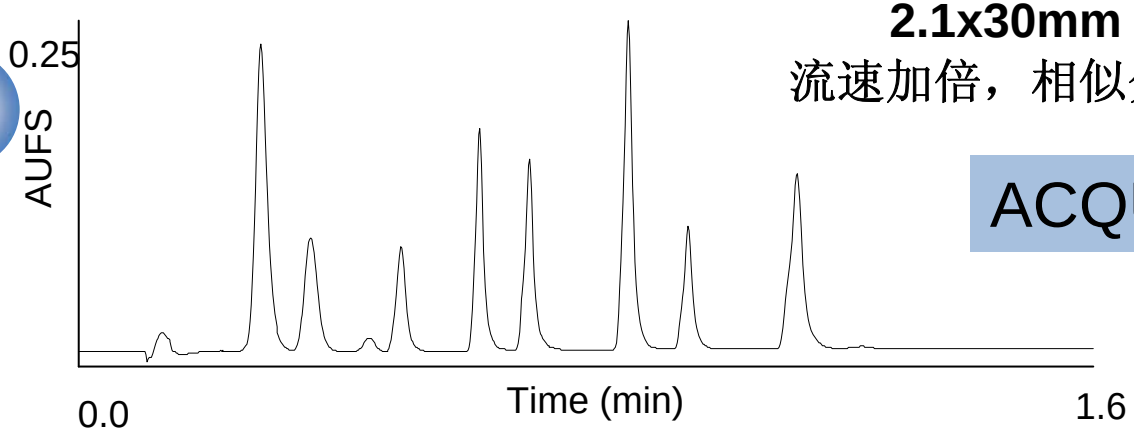


5

2.1x30mm 1.7µ Hybrid C18

流速加倍，相似分离度，更短分离时间

ACQUITY (UPLC)™



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实例:三环抗抑郁药

- 等度分析
  - —**HPLC** 对比 **UPLC**
- 方法传递
  - —**HPLC 5 µm** 颗粒到 **UPLC BEH 1.7 µm** 颗粒
- 优点
  - —减少分析时间 **(**提高速度**)**
  - —增加灵敏度
  - —增加分离度

HPLC 等度分析

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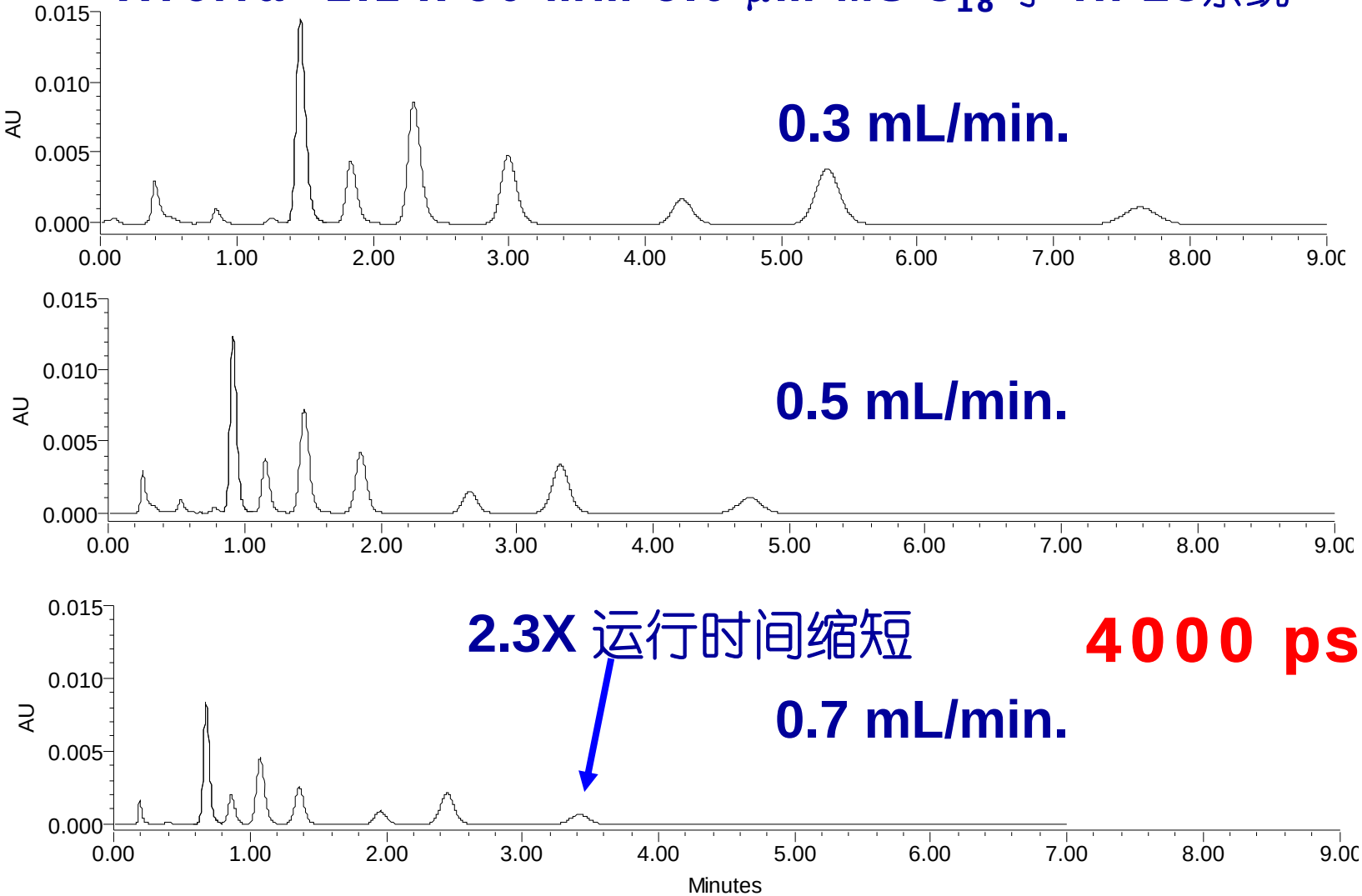
XTerra® 2.1 x 50 mm 5.0 μm MS C_{18} 于 HPLC系统



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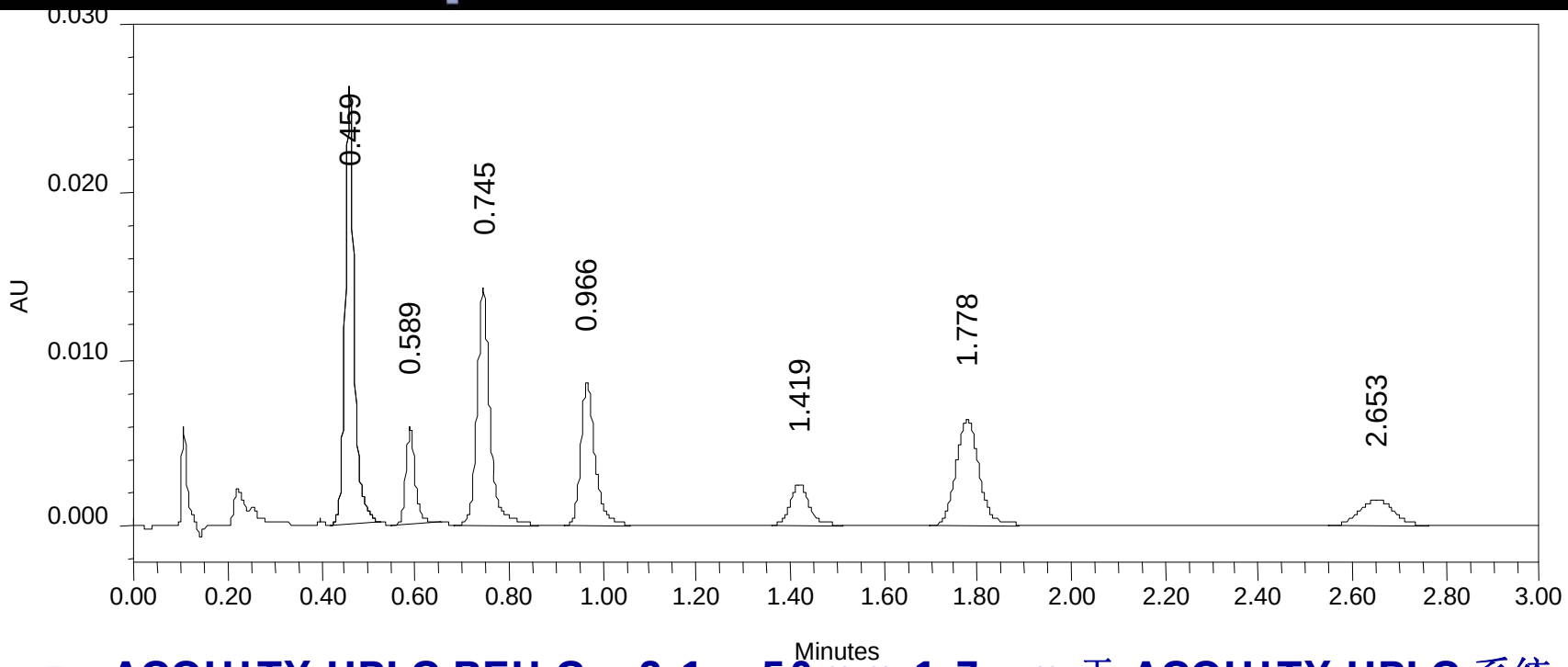
ACQUITY UPLC等度分析



- **ACQUITY UPLC BEH C_{18} 2.1 x 50mm 1.7 µm 于 ACQUITY UPLC 系统**
  - —流速增至 **1.1 mL/min**
  - —**XTerra** 柱不能做
    - o 导致反压 **>6500 psi**

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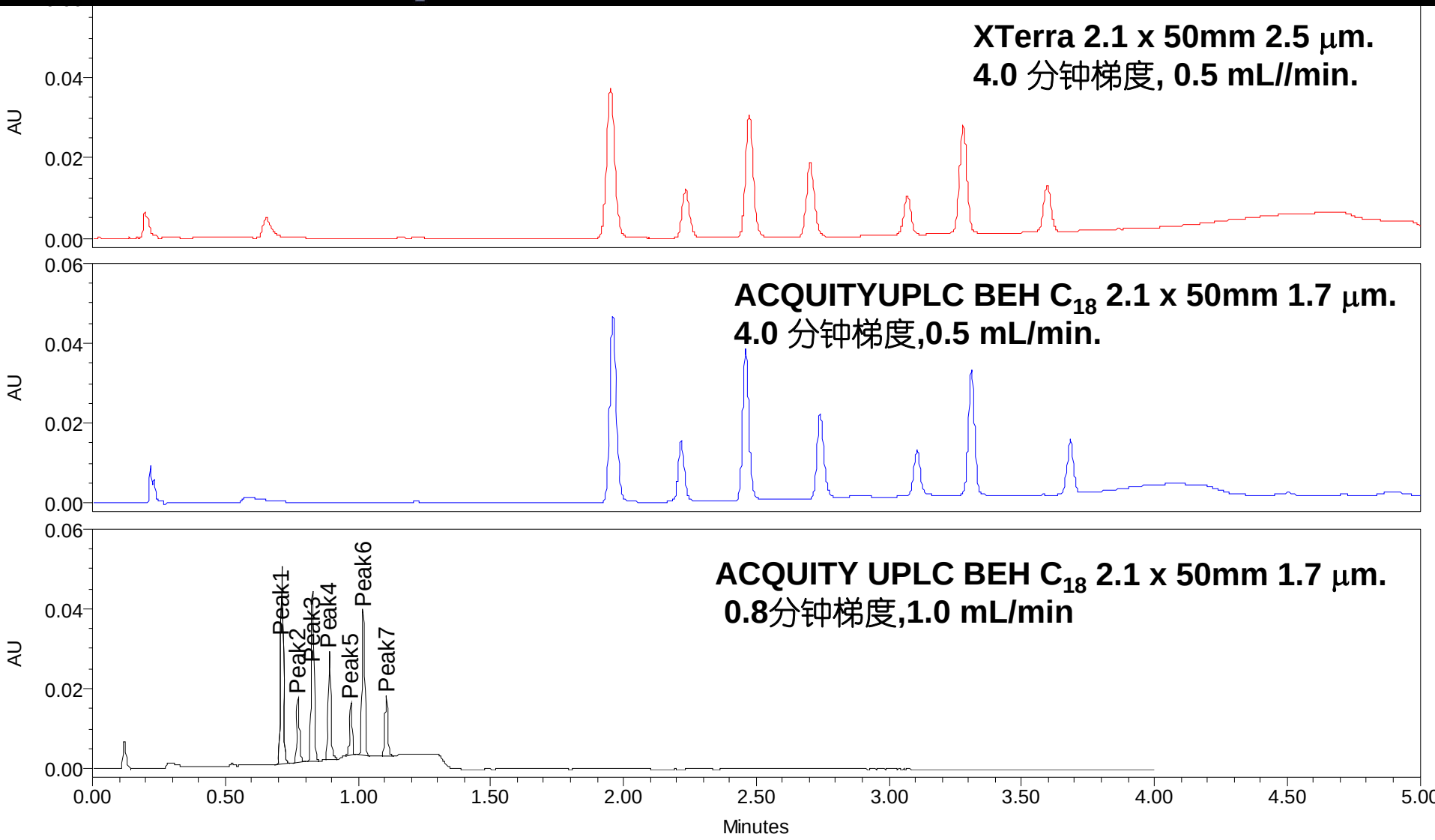
XTerra 用于ACQUITY UPLC系统



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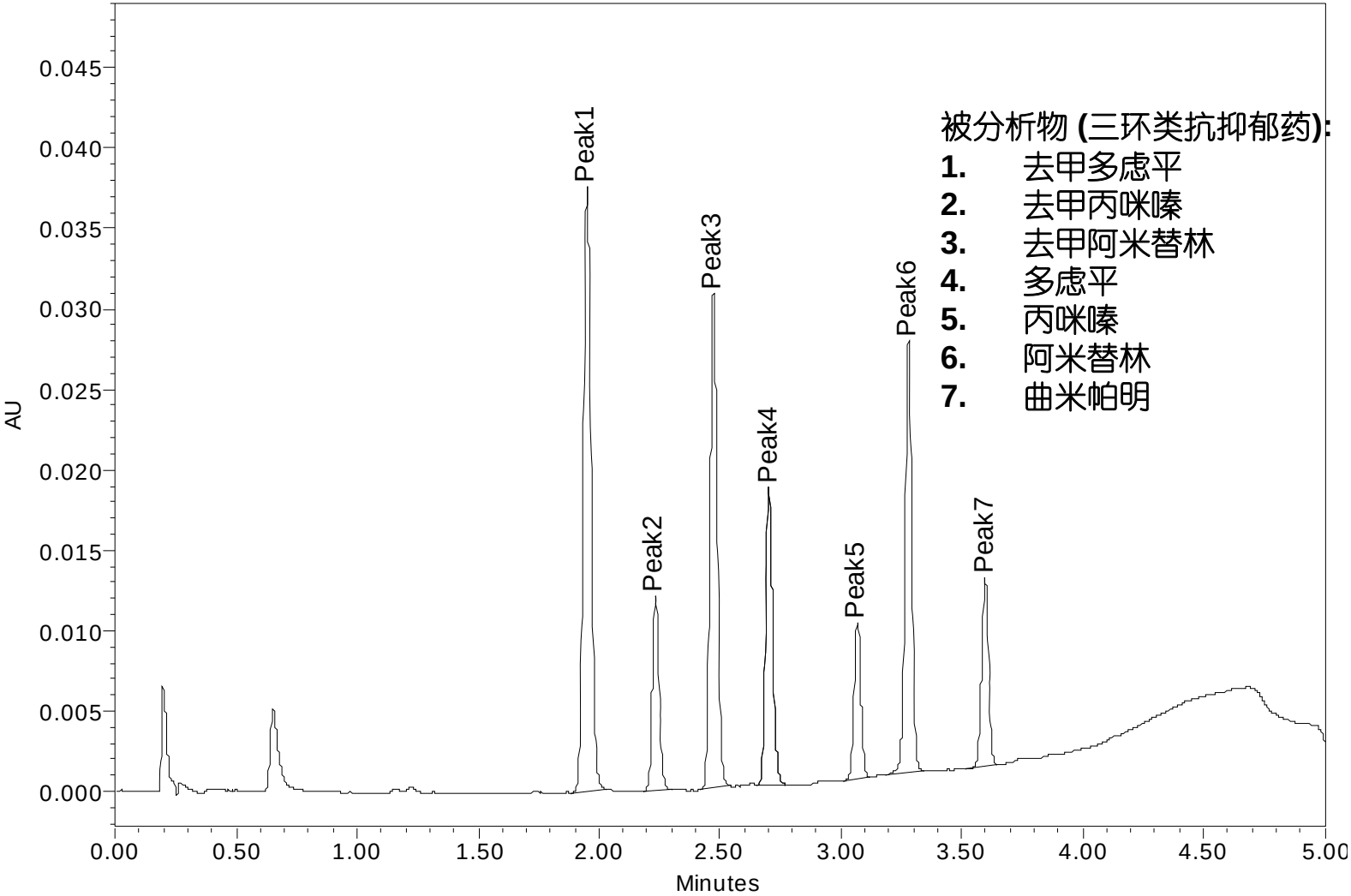
XTerra 2.1 x 50 mm 用于 ACQUITY UPLC 系统



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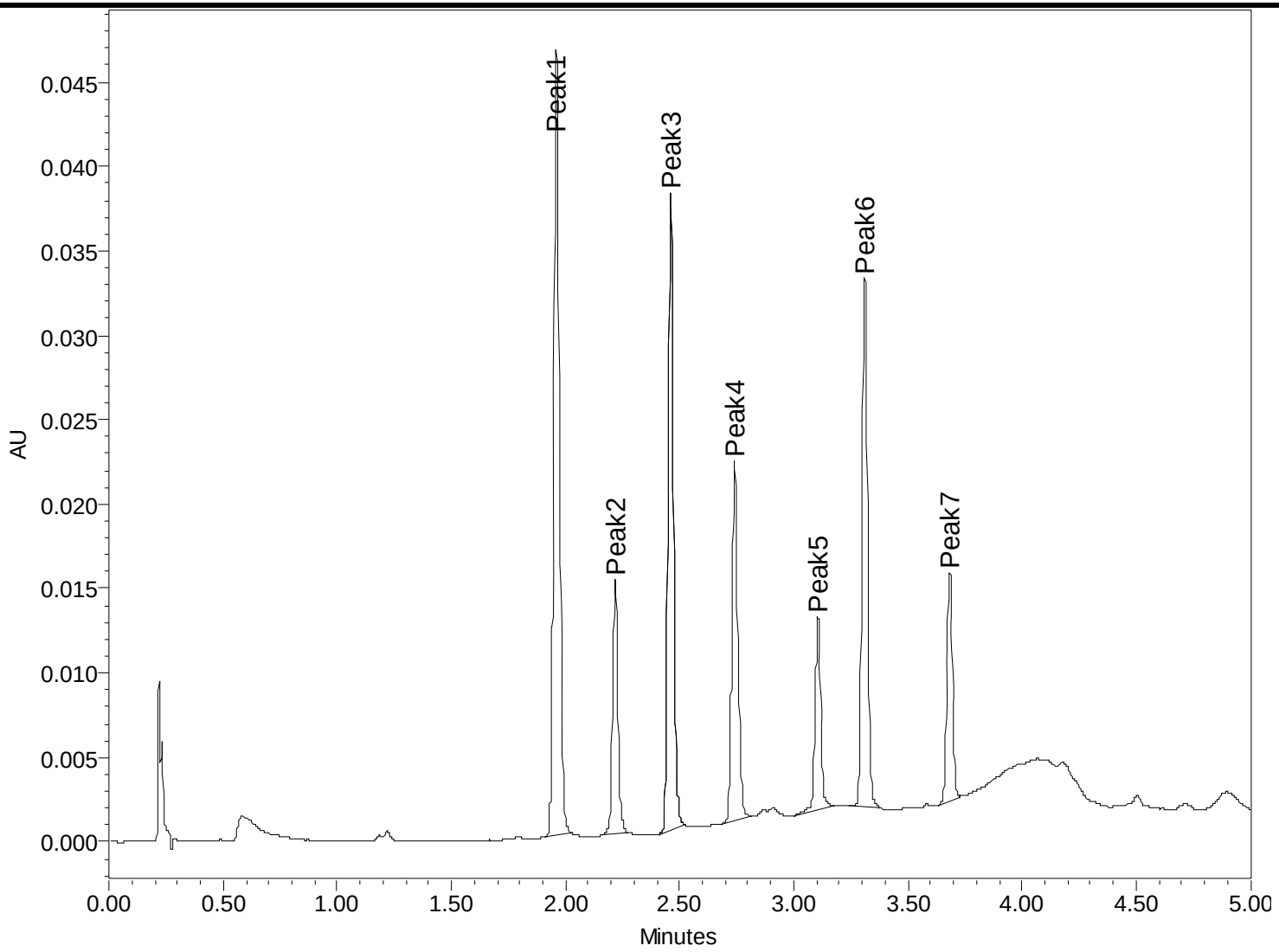
ACQUITY UPLC BEH C_{18} 2.1 x 50 mm 用于 ACQUITY UPLC 系统



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XTerra 2.5 µm 柱与 ACQUITY UPLC 1.7 µm 柱的比较

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	Name	R. T.	Area	Height	USP Resolution	Peak Capacity 4Sigma	5 Sigma	Width @ 13.4%	width in seconds @ 13.4%
Xterra 2.5um	Nordoxepin	1.951	81126	37589		69.70	16809	0.0582	3.49
Xterra 2.5um	Desipramine	2.234	23818	12136	5.19	76.53	26749	0.0530	3.18
Xterra 2.5um	Nortriptyline	2.474	62884	30809	4.53	73.92	30779	0.0549	3.29
Xterra 2.5um	Doxepin	2.703	38054	18567	4.21	72.42	36005	0.0560	3.36
Xterra 2.5um	Imipramine	3.069	18669	9737	6.94	78.34	53769	0.0517	3.10
Xterra 2.5um	Amitriptyline	3.280	52903	26883	4.09	76.74	57805	0.0528	3.17
Xterra 2.5um	Trimipramine	3.597	22891	11774	6.11	77.16	71551	0.0525	3.15
					Average	**74.97**	**41924**		
	Name	**R.T.**	**Area**	**Height**	**USP Resolution**	**Peak Capacity 4Sigma**	**5 Sigma**	**Width @ 13.4%**	**width in seconds**
ACQUITY 1.7um	Nordoxepin	1.960	81613	46507		85.56	25484	0.0473	2.84
ACQUITY 1.7um	Desipramine	2.217	24695	15177	5.83	92.37	33455	0.0438	2.63
ACQUITY 1.7um	Nortriptyline	2.460	62689	37797	5.71	90.68	44729	0.0446	2.68
ACQUITY 1.7um	Doxepin	2.741	38559	21248	6.18	80.54	45538	0.0503	3.02
ACQUITY 1.7um	Imipramine	3.106	20229	11482	7.97	88.21	56985	0.0459	2.75
ACQUITY 1.7um	Amitriptyline	3.311	52040	31246	4.63	91.04	84679	0.0444	2.67
ACQUITY 1.7um	Trimipramine	3.684	22749	13522	8.35	89.73	104829	0.0451	2.70
					Average	**88.30**	**56528**		

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ACQUITY UPLC 柱对速度的优化

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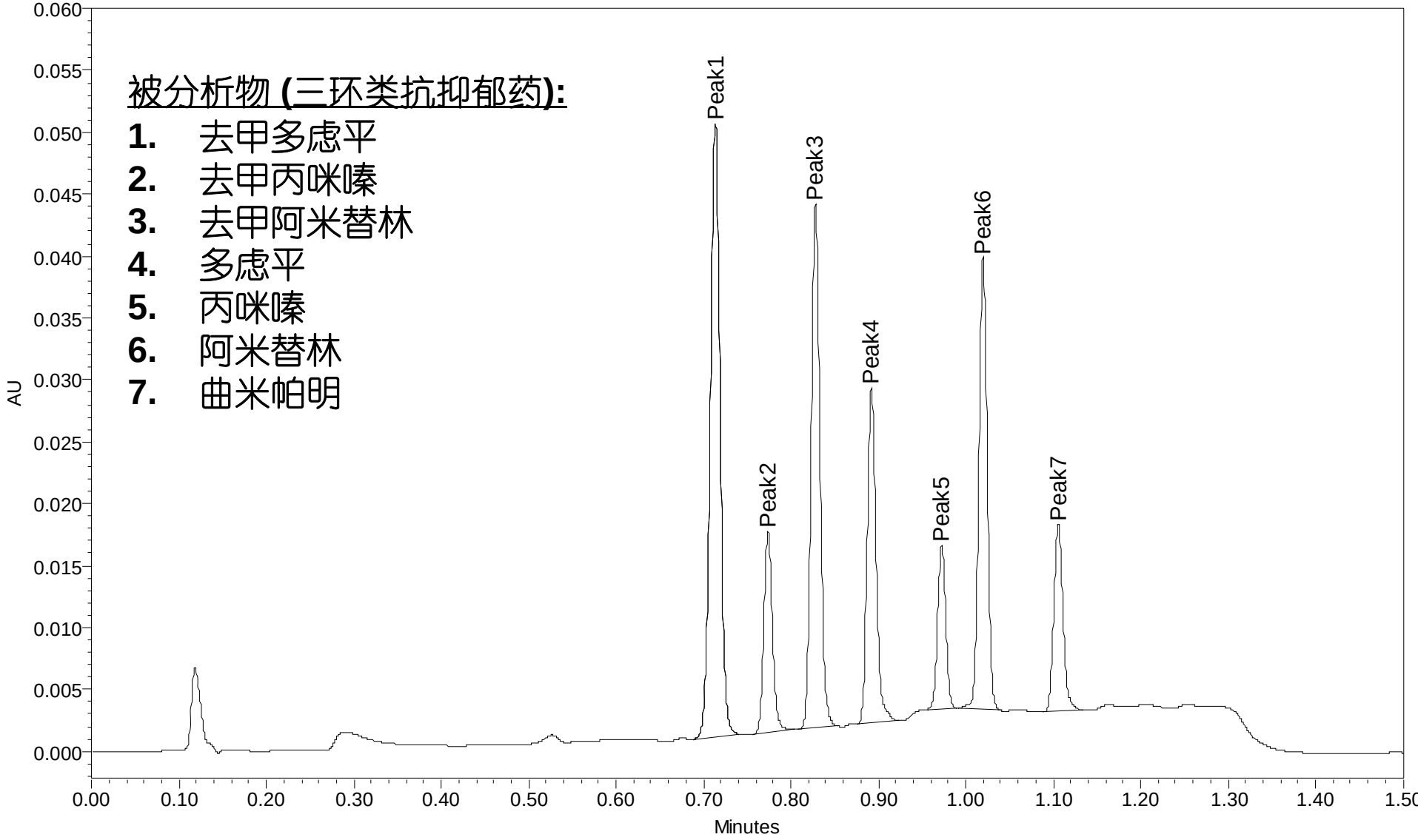

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灵敏度和分离度的优势
从HPLC 到 UPLC

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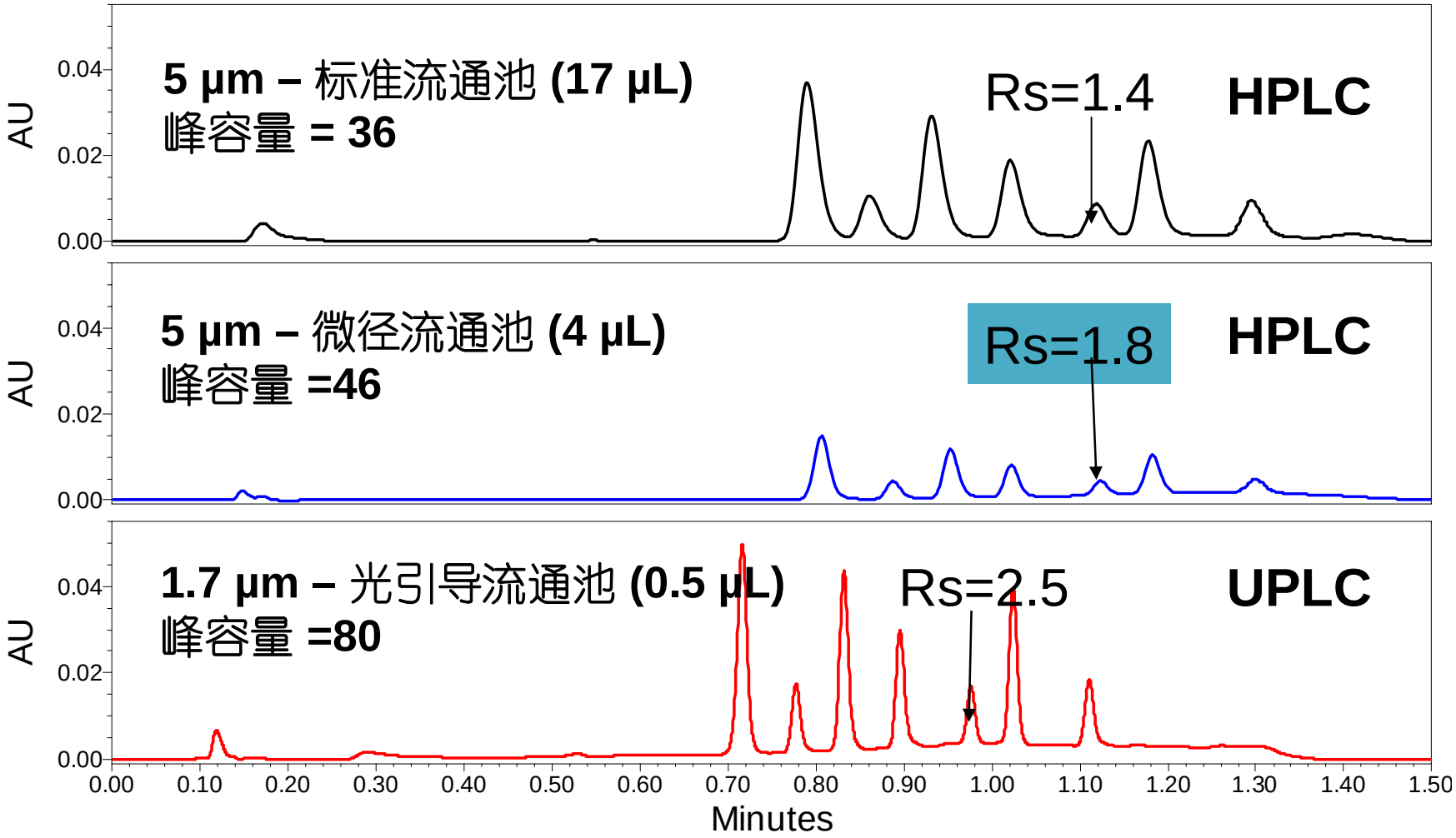

2.1 x 50 mm ACQUITY UPLC 柱, 1.0 mL/min,在 0.8 分钟内,5% 到 35% ACN

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灵敏度和分离度的优势
从HPLC 到 UPLC

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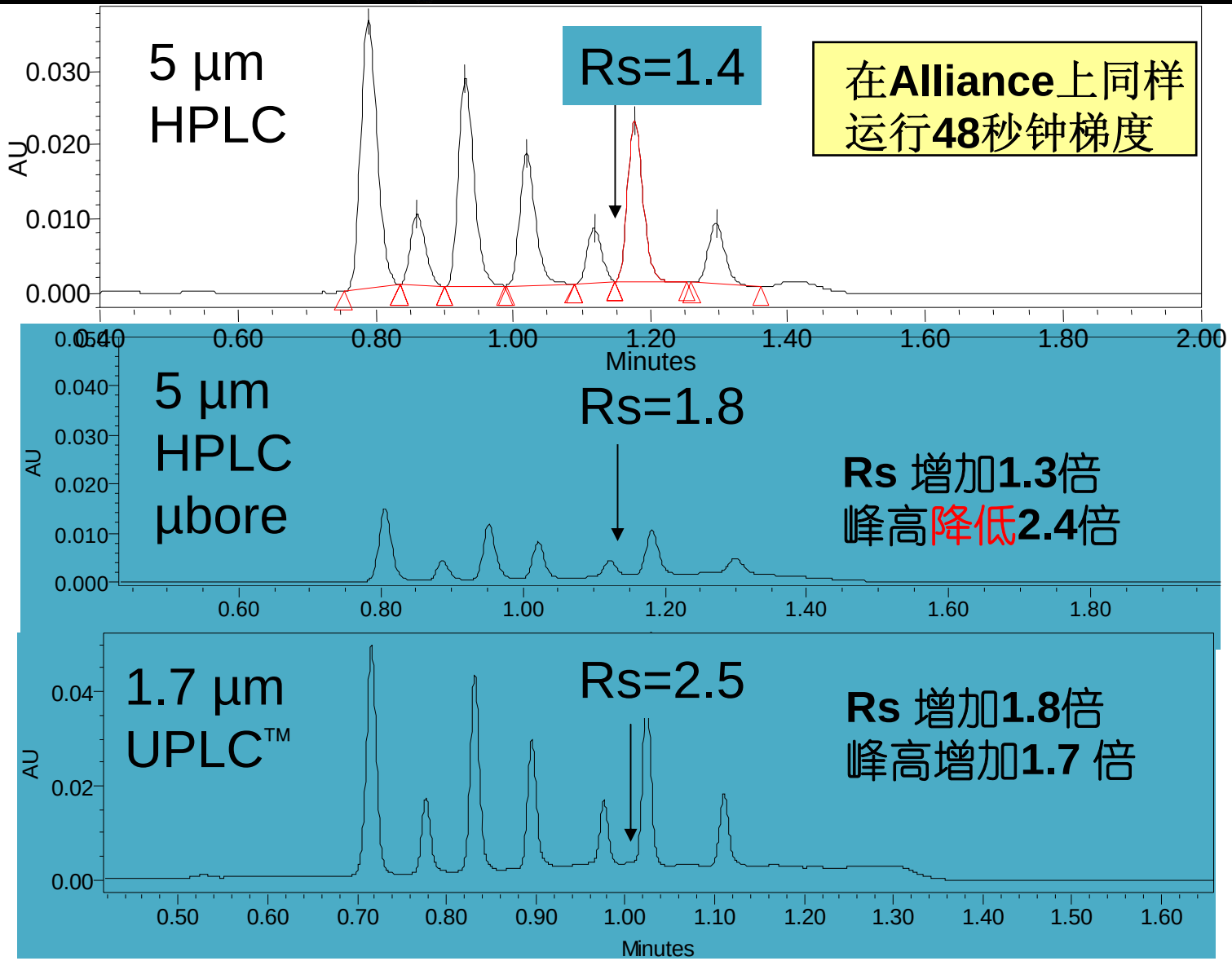

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UPLC 小结

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- 分离分析的质量受到色谱柱化学的驱动和限制
  - —优化的化学品能够使色谱过程得到增加速度、灵敏度和分离度的益处
- 颗粒度是色谱柱性能的首要考虑因素
- 小于**2 μm** 的颗粒需要在比传统**HPLC**高出两倍以上的压力下运行
- **ACQUITY UPLC**系统将仪器和柱化学结合,可以获得色谱理论的益处

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