Comparison of SFC, HPLC, and Chiral techniques for the separation of diastereomers of a diverse set of small molecules

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Purpose of the study

- **Diastereomer separation has a key importance in the Pharmaceutical Industry.**
  - Isomer separation generally favors unmodified silica columns[1], but modified stationary phases, including Chiral columns, using NPLC, RPLC or SFC conditions, frequently in isocratic mode, have also been reported for diastereomer separation.
- **New developments in SFC instrumentation (SFC-MS, open bed fraction collector for batch purification) has led to renewed interest in SFC for complex separations.**

1 Introduction to Modern Liquid Chromatography, L..R. Snyder, J.J. Kirkland, J.W. Dolan, 3rd edition, (2010).

Design and Goals

- **Success rates for separation of 258 synthetic diastereomer pairs were compared using three techniques, and two stationary phases per technique:**

1. **Reverse Phase Non-Chiral HPLC**
2. **Reverse Phase Chiral HPLC Separation**
3. **Non-Chiral SFC**

Selection of stationary phases

Column dimension: 4.6 x 150 mm, 5 µm

Selection : Literature and Experimental data

Selection of stationary phases

Literature data - Columns selection for HPLC

- **1. Reverse Phase Non-Chiral HPLC Separation**

  RP columns under HPLC conditions are tested, and documented by vendors, with consideration of their orthogonality[1] two commonly used columns were selected:

  XBridge™ C18 (Waters Corporation, Milford MA, USA)

  Synergi 4 μm Polar RP (Phenomenex, Torrance CA, USA)

- **2. Reverse Phase Chiral HPLC Separation[2]**

  Ultron ES-OVM (Shinwa Kyoto, Japan), protein based

  Chiralcel OJ-RH (Daicel Chemical Industries Tokyo,Japan), cellulose based

[1] L. R. Snyder, J. W. Dolan, and P. W. Carr, The hydrophobic subtraction model of reversed phase column selectivity, J. Chromat. A, 1060, 77-116, (2004).

[2] K. Valko (Ed.) Separation Methods in Drug Synthesis and Purification, Handbook of Analytical Separation Volume 1, Recent development in liquid chromatographic enantioseparation. M. Lammerhofer and W.Lindner 337-437, (2000).

Selection of stationary phases
Experimentation required for column selection - SFC

- Literature data[1] and our own observations suggested that retention factors can shift significantly on certain stationary phases when ammonium acetate additive is used.
  - This effect may have resulted from interaction between the additive and the stationary phases and/or ion pair formation between the additive and the analyte.

[1] J. Zheng, T. Glass , L.T. Taylor, J. David Pinkston, Study of the elution mechanism of sodium aryl sulfonates on bare silica and cyano bonded phase with methanol-modified carbon dioxide containing an ionic additive J. Chromat. A, 1090, 155-164, (2005).

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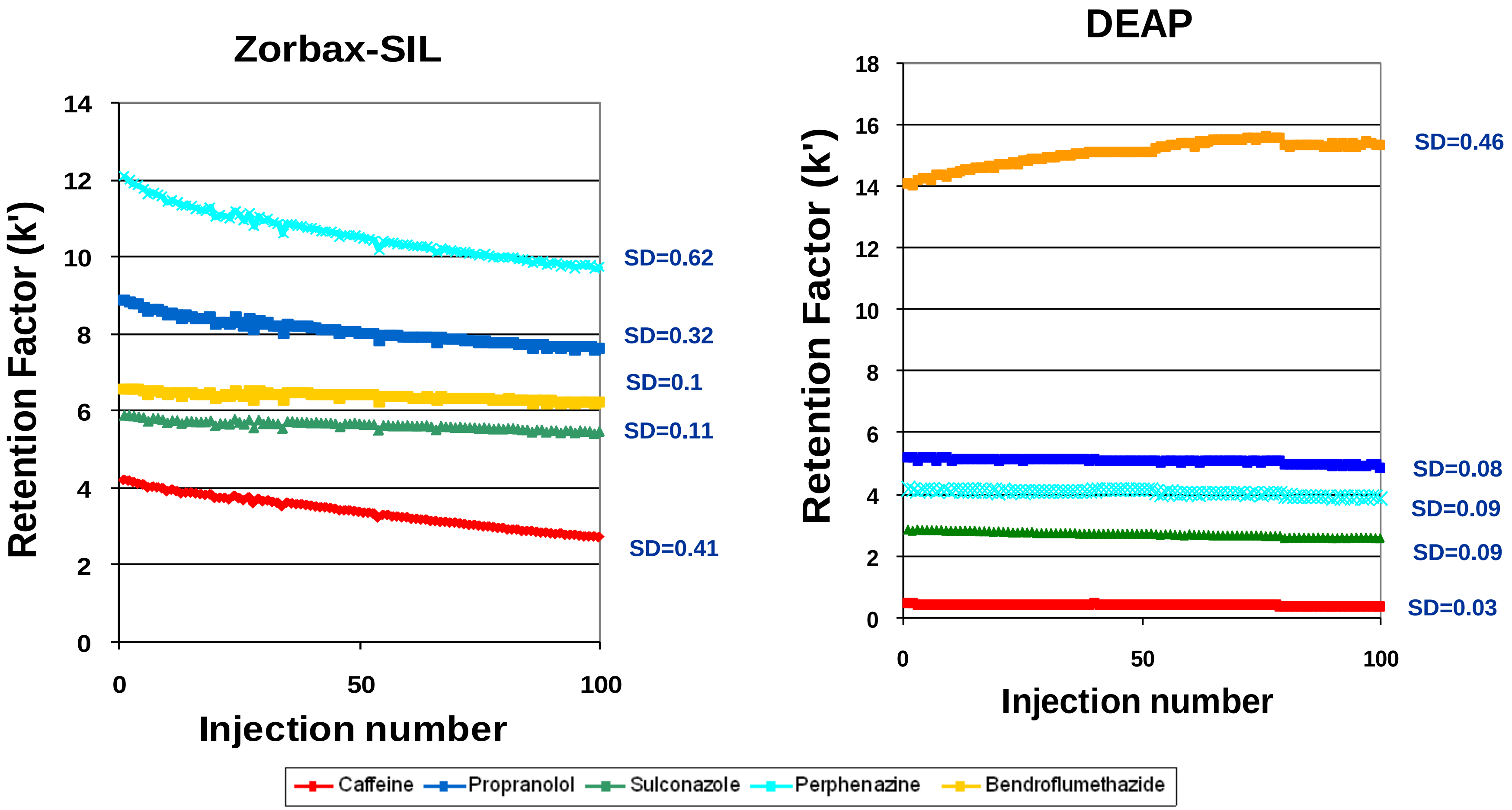
Examples of Retention Factor Shift

Retention Factors for a 5 component text mixture injected 100 times consecutively and eluted using the same linear gradient.



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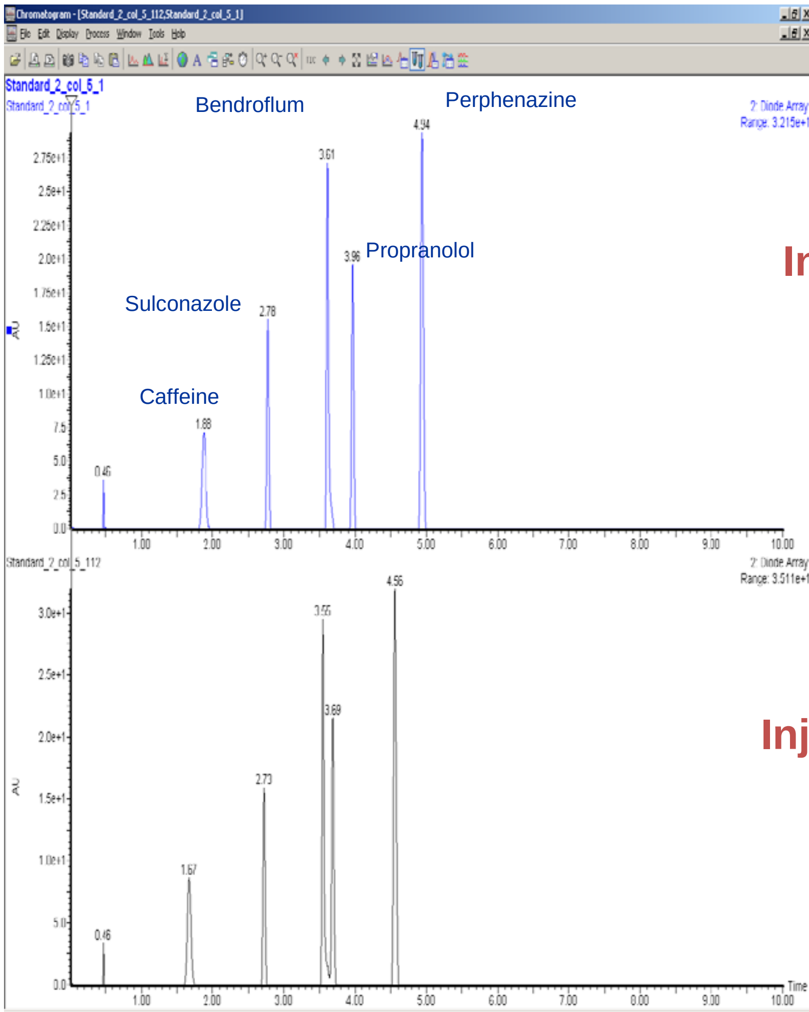
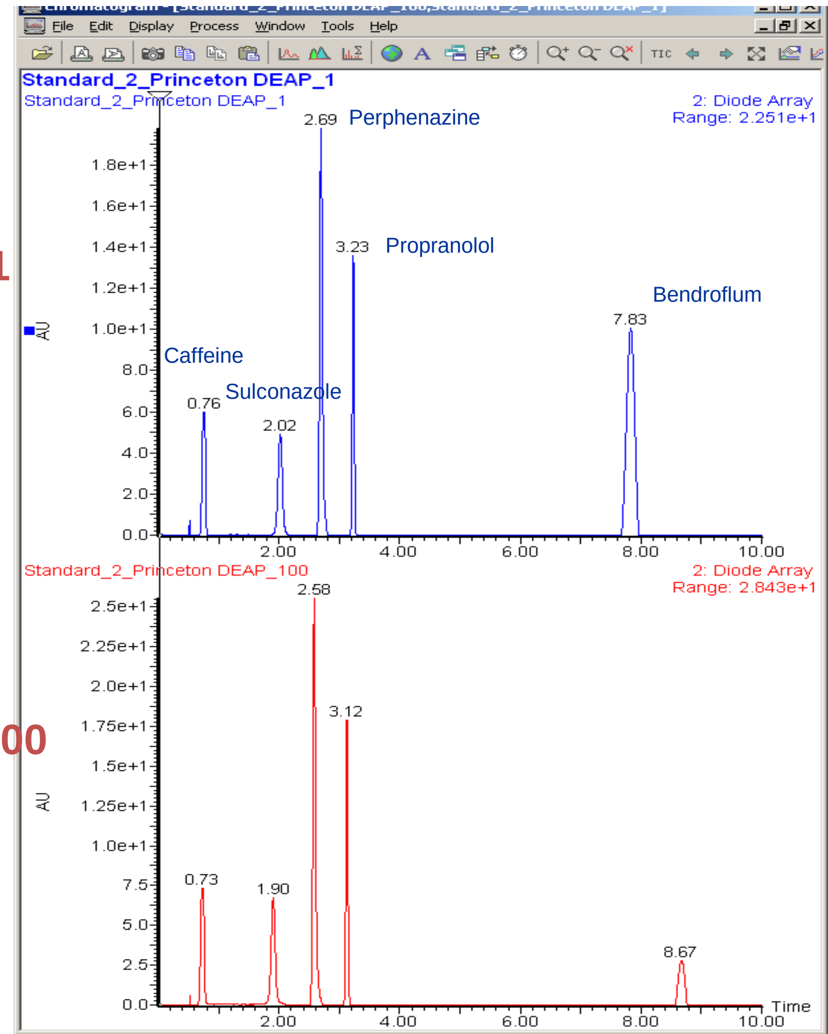
Retention factor shift overtime

Zorbax SIL



Princeton DEAP



Inj#1

Inj# 100

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Process for selecting only two columns for SFC

1. **Start with 24 columns**
   a) Retention factor reproducibility on 24 columns
   b) Orthogonality consideration on 24 columns
   c) Column selectivity parameters from the literature
2. **Reduce column number to 10 columns**
   a) Separation of a subset of 33 diastereomeric mixtures on 10 selected columns
3. **Selection of the final two columns**

Columns Used in the Study

Short Name	Full Name	Vendor	Short Name	Full Name	Vendor
Kromasil Sil	Kromasil 60-5SIL	Eka-AkzoNobel, Eka Chemicals AB,Sweden	SiliCycle XDB1-CN	SiliCycle®SiliaChrom ™ XDB1CN	SiliCycle Inc., Quebec City, Canada
Zorbax Sil	Zorbax SIL	Agilent Technologies Wilmington, DE, USA	SiliCycle HILIC	SiliCycle®SiliaChrom ™ HILIC	SiliCycle Inc., Quebec City, Canada
Zorbax Rx Sil	Zorbax Rx- SIL	Agilent Technologies Wilmington, DE, USA	Viridis EP	Viridis™ SFC 2-Ethylpyridine	Waters Corporation, Milford MA, USA
Luna Sil	Luna Silica (2)	Phenomenex, Torrance CA, USA	Princeton EP*	PrincetonSFC 2-Ethylpyridine	Princeton Chromatography, Princeton NJ, USA
Viridis Sil	Viridis™ SFC Silica	Waters Corporation, Milford MA, USA	Zorbax NH2	Zorbax NH2	Agilent Technologies Wilmington, DE, USA
Princeton Sil	PrincetonSFC SILICA	Princeton Chromatography, Princeton NJ, USA	Princeton DEAP	PrincetonSFC DEAP	Princeton Chromatography, Princeton NJ, USA
YMC PVA-Sil	YMC-Pack PVA-SIL-NP	YMC Co., Ltd. Japan	Princeton HA-Dipyridyl	PrincetonSFC HA-Dipyridyl	Princeton Chromatography, Princeton NJ, USA
Atlantis HILIC	Atlantis® HILIC Silica	Waters Corporation, Milford MA, USA	Princeton DNP	PrincetonSFC DNP	Princeton Chromatography, Princeton NJ, USA
Xbridge HILIC	XBridge™ HILIC	Waters Corporation, Milford MA, USA	Zorbax TMS	Zorbax TMS	Agilent Technologies Wilmington, DE, USA
Luna HILIC	Luna 5um HILIC	Phenomenex, Torrance CA, USA	Synergi Polar RP	Synergi 4 um Polar RP	Phenomenex, Torrance CA, USA
Princeton CN-Diol	PrincetonSFC 2CN:Diol	Princeton Chromatography, Princeton NJ, USA	Xbridge Amide	XBridge™ Amide	Waters Corporation, Milford MA, USA
Zorbax SB-CN	Zorbax SB-CN	Agilent Technologies Wilmington, DE, USA	Cosmosil PYE	Cosmosil 5U PYE	Nacalai USA. Inc.,San Diego, CA, USA

Retention factor reproducibility on 24 columns

24 columns, selected from 4 column-type categories, were evaluated for retention reproducibility. (4.6 x 150 mm, 5 µm**)**

- **A test mix of 5 components was used**
  - **Sulconazole** (weak base, not ionized in ammonium acetate)
  - **Caffeine** (weak base, not ionized in ammonium acetate)
  - **Bendroflumethiazide** (weak acid, not ionized in ammonium acetate)
  - **Propanolol** (strong base, protonated in ammonium acetate)
  - **Perphenazine** (strong base, protonated in ammonium acetate)
- **The test mix was injected on each column 100 times consecutively and eluted using the same linear gradient method.**
  - **CO_2 / Methanol + 10 mM NH_4OAc**
  - **5% to 60% Methanol from 1-7 minutes + 1.5 min @ 60%**
  - **Flow rate 5 g/min, Inlet pressure 100 bar**

Retention factors of 100 injections of all 5 components on each columns were recorded and the standard deviations (SD) were calculated and compared.

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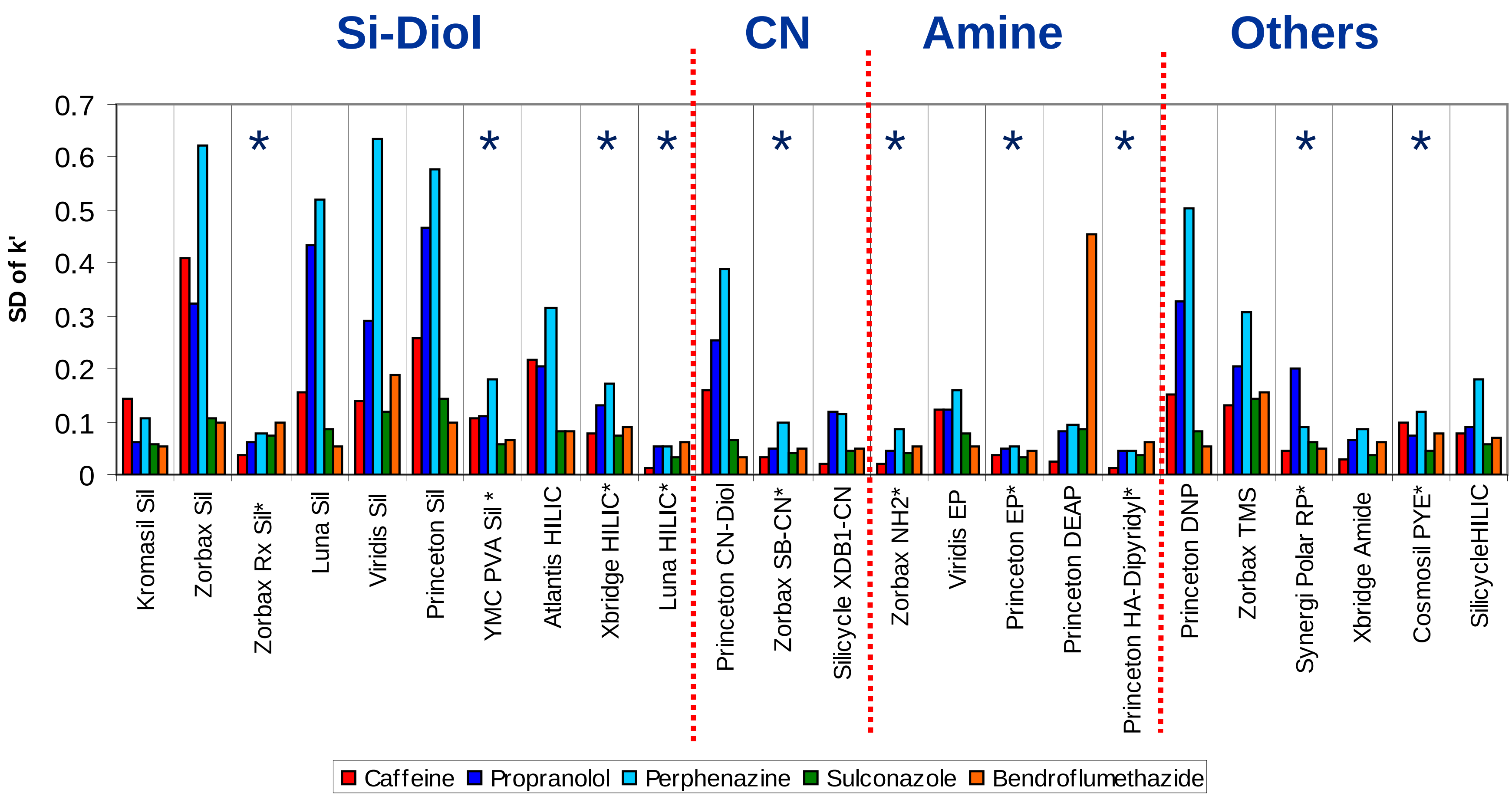
Retention factor reproducibility on 24 columns
Comparison of standard deviations of k'



Significant retention factor drift is observed on some columns over time.

SD of k' = Standard Deviation of the retention factor based on 100 injection

*** = Selected for further study**

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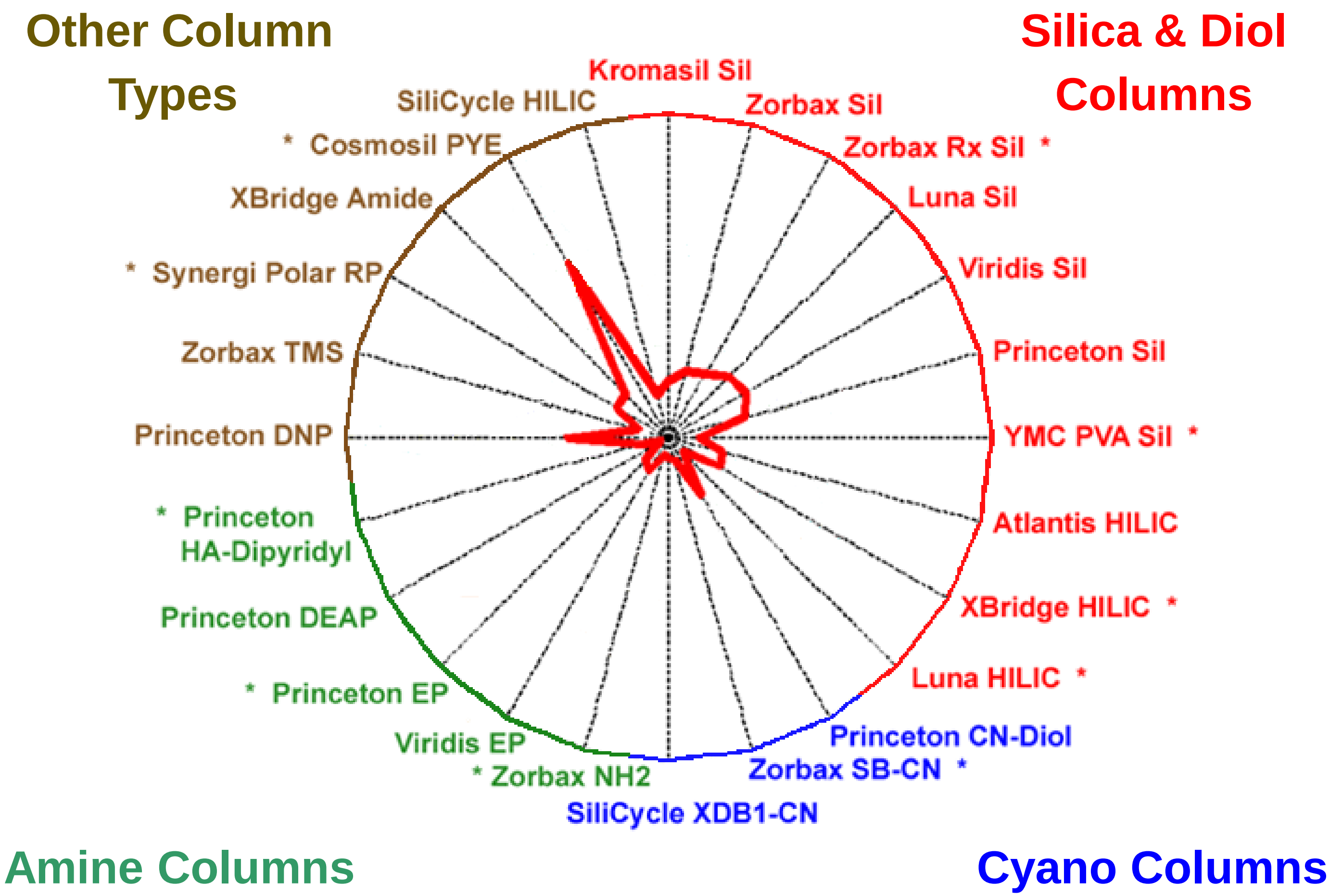
Retention Orthogonality for 24 Columns

Retention Factors for 5 components on each of 24 columns



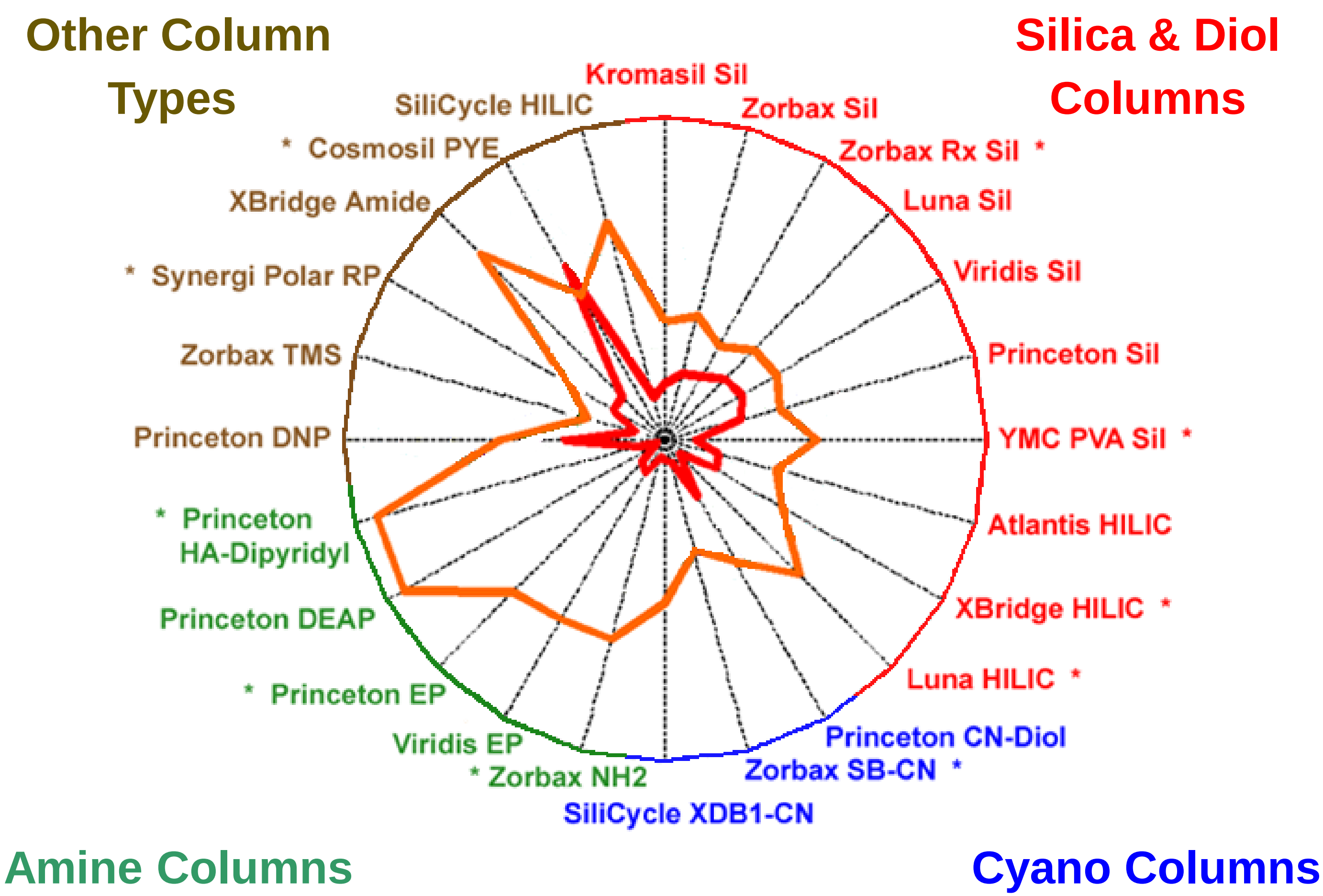
Retention factors (distance from center), vary significantly from column to column, even for columns with nominally similar chemistry.

Ten columns (*) were chosen for further study.

<u>Test Compounds:</u>
Caffeine
Sulconazole
Propanolol
Perphenazine
Bendroflumethiazide

Retention Orthogonality for 24 Columns

Retention Factors for 5 components on each of 24 columns



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Retention Orthogonality for 24 Columns

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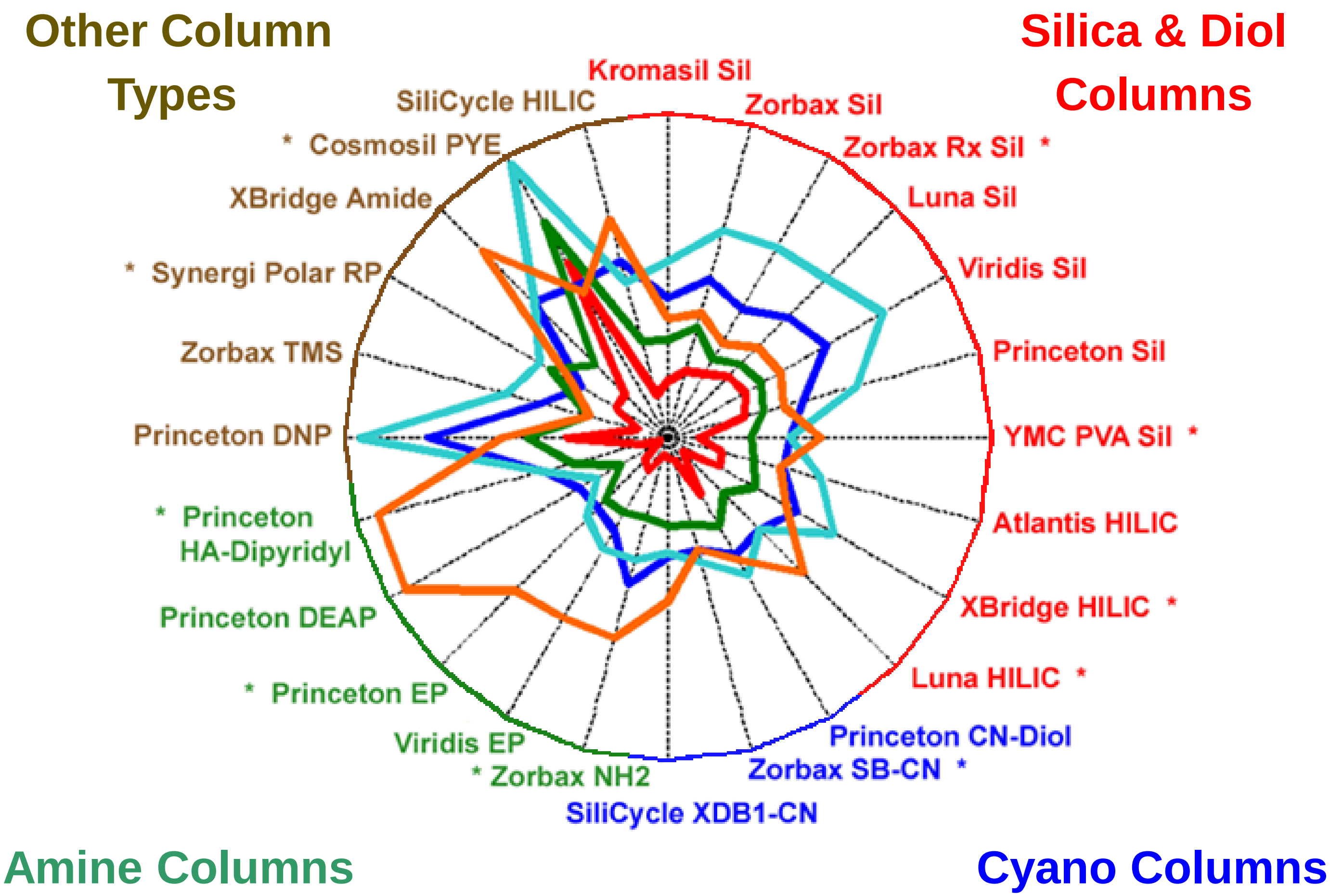

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Process for selecting only two columns for SFC

1. **Start with 24 columns**
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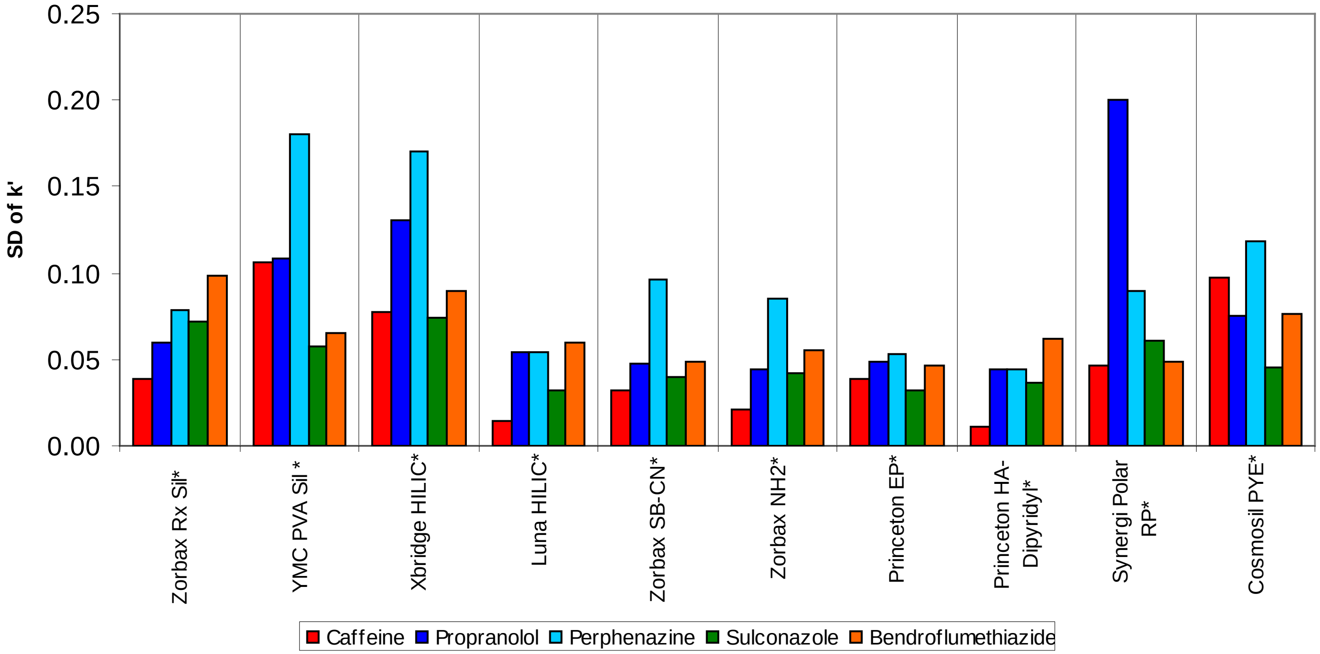
   c) **Column selectivity parameters from the literature**
2. **Reduce column number to 10 columns**
   a) **Separation of a subset of 33 diastereomeric mixtures on 10 selected columns**
3. **Selection of the final two columns**

Column selectivity parameters from the literature[1]

Reduced column number - 10 selected columns.

(Retention factor reproducibility, orthogonality consideration and literature data on 24 columns)



[1] **C. West, E. Leslellier, Orthogonal screening system of columns for supercritical fluid chromatography J. Chromatogr. A., 1203, 105-113, (2008).**

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Process for selecting only two columns for SFC

1. Start with 24 columns
    a) Retention factor reproducibility on 24 columns
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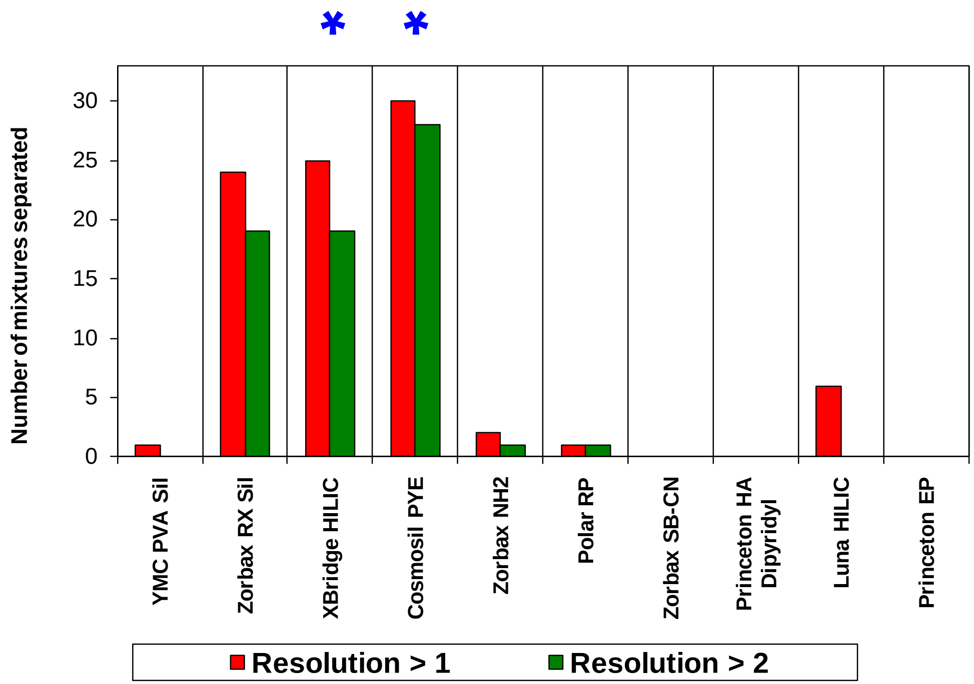
    c) Column selectivity parameters from the literature
2. Reduce column number to 10 columns
    a) Separation of a subset of 33 diastereomeric mixtures on 10 selected columns
3. Selection of the final two columns

Separation of a subset of 33 diastereomeric mixtures on 10 selected columns

Separation success rates with consideration of orthogonality were used to select the final two columns.



*** Cosmosil PYE**

*** Xbridge HILIC**

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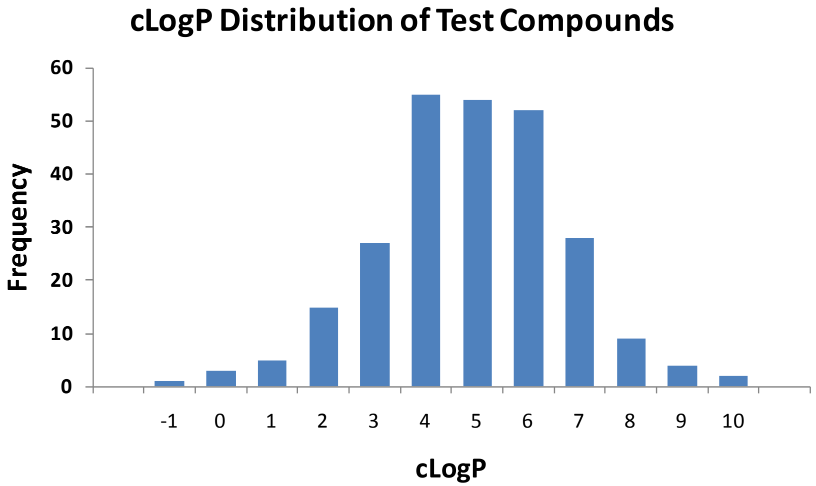
Final Columns Used for the Separation Study

- **Reverse Phase Non-Chiral HPLC**
  - **XBridge™ C18** (Waters Corporation, Milford MA, USA)
  - **Synergi 4 µm Polar RP** (Phenomenex, Torrance CA, USA)
- **Reverse Phase Chiral HPLC**[2]
  - **Ultron ES-OVM** (Shinwa Kyoto, Japan), protein based
  - **Chiralcel OJ-RH** (Daicel Chemical Industries Tokyo,Japan), cellulose based
- **Non-Chiral SFC**
  - **XBridge™ HILIC** (Waters Corporation, Milford MA, USA)
  - **Cosmosil PYE** (Nacalai USA. Inc.,San Diego, CA, USA)

Diastereomer Separation Study

- **258 synthetic diastereomer mixtures were analyzed.**

ACD cLogP data for these mixtures was consistent with typical "drug like" compounds commonly encountered in pharmaceutical research[1]



- **All mixtures were analyzed on six columns**
  - **2 Reverse Phase Non-Chiral HPLC**
  - **2 Reverse Phase Chiral HPLC**
  - **2 Non-Chiral SFC**

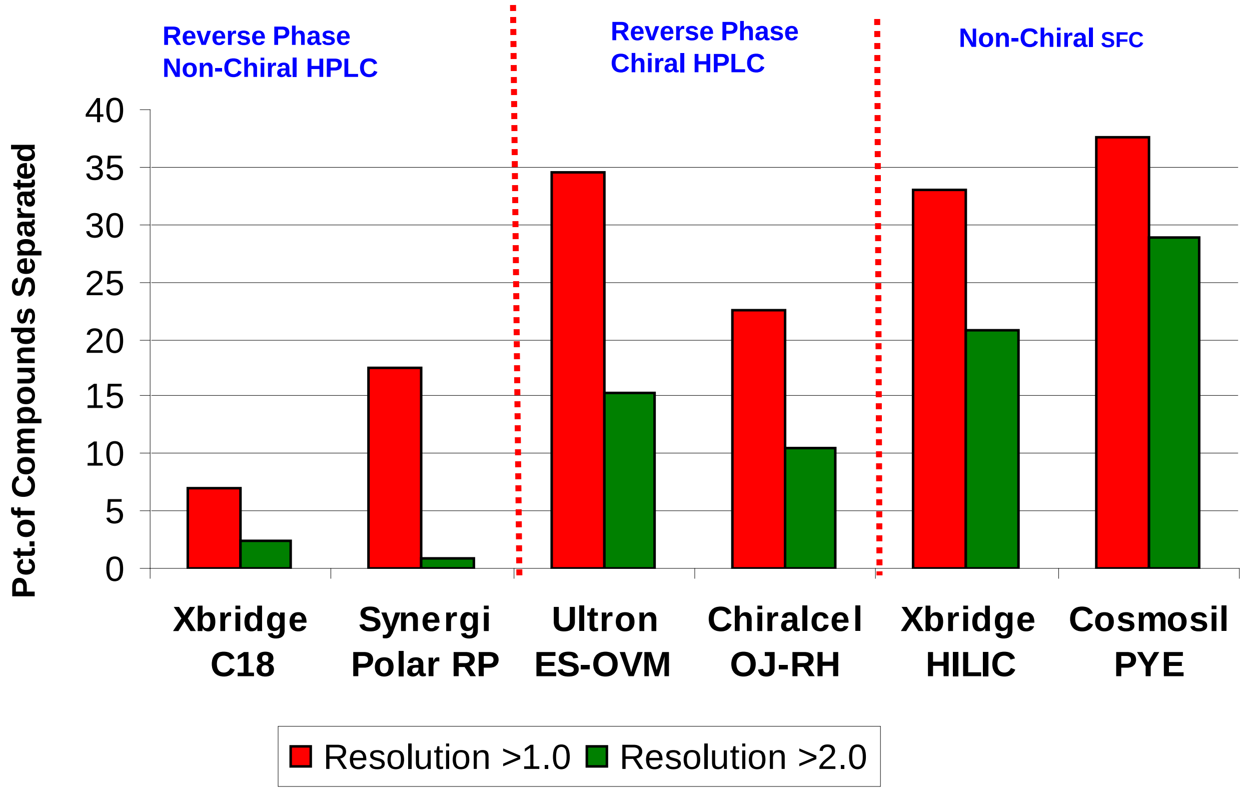
[1] T.I. Oprea, Property Distribution of drug-related chemical databases. J. Computer-Aided Molecular Design, 14, 251-264, (2000).

Methods for diastereomer separation study

Type	Column	Organic Modifier	Buffer	Flow Rate (ml/min)	Gradient Time (min)	Gradient retention factor[1] (k*)	Gradient Steepness [1] (G_S) (%/min)	Temp (^{0}C)	Gradient Range (% Organic)	MS Ionization
HPLC Non-Chiral	Xbridge C-18 4.6 x 150	CH_3OH	NH_4OAc	1.2	10	2.2	9.2	25	20-95	ESI^+
HPLC Non-Chiral	Polar RP 4.6 x 150	CH_3OH	NH_4OAc	1.2	10	2.2	9.2	25	20-95	ESI^+
HPLC Chiral	Ultron ES-OVM 4.6 x 150	CH_3CN	NH_4OAc	1.0	17	5.8	3.5	35	10-50	ESI^+
HPLC Chiral	Chiralcel OJ-RH 4.6 x 150	CH_3OH	TFA	1.0	11	1.8	11.4	35	10-95	ESI^+
SFC Non-Chiral	Cosmosil PYE 4.6 x 150	CH_3OH	NH_4OAc	5.0	6	4.7	4.2	35	5-60	$APCI^+$
SFC Non-Chiral	Xbridge HILIC 4.6 x 150	CH_3OH	NH_4OAc	5.0	6	4.7	4.2	35	5-60	$APCI^+$

[1] L.R. Snyder, J.J. Kirkland, J.L.Glajch Practical HPLC Method Development , Second edition (1997).

Diastereomer Separation Results



Diastereomer pairs were separated for all 258 compounds on one or more of the 6 columns used.

Non-Chiral SFC separated a higher percentage of diastereomers with resolution greater than one relative to the other two techniques studied.

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Summary

SFC methods using relatively inexpensive non-chiral columns provided equivalent or superior diastereomer separation compared with the methods using more expensive chiral columns.

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Acknowledgements

The authors thank the SATT Synthesis Analysis and Technology Team for their support.

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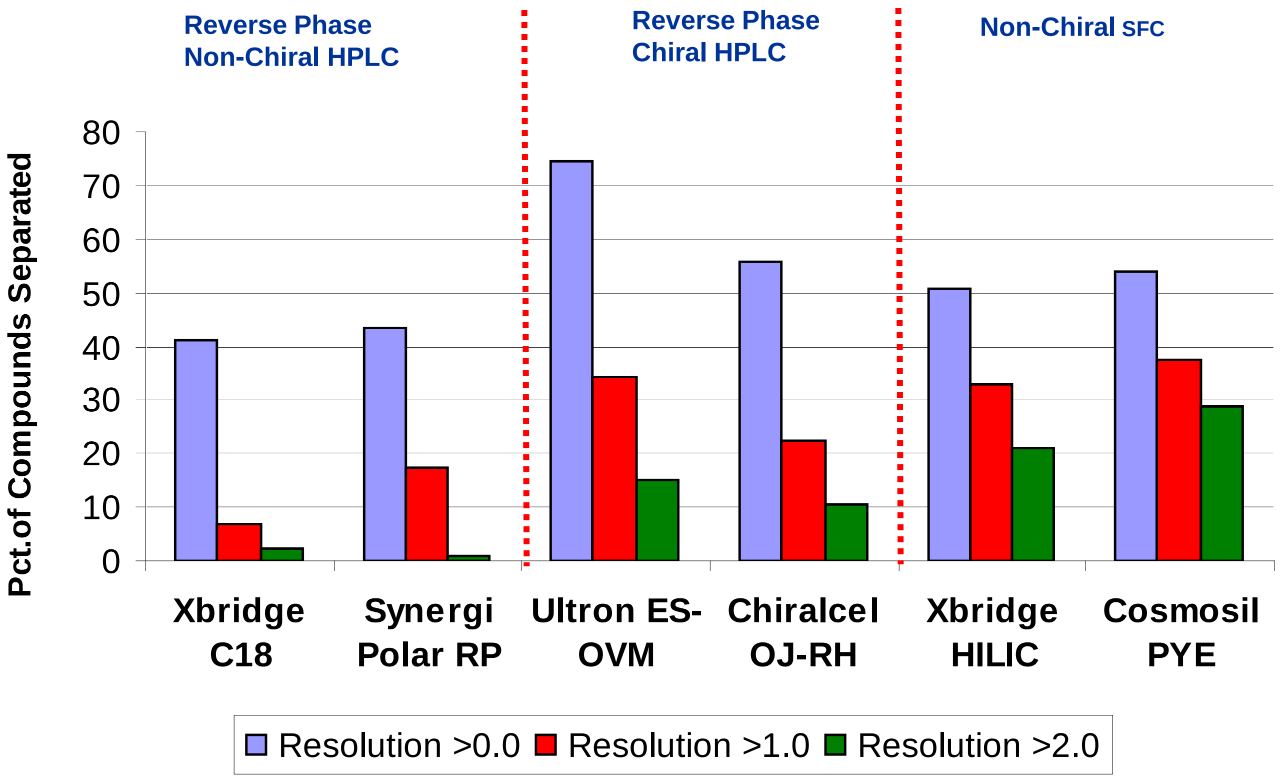
Additional slides for possible questions

Following slides only needed in the event of specific questions.

Diastereomer Separation Results



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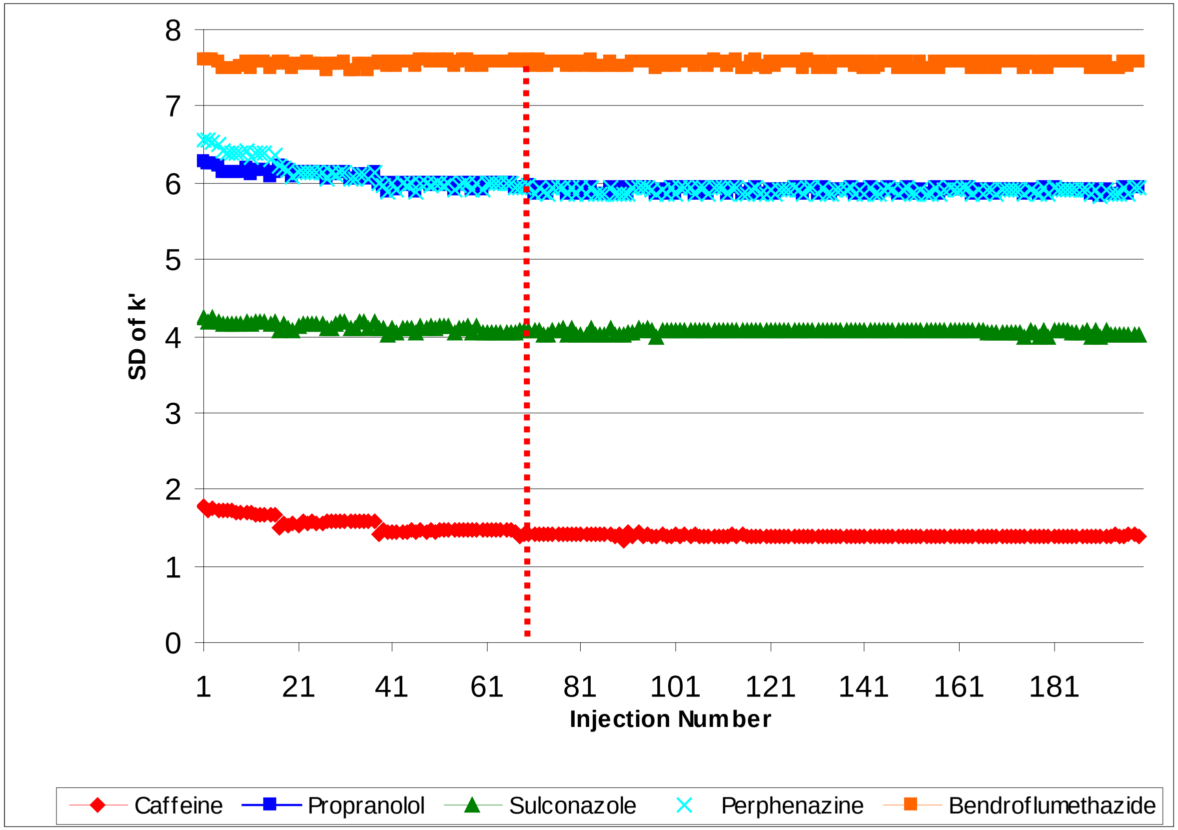
Change of retention factors on YMC PVA Sil Column



Based on 200 injection

	Caffeine	Propranolol	Sulconazole	Perphenazine	Bendroflumetha:
stdev inj1-100	0.11	0.11	0.06	0.19	0.04
stdev inj100-200	0.01	0.04	0.02	0.04	0.04
stdev inj1-200	0.09	0.10	0.05	0.15	0.04

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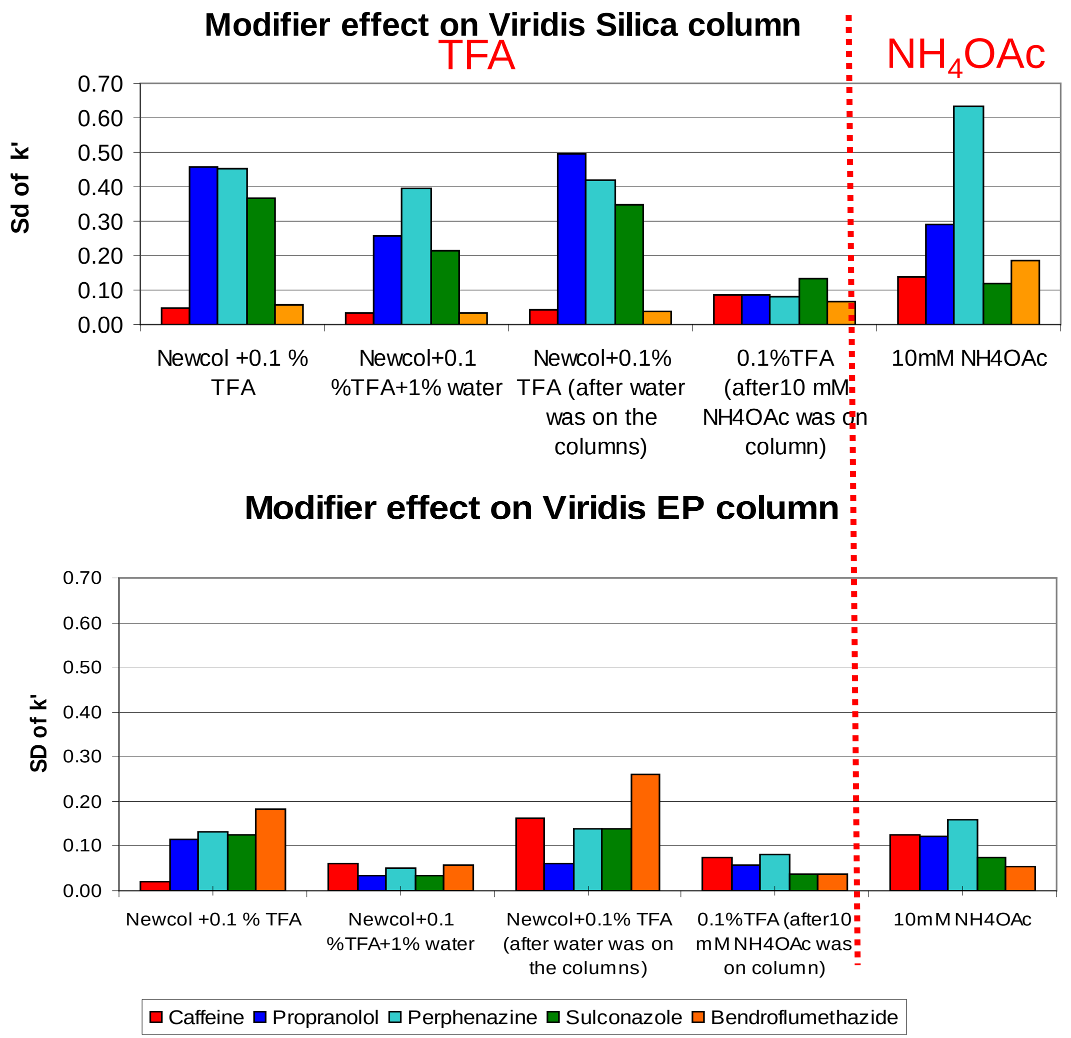
Modifier effect – Silica column and Modified silica surface

Silica Surface

Modified silica Surface



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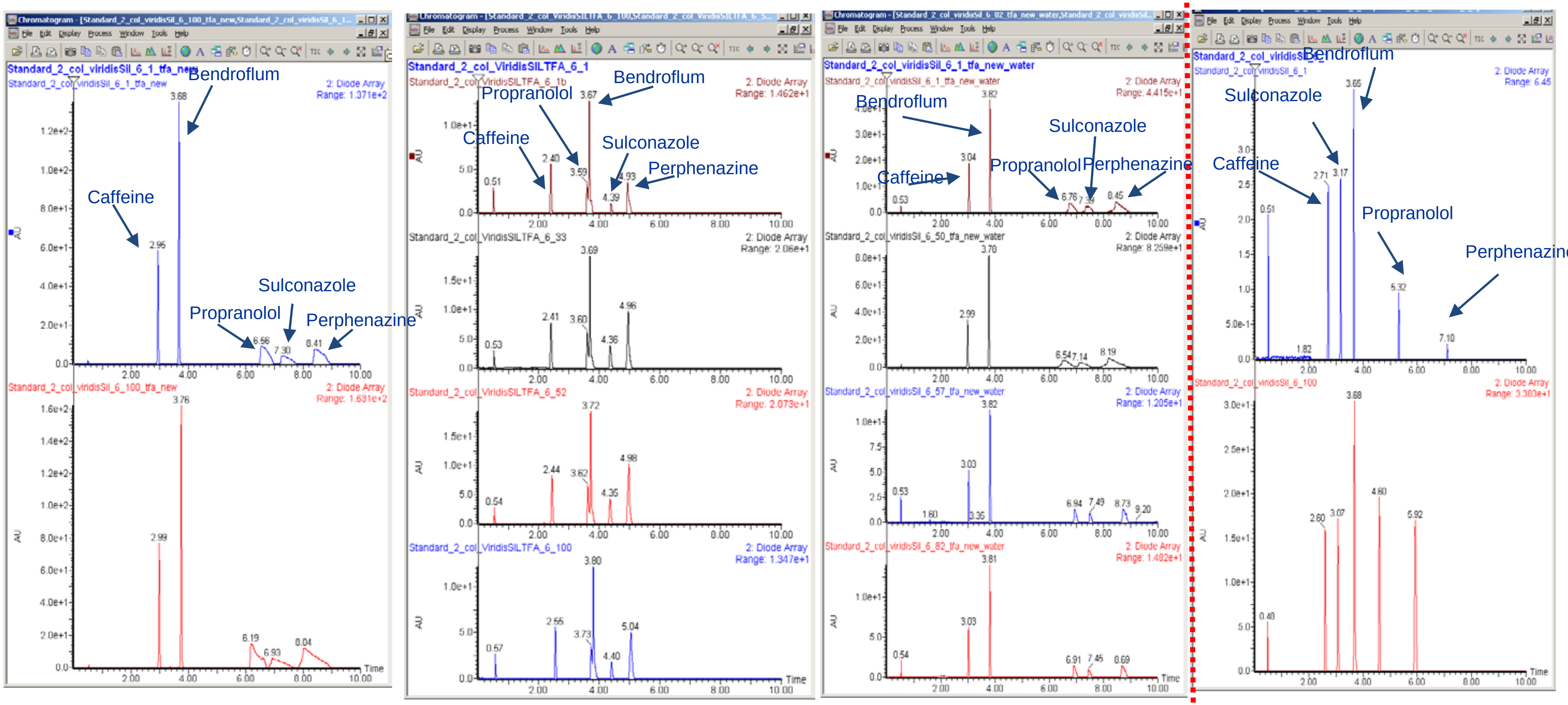
Modifier effect – Silica surface Viridis Sil

0.1 % TFA | **0.1% TFA+on column run NH$_4$OAc additive before** | **0.1% TFA+1 %Water** | **NH$_4$OAc additive**



Memory effect of the Silica column after using Ammonium salt additive

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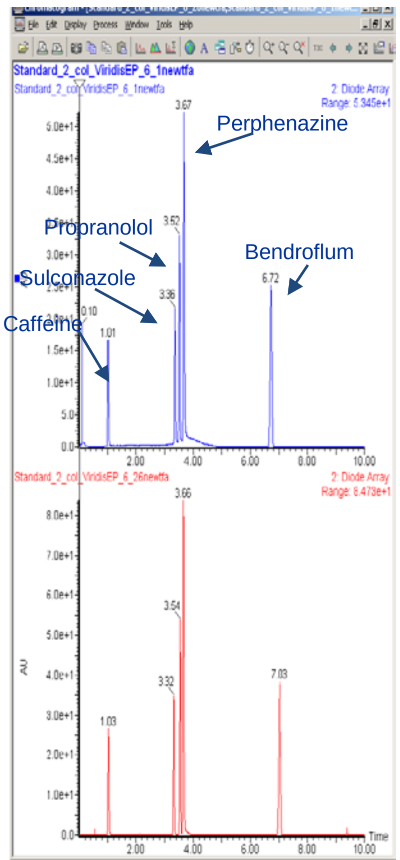
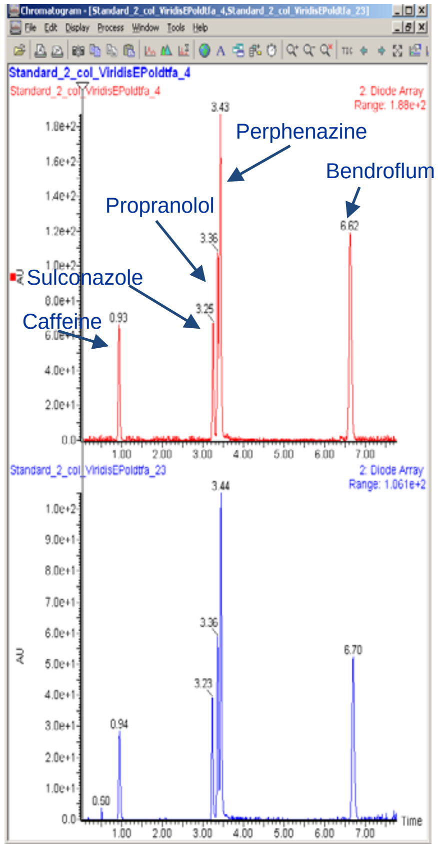
Modifier effect – Bonded Silica surface Viridis EP

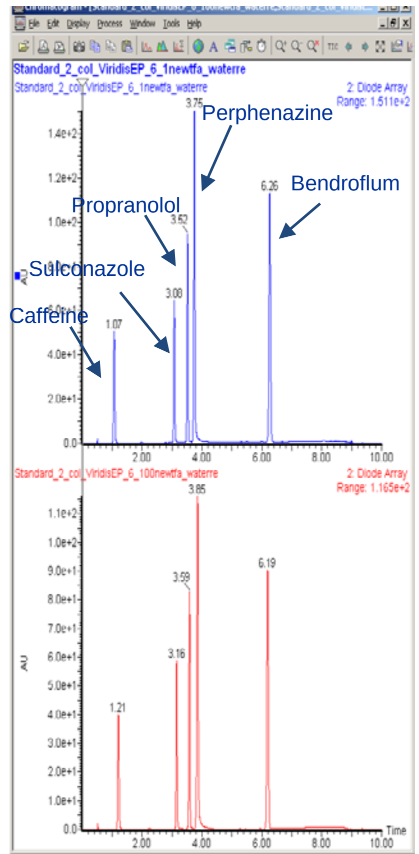
0.1 % TFA



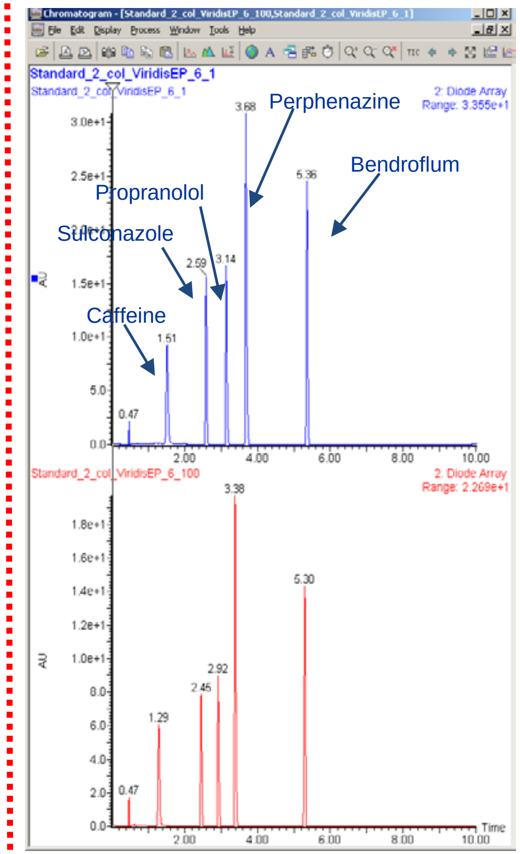
0.1% TFA+on column run
NH_4OAc additive before



0.1% TFA+1 %Water



NH_4OAc additive



No memory effect on bonded silica surface after using Ammonium salt additive

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