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

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Research and Development
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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.**

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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 \(\mu\)m 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

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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.

Examples of Retention Factor Shift

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

Zorbax-SIL

DEAP

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

Zorbax SIL

Princeton DEAP

Inj#1

Inj# 100
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

<table>
<thead>
<tr>
<th>Short Name</th>
<th>Full Name</th>
<th>Vendor</th>
<th>Short Name</th>
<th>Full Name</th>
<th>Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kromasil Sil</td>
<td>Kromasil 60-5SIL</td>
<td>Eka-AkzoNobel, Eka Chemicals AB, Sweden</td>
<td>SiliCycle XDB1-CN</td>
<td>SiliCycle@SiliaChrom™ XDB1CN</td>
<td>SiliCycle Inc., Quebec City, Canada</td>
</tr>
<tr>
<td>Zorbax Sil</td>
<td>Zorbax SIL</td>
<td>Agilent Technologies Wilmington, DE, USA</td>
<td>SiliCycle HILIC</td>
<td>SiliCycle@SiliaChrom™ HILIC</td>
<td>SiliCycle Inc., Quebec City, Canada</td>
</tr>
<tr>
<td>Zorbax Rx Sil</td>
<td>Zorbax Rx- SIL</td>
<td>Agilent Technologies Wilmington, DE, USA</td>
<td>Viridis EP</td>
<td>Viridis™ SFC 2-Ethylpyridine</td>
<td>Waters Corporation, Milford MA, USA</td>
</tr>
<tr>
<td>Luna Sil</td>
<td>Luna Silica (2)</td>
<td>Phenomenex, Torrance CA, USA</td>
<td>Princeton EP*</td>
<td>PrincetonSFC 2-Ethylpyridine</td>
<td>Princeton Chromatography, Princeton NJ, USA</td>
</tr>
<tr>
<td>Viridis Sil</td>
<td>Viridis™ SFC Silica</td>
<td>Waters Corporation, Milford MA, USA</td>
<td>Zorbax NH2</td>
<td>Zorbax NH2</td>
<td>Agilent Technologies Wilmington, DE, USA</td>
</tr>
<tr>
<td>Atlantis HILIC</td>
<td>Atlantis® HILIC Silica</td>
<td>Waters Corporation, Milford MA, USA</td>
<td>Princeton DNP</td>
<td>PrincetonSFC DNP</td>
<td>Princeton Chromatography, Princeton NJ, USA</td>
</tr>
<tr>
<td>Xbridge HILIC</td>
<td>XBridge™ HILIC</td>
<td>Waters Corporation, Milford MA, USA</td>
<td>Zorbax TMS</td>
<td>Zorbax TMS</td>
<td>Agilent Technologies Wilmington, DE, USA</td>
</tr>
<tr>
<td>Luna HILIC</td>
<td>Luna 5um HILIC</td>
<td>Phenomenex, Torrance CA, USA</td>
<td>Synergi Polar RP</td>
<td>Synergi 4 um Polar RP</td>
<td>Phenomenex, Torrance CA, USA</td>
</tr>
<tr>
<td>Princeton CN-Diol</td>
<td>PrincetonSFC 2CN:Diol</td>
<td>Princeton Chromatography, Princeton NJ, USA</td>
<td>Xbridge Amide</td>
<td>XBridge™ Amide</td>
<td>Waters Corporation, Milford MA, USA</td>
</tr>
<tr>
<td>Zorbax SB-CN</td>
<td>Zorbax SB-CN</td>
<td>Agilent Technologies Wilmington, DE, USA</td>
<td>Cosmosil PYE</td>
<td>Cosmosil 5U PYE</td>
<td>Nacalai USA, Inc., San Diego, CA, USA</td>
</tr>
</tbody>
</table>
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.
  - CO₂ / Methanol + 10 mM NH₄OAc
  - 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 column were recorded and the standard deviations (SD) were calculated and compared.
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
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**
Retention Orthogonality for 24 Columns
Retention Factors for 5 components on each of 24 columns

Other Column Types

Silica & Diol 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.

Amine Columns

Cyano Columns

Test Compounds:
Caffeine
Sulconazole
Propanolol
Perphenazine
Bendroflumethiazide
Retention Orthogonality for 24 Columns
Retention Factors for 5 components on each of 24 columns

Other Column Types
- Amine Columns
- Cyano Columns
- Silica & Diol 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.

Test Compounds:
- Caffeine
- Sulconazole
- Propanolol
- Perphenazine
- Bendroflumethiazide
Retention Orthogonality for 24 Columns
Retention Factors for 5 components on each of 24 columns

Other Column Types

- Amine Columns
  - SiliCycle HILIC
  - Zorbax TMS
  - Princeton DNP
  - Princeton DEAP
  - Princeton EP

- Cyano Columns
  - XBridge Amide
  - Synergi Polar RP
  - Princenton HA-Diprydyl

- Silica & Diol Columns
  - Kromasil Sil
  - Zorbax Sil
  - Zorbax RxSil
  - Luna Sil
  - Viridis Sil
  - Princeton Sil
  - YMC PVA Sil
  - Atlantis HILIC
  - XBridge HILIC
  - Luna HILIC
  - Princeton CN-Diol
  - SiliCycle XDB1-CN

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.

Test Compounds:
- Caffeine
- Sulconazole
- Propranolol
- Perphenazine
- Bendroflumethiazide
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
Column selectivity parameters from the literature\textsuperscript{1}

Reduced column number - 10 selected columns.

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

\[ SD_{k'} \]

Caffeine \quad Propranolol \quad Perphenazine \quad Sulconazole \quad Bendroflumethiazide

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

## Methods for diastereomer separation study

<table>
<thead>
<tr>
<th>Type</th>
<th>Column</th>
<th>Organic Modifier</th>
<th>Buffer</th>
<th>Flow Rate (ml/min)</th>
<th>Gradient Time (min)</th>
<th>Gradient retention factor(^1) (k*)</th>
<th>Gradient Steepness (^1) (G*) (%/min)</th>
<th>Temp (°C)</th>
<th>Gradient Range (% Organic)</th>
<th>MS Ionization</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC Non-Chiral</td>
<td>Xbridge C-18 4.6 x 150</td>
<td>CH(_3)OH</td>
<td>NH(_4)OAc</td>
<td>1.2</td>
<td>10</td>
<td>2.2</td>
<td>9.2</td>
<td>25</td>
<td>20-95</td>
<td>ESI*</td>
</tr>
<tr>
<td>HPLC Non-Chiral</td>
<td>Polar RP 4.6 x 150</td>
<td>CH(_3)OH</td>
<td>NH(_4)OAc</td>
<td>1.2</td>
<td>10</td>
<td>2.2</td>
<td>9.2</td>
<td>25</td>
<td>20-95</td>
<td>ESI*</td>
</tr>
<tr>
<td>HPLC Chiral</td>
<td>Ultron ES-OVM 4.6 x 150</td>
<td>CH(_3)CN</td>
<td>NH(_4)OAc</td>
<td>1.0</td>
<td>17</td>
<td>5.8</td>
<td>3.5</td>
<td>35</td>
<td>10-50</td>
<td>ESI*</td>
</tr>
<tr>
<td>HPLC Chiral</td>
<td>Chiralcel OJ-RH 4.6 x 150</td>
<td>CH(_3)OH</td>
<td>TFA</td>
<td>1.0</td>
<td>11</td>
<td>1.8</td>
<td>11.4</td>
<td>35</td>
<td>10-95</td>
<td>ESI*</td>
</tr>
<tr>
<td>SFC Non-Chiral</td>
<td>Cosmosil PYE 4.6 x 150</td>
<td>CH(_3)OH</td>
<td>NH(_4)OAc</td>
<td>5.0</td>
<td>6</td>
<td>4.7</td>
<td>4.2</td>
<td>35</td>
<td>5-60</td>
<td>APCI*</td>
</tr>
<tr>
<td>SFC Non-Chiral</td>
<td>Xbridge HILIC 4.6 x 150</td>
<td>CH(_3)OH</td>
<td>NH(_4)OAc</td>
<td>5.0</td>
<td>6</td>
<td>4.7</td>
<td>4.2</td>
<td>35</td>
<td>5-60</td>
<td>APCI*</td>
</tr>
</tbody>
</table>

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

The authors thank the SATT Synthesis Analysis and Technology Team for their support.
Additional slides for possible questions

Following slides only needed in the event of specific questions.
Diastereomer Separation Results

- **Reverse Phase Non-Chiral HPLC**
  - Xbridge C18
  - Synergi Polar RP
  - Ultron ES-OVM
  - Chiralcel OJ-RH
  - Xbridge HILIC
  - Cosmosil PYE

- **Reverse Phase Chiral HPLC**
  - Resolution >0.0
  - Resolution >1.0
  - Resolution >2.0

- **Non-Chiral SFC**
  - Resolution >0.0
  - Resolution >1.0
  - Resolution >2.0
Change of retention factors on YMC PVA Sil Column

Based on 200 injection

<table>
<thead>
<tr>
<th></th>
<th>Caffeine</th>
<th>Propranolol</th>
<th>Sulconazole</th>
<th>Perphenazine</th>
<th>Bendroflumethazine</th>
</tr>
</thead>
<tbody>
<tr>
<td>stdev inj1-100</td>
<td>0.11</td>
<td>0.11</td>
<td>0.06</td>
<td>0.19</td>
<td>0.04</td>
</tr>
<tr>
<td>stdev inj100-200</td>
<td>0.01</td>
<td>0.04</td>
<td>0.02</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>stdev inj1-200</td>
<td>0.09</td>
<td>0.10</td>
<td>0.05</td>
<td>0.15</td>
<td>0.04</td>
</tr>
</tbody>
</table>
Modifier effect – Silica column and Modified silica surface

Modifier effect on Viridis Silica column

<table>
<thead>
<tr>
<th>Modifier effect</th>
<th>Sd of k’</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFA</td>
<td></td>
</tr>
<tr>
<td>NH₄OAc</td>
<td></td>
</tr>
</tbody>
</table>

Silica Surface

- Newcol +0.1% TFA
- Newcol +0.1% TFA +1% water
- Newcol +0.1% TFA (after water was on the columns)
- 0.1% TFA (after 10 mM NH₄OAc was on column)
- 10 mM NH₄OAc

Modifier effect on Viridis EP column

<table>
<thead>
<tr>
<th>Modifier effect</th>
<th>Sd of k’</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFA</td>
<td></td>
</tr>
<tr>
<td>NH₄OAc</td>
<td></td>
</tr>
</tbody>
</table>

Modified silica Surface

- Newcol +0.1% TFA
- Newcol +0.1% TFA +1% water
- Newcol +0.1% TFA (after water was on the columns)
- 0.1% TFA (after 10 mM NH₄OAc was on column)
- 10 mM NH₄OAc

Chemicals:
- Caffeine
- Propranolol
- Perphenazine
- Sulconazole
- Bendroflumethazine
Modifier effect – Silica surface Viridis Sil

0.1 % TFA

0.1% TFA+on column run

NH₄OAc additive before

0.1% TFA+1 %Water

NH₄OAc additive

Memory effect of the Silica column after using Ammonium salt additive
Modifier effect – Bonded Silica surface Viridis EP

0.1 % TFA

0.1% TFA+ on column run

NH₄OAc additive before

0.1% TFA+ 1 %Water

NH₄OAc additive

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